Conjugated linoleic acid modulates immune responses in patients with mild to moderately active Crohn's disease


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SUMMARY

Background & aims: Conjugated linoleic acid (CLA) has demonstrated efficacy as an immune modulator and anti-inflammatory compound in mouse and pig models of colitis. We investigated the immunoregulatory efficacy of CLA in patients with mild to moderate Crohn's disease (CD).

Methods: Thirteen patients with mild to moderately active CD were enrolled in an open-label study of CLA (6 g/d orally) for 12 weeks. Peripheral blood was collected at baseline, 6 and 12 weeks after treatment initiation for isolation of peripheral blood mononuclear cells for functional analyses of lymphocyte populations.

Results: CLA significantly suppressed the ability of peripheral blood CD4+ and CD8+ T cell subsets to produce IFN-γ, TNF-α and IL-17 and lymphoproliferation at week 12. There was a statistically significant drop in CDAI from 245 to 187 (P < 0.013) and increase in IBDQ from 141 to 165 (P < 0.017) on week 12.

Conclusion: Oral CLA administration was well tolerated and suppressed the ability of peripheral blood T cells to produce pro-inflammatory cytokines, decreased disease activity and increased the quality of life of patients with CD.

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1. Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing immune-mediated inflammatory disease of the gastrointestinal tract characterized by two clinical and histopathologic manifestations: Crohn's disease (CD) and ulcerative colitis (UC). CD results in transmural lesions that can affect the entire gastrointestinal tract, whereas in UC lesions are continuous, reside within the mucosal layer and are localized in the colon. The etiology of IBD is unknown although there is increasing evidence of an interplay between genetic susceptibility factors, environmental triggers and immune dysregulation. Dietary supplementation with anti-inflammatory agents provides a unique and potentially safer way of treating mucosal disease and has lead to the evolution of the field of nutritional immunology.

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of octadecadienoic acid. Several CLA isomers, including cis-9, trans-11 CLA, are naturally found in milk, cheese and ruminant products. In addition to the well characterized anti-inflammatory and anti-oxidant properties that have been characterized in animal models including arthritis, type I hypersensitivity and intestinal inflammation, dietary CLA-supplementation suppresses colonic inflammation and up-regulates colonic PPAR-γ expression in pigs with bacterial-induced colitis. In addition, activation of colonic peroxisome proliferator-activated receptor (PPAR) γ by CLA mediates protection from experimental IBD in mice and n-3 PUFA antagonizes the effects of CLA on PPAR-γ in a pig model of DSS colitis. Dietary CLA has been shown to ameliorate inflammation-driven colorectal cancer by activating immune and epithelial cell PPAR-γ in a mouse model. There have been reports documenting increases of PPAR-γ expression and activity in adipocytes, skeletal muscle, and colonic...
mucosa\textsuperscript{12} and macrophages\textsuperscript{13,14} with CLA treatment or CLA-rich diets. However, a reduction in PPAR γ expression in adipocytes by CLA has also been reported.\textsuperscript{15,16} This may be due to cell-type specificity of the response to CLA or isomer specificity since the t10, c12 reduced PPAR γ expression in adipocytes but the c9, t11 failed to show the same suppressive effect on PPAR γ activity.\textsuperscript{17} Interestingly, some probiotic bacteria produce c9, t11 CLA and other anti-inflammatory metabolites locally in the gut that targets PPAR γ in colonic lamina propria macrophages to suppress colitis.\textsuperscript{18}

Modulation of gut inflammation through PPAR γ-dependent mechanisms has a precedent in IBD therapy, the intestinal anti-inflammatory effect of 5-aminosalicylate, a broadly used IBD therapy, is mediated by PPAR γ\textsuperscript{19} and rosiglitazone, a PPAR γ ligand used in diabetes has been shown to be effective in the treatment of ulcerative colitis.\textsuperscript{20} However, the universal application of rosiglitazone or other (thiazolidinediones) TZDs to IBD treatment is unlikely due to reports of increased cardiovascular effects including myocardial infarction and heart failure and the restrictions on rosiglitazone (Avandia) use set by the U.S. Food and Drug Administration (FDA).\textsuperscript{21}

