Atherosclerosis 211 (2010) 96–102

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Conjugated linoleic acid suppresses the migratory and inflammatory phenotype of the monocyte/macrophage cell

Sarah McClelland[a], Clare Cox[a], Roisin O'Connor[b], Monica de Gaetano[a], Cathal McCarthy[a], Lorna Cryan[c], Des Fitzgerald[a], Orina Belton[a,*]

[a] School of Biomedical and Biomolecular Science, UCD Conway Institute, University College Dublin, Ireland
[b] Wolfson Institute for Biomedical Research, University College London, UK
[c] Vascular Biology Programme, Boston Children's Hospital, Boston, MA, USA

ARTICLE INFO

Article history:
Received 19 November 2009
Received in revised form 18 January 2010
Accepted 2 February 2010
Available online 10 February 2010

Keywords:
Conjugated linoleic acid
Monocyte migration
PPARs
Cyclooxygenase

ABSTRACT

Objective: We have previously shown that conjugated linoleic acid (CLA) regresses pre-established murine atherosclerosis. Although the exact underlying mechanisms are unclear, accumulation of macrophages and expression of inflammatory markers were reduced in atherosclerotic plaques of CLA-fed mice, implicating the monocyte/macrophage as a target through which CLA may mediate anti-atherosclerotic effects. CLA mediates its effect at least in part via activation of the nuclear receptor, peroxisome proliferator activator receptor-γ (PPARγ). In this study we investigate if CLA mediates anti-atherogenic effects via modulation of monocyte/macrophage function and provide evidence for an additional PPARγ-independent mechanism for CLA.

Methods and results: Migration of the human monocyte cell line THP-1, and primary blood monocytes (HPBMCs) was assessed using transwell migration assays. Monocyte chemoattractant protein-1 (MCP-1) mediates chemotaxis via interaction with the chemokine (C–C motif)-2 receptor (CCR-2), which is expressed on the monocyte cell surface, and is negatively regulated by PPARγ agonists. Incubation of THP-1 monocytes with CLA-isomers and a PPARγ agonist inhibited MCP-1-induced monocyte migration. Prior to monocyte recruitment, activated platelets accumulate and release the contents of their secretory granules ("platelet-releasate"). Here we demonstrate that platelet-releasate is a monocyte chemoattractant, and CLA, but not the PPARγ agonist, inhibits platelet-releasate-induced migration of THP-1 and HPBMC monocytes. CLA-treatment also suppressed the inflammatory macrophage phenotype, demonstrated by decreased induction of monocyte migration by CLA-treated macrophage-conditioned-media, as well as by decreased cyclooxygenase (COX)-2 and cytosolic phospholipase-A_2 ($cPLA_2$) expression and MCP-1, prostaglandin E_2 (PGE_2) and matrix metalloprotease (MMP)-9 generation.

Conclusions: CLA-isomers inhibit monocyte migration and reduce the inflammatory output of the macrophage. These mechanisms may contribute to the potent anti-atherosclerotic effects of CLA *in vivo*.

1. Introduction

Conjugated linoleic acid (CLA) is the term denoting a group of naturally occurring isomers of linoleic acid (18:2, n6), differing in the position or geometry of their double bonds. The predominant biologically active isomers are *cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA. While differential isomeric effects have been reported [1] there is a general consensus that CLA inhibits atherogenesis in animal models [2,3], however the underlying mechanism(s) remains to be elucidated.

We have