CLA has also demonstrated efficacy in modulating immune responses to vaccination and challenge in pig models, which have the advantage of closely resembling the human immune system. Specifically, dietary CLA-supplementation enhanced cellular immunity by modulating phenotype and effector functions of CD8+ T cells\textsuperscript{22} and enhanced anti-viral responses in pig models.\textsuperscript{23,24} These findings highlight the importance of CLA as a unique compound that can suppress inflammatory lesions in the gut while stimulating adaptive cellular immune responses to viral and bacterial pathogens. This is in contrast with current IBD therapies that have potent immune suppressive effects that can increase the risk of patients to infections. The objective of the present study was to characterize the systemic immune modulatory activity of CLA in patients with active CD and as a secondary endpoint to explore its effects on ameliorating disease activity and quality of life.

2. Research design and methods

2.1. Patients

A total of 24 patients with mild to moderately active CD (estimated CDAI > 150 - < 450) were screened for enrollment into the study between August 2009 and February 2011. Patients were required to be on stable medications 2 months prior to entry and could not be on prednisone at the time of screening. There were 11 screening failures. Thirteen patients completed the study and 1 patient withdrew prior to the six-week evaluation due to side effects related to joint pain. Clinical and demographic characteristics of the patients are included in Table 1. All patients had well-documented CD on the basis of prior colonoscopy, surgery, and/or small bowel imaging. Patients were allowed to continue their baseline CD medications with the exception of oral prednisone and oral dietary supplements.

2.2. Study design

This was an open label pilot study. Patients were screened for inclusion criteria for the study which included well-documented mild to moderate CD. Patients underwent a screening visit which included a complete history and physical and blood work. If all the admission criteria were met a diary was dispensed to capture 1 week of symptom data for CDAI calculation. The baseline visit was carried after 7 days with blood work, clinical assessment and calculation of the CDAI,\textsuperscript{26} completion of a quality of life index, IBDQ\textsuperscript{27} and CLA was dispensed.

2.3. Oral administration of CLA

CLA was self-administered on a daily basis by the subject. The subject was required to take the prescribed dose (6 capsules) one time daily. On study day one the subject received a 6 week supply of CLA (50% cis9, trans-11, 50% trans10, cis12–C18:2) as softgel capsules (6 g/d). The purity of the CLA in the capsules was 77.7% (39% cis9, trans-11, 38.7% trans10, cis12–CLA). The oil was also comprised of 13% oleic acid, 2.7% palmitic acid, 2.6% stearic acid and 0.2% linoleic acid. CLA is an isomeretic fatty acid mixture of same length as linoleic acid which is one of the essential fatty acids required for normal human growth and development. At the 6-week study visit all unused capsules were collected and counted to assess compliance and the remaining 6 weeks of CLA was dispensed.

2.4. Physical assessment

A complete physical assessment was performed during the following visits: screening, baseline, week 6 and week 12 and for unscheduled visits or early withdrawal. At each visit, blood was collected via venipuncture for clinical laboratory assessments. Subjects maintained a diary of liquid stools, abdominal pain, and general well-being for the entire course of the study. The CDAI was calculated at baseline, week 6 and week 12 based on subject diary responses, laboratory data, and physical assessment. At the baseline, week 6 and week 12 visits, a 24-h dietary recall questionnaire was obtained. Patients completed a quality of life questionnaire (IBDQ) at the baseline visit and at the week 12 visit.

2.5. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by using a gradient centrifugation procedure.\textsuperscript{22} Briefly, PBMCs were isolated by overlaying lymphoprep (Mediatech, Herndon, VA) with whole blood diluted 1:4 in phosphate-buffered saline (PBS). Mononuclear cells located in the interface between the diluted plasma and the lymphoprep were recovered by using a sterile Pasteur pipette. PBMCs were washed twice with PBS and resuspended in complete medium.\textsuperscript{22} Complete medium was prepared by supplementing RPMI-1640 with 25 mmol HEPES

Table 1

| Age range | 25–61 (mean 40) |
| M/F | 2/11 |
| Smoking status | Former 4, Never 9 |
| Prior surgery | Yes 10, No 3 |
| Disease distribution | Small bowel (SB) 6, Colon 2, SB + Colon 5 |
| Disease duration | 2–24 years (mean 12.1) |
| Concomitant medications | Mesalamine 6, Sulfasalazine 1, Adalimumab 1, Methotrexate 2, Certolizumab 2, No medications 1 |

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buffer/L (Sigma, St. Louis, MO), 100 units penicillin/mL (Sigma), 0.1 mg streptomycin/mL (Sigma), 5 x 10^{-5} mol 2-mercaptoethanol/L (Sigma), 1 mmol essential amino acids/L (Medi tech), 1 mmol nonessential amino acids/L (Sigma), 2 mmol L-glutamine/L (Sigma), 1 mmol sodium pyruvate/L (Sigma) and 10% fetal bovine serum.28

2.6. PBMC proliferation assays

The proliferation assays were performed on PBMCs isolated from all of the subjects on weeks 0, 6, and 12. To measure total lymphocyte proliferation, we used a lymphocyte blastogenesis test (LTB) based on the incorporation of titrated thymidine. U-bottomed, 96-well microtiter plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ) were seeded with 100 μL PBMCs at 3 x 10^6 cells/mL and 100 μL of media alone (non-stimulated wells), media containing concanavalin A (ConA; 5 μg/mL) (Sigma) and media containing phorbol myristyl acetate (PMA; 50 ng/mL). For anti-CD3/CD28 stimulation, wells were incubated with 5 μg/mL of anti-human CD3 antibody and 2.5 μg/mL of anti-human CD28 antibody in PBS for 1 h at 37 °C in a volume of 50 μL. Wells were washed before seeding cells in 200 μL complete medium. Plates were incubated for 4 days at 37 °C in a 5% CO2 humidified atmosphere. After 4 days of culture, 0.5 μCi methyl-[3H] thymidine (specific radioactivity: 6.7 Ci mmol^{-1}; PerkinElmer, Waltham, MA) in 10 μL medium was added to each well, and the plates were incubated for an additional 20 h. Twenty hours after thymidine addition, cells were harvested onto glass fiber filters with a Combicell harvester (Skatron Instruments, Sterling, VA), and incorporated radioactivity was measured by liquid scintillation counting (LS 6500; Beckman Instruments, Palo Alto, CA).28 Overall lymphocyte proliferation results were expressed as stimulation indexes, which were calculated by dividing the counts per minute of antigen-stimulated wells by the counts per minute of unstimulated wells.

To determine subset-specific proliferation, PBMCs were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), a fluorescent dye used for tracking cell division. CFSE is an ester that diffuses into cells, where it reacts with amine groups, becoming fluorescent. The label is stably retained by proteins and, after cell division; it is equally distributed between daughter cell populations. Briefly, 5 x 10^7 cells were incubated for 10 min at room temperature in 1 mL complete RPMI containing 1 mmol CFSE/L. After 2 washes, cells were re-suspended in complete RPMI and enumerated, and the cell concentration was normalized to 3 x 10^7/mL. Cells were cultured with media alone or stimulated with anti-CD3/CD28, PMA or ConA. On day 5, cells were stained with primary anti-human CD4, anti-human CD8a, and anti-human CD3 antibodies. Data acquisition was performed in a BD LSRII flow cytometer and data analysis was conducted with BD FACSDiva analysis software (BD Biosciences, San Jose, CA).24,28

2.7. Immunophenotyping of peripheral blood mononuclear cells

PBMCs were labeled with anti-human primary antibodies. The primary antibodies were anti-human CD4-PerCP-Cy5.5, anti-human CD8a-PE, and anti-human CD3-PE-Cy5 antibodies. They were then stained with Intracellular anti-human IFN-γ, PE-Cy7, anti-human TNFα-PE, anti-human IL-2-PE, anti-human IL-17P, and anti-human TGFβ-1-APC antibodies.29 Flow cytometric data acquisition and analyses were performed on a BD LSRII flow cytometer and FACSDiva software.

2.8. Cytometric bead array (CBA) assay

CBA kit (BD Biosciences, San Jose, CA) was performed on serum collected at each subject visit (Week 0, Week 6, and Week 12). The CBA technique utilizes micro particles or beads labeled with discrete fluorescence intensity. The maximum emission of capture beads is at 650 nm on RED parameter. Cytokine specific capture antibody is covalently attached to beads. The captured cytokines are detected using specific antibodies with phycoerythrin (PE). The intensity of fluorescence of yellow parameter is proportional to the amount of cytokine present in test samples. Cytokines were determined in the test samples according to the manufacturer instructions. Briefly, test samples (50 μL) and PE detection antibody were incubated with capture bead reagent for 3 h in the dark at room temperature. All unbound antibodies are washed (1.0 mL wash buffer), re-suspended in 300 μL before acquisition. This test was performed to measure Human Inflammatory cytokine response over time quantitatively. Six cytokines were measured: Interleukin-8 (IL-8), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor-α (TNF-α), and Interleukin-12p70 (IL-12p70). Data acquisition was performed in a BD LSRII flow cytometer and data analysis was conducted with BD FCAP Array software (BD Biosciences, San Jose, CA).

2.9. Ethical considerations

The study was approved by the Biomedical Institutional Review Boards at the University of North Carolina at Chapel Hill, Wake Forest University, Winston-Salem, NC; and Virginia Tech, Blacksburg, Virginia. All patients gave written informed consent prior to enrollment in the study.

2.10. Statistical analyses

Analysis of variance (ANOVA) was performed using the general linear model procedure of Statistical Analysis Software (SAS), and probability value (P) < 0.05 was considered to be significant. When the model was significant, ANOVA was followed by Fisher’s Protected Least Significant Difference multiple comparison method.

3. Results

3.1. Patients

A total of 23 patients were screened for entry into this open label trial. There were 9 screening failures due to CDAI measurements under 200 and scheduling conflicts. There were 2 additional screening failures due to a concomitant flare of gout in one subject and introduction of an excluded medication in a second patient. One subject withdrew at 11 weeks due to lack of efficacy and increasing joint activity, however, data was included using the withdrawal visit at week 11 for the week 12 data. There were two missing data points: one patient did not return a diary for the final visit so a week 12 CDAI could not be calculated and one patient did not fill out the IBDQ questionnaire at the final visit. The mean CDAI score was 245 (SD 55) at the baseline visit. The mean IBDQ at the baseline visit was 141 (SD 33.5). The CDAI was well tolerated and patients were compliant in terms of dosing as measured by pill counts. There were no laboratory abnormalities noted during the study.

3.2. Intracellular cytokines in peripheral blood subsets

Flow cytometric examination of cytokine production by peripheral blood lymphocyte subset indicators that oral CLA...
treatment significantly suppressed the ability of CD4+ and CD8+ T cell subsets to produce TNF-α, IFN-γ and IL-17 (Fig. 1), suggesting a suppressed ability of T cell subsets to induce inflammatory responses as well as CD4+ T helper (Th)1 and Th17 differentiation. Oral CLA treatment also increased the ability of CD4+ and CD8+ T cells to produce IL-2 on week 6 of the intervention (Fig. 1C and G).

There were no differences in the intracellular levels of TGF-β in CD4+ or CD8+ T cell subsets over time.

3.3. Systemic cytokine concentrations

To examine the effect of oral CLA treatment on systemic cytokine concentrations, cytometric bead arrays were performed on serum samples from the study time points. In serum, oral CLA treatment increased the concentrations of IL-6 at week 12 of intervention (data not shown), but had no effect on serum concentrations of IL-8, IL-1β, IL-10, TNF-α, or IL-12p70.

3.4. Lymphocyte blastogenesis assay

A lymphocyte blastogenesis assay was performed on PBMC’s isolated from whole blood to measure overall proliferation of PBMCs. Lymphocyte proliferation was expressed as stimulation indexes, which were calculated by dividing counts per minute of stimulated wells by the counts per minute of non-stimulated wells. Oral CLA supplementation increased the overall proliferation of PBMCs following anti-CD3/CD28 stimulation at week 6 and 12 (Fig. 2A) as well as ConA- and PMA-induced proliferation at week 6 only (Fig. 2B and C).

3.5. CFSE lymphoproliferation assay

To measure subset-specific proliferation, we used the CFSE proliferation assay. In contrast to the LBT, which measures overall cell proliferation over a 12-h period, the CFSE assay measures cumulative proliferation of distinct cell subsets over a 5-day culture. Thus, CFSE is a more sensitive assay. The CFSE results indicate that at week 12 oral CLA-supplementation decreased anti-CD3/CD28- and PMA-induced proliferation of CD4+ T cell subsets when compared to week 0 or 6 of the intervention (Fig. 3C). In addition, background proliferation (i.e., proliferation of unstimulated cells) of CD3+ and CD4+ cells was significantly higher on day 0 than at 12 weeks post-treatment (Fig. 3A and C). Cell proliferation in the absence of stimulation correlates with the state of cell activation. Thus our results suggest that CLA modulates CD3+ and CD4+ T cell phenotype towards a resting state.

3.6. Disease activity and quality of life measures

The mean CDAI at entry into the study was 245 (SD 55), at 6 weeks 212.9 (SD 80.9) and at 12 weeks 187 (SD 55.6). Using a paired students t test the difference between the entry CDAI and the 12-week CDAI was statistically significant ($P = 0.013$) (95% confidence interval 14.44–101.8). This represented a 58-point drop, which may not be clinically significant. Three patients dropped their CDAI greater than 100 points from baseline and 3 patients dropped greater than 70 points from baseline. C reactive protein ranged from 0 to 3.4 at baseline and from 0 to 4.4 at week 12. The mean CRP at baseline was 1.525 and at week 12 was 1.758. There was no significant difference between the CRP at baseline and at week 12 ($P = 0.6599$).

A quality of life questionnaire was completed at baseline and week 12 (IBDQ). Each of 32 items is rated on a scale of 1–7 with a range of score from 32 (very poor quality of life) to 224 (optimal quality of life). The mean quality of life at baseline was 141 (SD 33.5), with an increase to 165 (SD 44.5) at the end of the study. This is a statistically significant improvement in measured quality of life ($P = 0.017$).

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because the preponderant theory is that dysregulated CD4+ glandin E2 (PGE2) release from tracheae of guinea pigs. In addition, the effector function of CD4+ mediated in occurring and potentially safer compounds such as CLA. Accord-

4. Discussion

Current pharmacological therapies for CD are modestly successful for the long-term management of the disease and result in significant side effects, including infections and cancer resulting from immunosuppression caused by these drugs. Dissatisfaction with current traditional therapies, the chronicity of the disease and concern over side effects have resulted in increased use of naturally occurring and potentially safer compounds such as CLA. Accordingly, the incidence of complementary and alternative medicine usage among IBD patients was estimated to be 49.5%. but limited information is available regarding their mechanisms of action, clinical efficacy and tolerability in humans with IBD. A previous study in obese humans demonstrated that CLA is safe for use for at least one year.

Mechanistically, CLA can bind to the ligand-binding domain of PPARγ and expression of PPARγ by immune and epithelial cells is required for the full spectrum of anti-inflammatory effects of CLA in several mouse models of experimental IBD pig models of bacterial-induced colitis and DSS colitis as well as a mouse model of inflammation-driven colorectal cancer. Another mechanism by which CLA can suppress inflammatory responses involves downregulation of eicosanoid synthesis such as suppression of prosta-

**Fig. 2.** Effect of oral conjugated linoleic acid (CLA) treatment on overall proliferation of peripheral blood mononuclear cells (PBMC) following ex vivo stimulation with anti-CD3/CD28, concanavalin A (ConA) and phorbol myristate acetate (PMA). Overall ex vivo proliferation of PBMCs was determined by using a lymphocyte blastogenesis test. Results are expressed as stimulation index. Values are means ± SEM, n = 13. Statistically significant differences (P < 0.05) over time are indicated with different letter superscripts.

Consistent with a modulatory effect of CLA on CD4+ T cell subsets, CLA prevented CD4+ T cell-induced colitis in a CD4+CD45RBhi adoptive transfer mouse model. A follow up study demonstrated that PPARγ was required for the prevention of CD4+ T cell-induced colitis using a similar co-transfer model of colitis. The suppressed CD4+ T cell lymphoproliferation to anti-CD3/CD28 stimulation in PBMCs at week 12 of CLA treatment further supports an effect of CLA on the CD4+ T cell population in peripheral blood of CD patients. This finding was in contrast to the increased overall lymphoproliferation ability on week 6 of CLA treatment, suggesting that CLA may have enhanced the proliferation of another PBMC subset at week 6. In this regard, the proliferative ability of peripheral blood CD8+ T cells following ex vivo stimulation with PMA was significantly increased on week 6. The increased lymphoproliferative ability of peripheral blood CD8+ T cells following CLA treatment was previously reported in pig models of vaccination and challenge. In pigs, this effect was accompanied by an in vivo expansion of CD8+ T cells and γδ T cells in peripheral blood as well as increased percentages of CD8αβ+ thymocytes.

Peripherally cytokine measurements IL-8, IL-1β, IL-10, TNF-α, or IL-12p70 with the exception of IL-6 were not affected by CLA administration. There was an increase in peripheral IL-6 at week 12 of therapy. Although IL-6 concentrations have been shown to be elevated in patients with active CD as compared to patients with inactive CD, these levels are poor markers of disease activity. IL-6 tends to parallel CRP levels which were not affected by CLA in this study. Tissue cytokine levels may be more meaningful in terms of gut immunomodulation. This was not looked at in this pilot study. Likely larger numbers of patients with active and inactive disease will need to be studied to further understand how CLA regulates mucosal immune responses and peripheral cytokine measurements.

The CDAI results indicate a trend towards a decrease in CDAI at weeks 6 and a statistically significant decline of 58 points by week 12 of the oral intervention with CLA. Of note, CLA showed a more marked clinical efficacy in those CD patients that started the trial with higher CDAI scores. A 100-point drop in CDAI scores occurred in 33% of patients by week 12 of intervention and a greater than 70 point drop in an additional 33% of patients. CDAI definitions of clinical response in CD therapeutic trials have used both 70-point CDAI and 100-point CDAI drops as clinically significant changes. Clinical remission is defined as a CDAI score of less than 150. Using this definition, 3 patients were in remission at the end of the trial. It remains unknown whether this was an effect of CLA or a synergistic
effect between CLA and the stable medication they received. In this study there was also a statistically significant improvement in quality of life at the end of the 12-week study period, correlating with the improvement in the CDAI. These results are comparable to those seen in trials with the PPAR γ agonist rosiglitazone. In the present study has shed new light on the clinical potential of this compound and provided insights on the possible mechanisms of immune modulation targeted by CLA in the human system. Based on these results, a double-blind, placebo-controlled, randomized trial with several doses of CLA is warranted.

Statement of authorship

JBR, KLI and RH designed the experiments, interpreted the results, contributed to write the paper and directed the project. KLI, MS, RB and HHH performed the human subject recruitment. WTH conducted the cytokine and lymphoproliferation studies and prepared figures.

Conflict of interest

No conflicts.

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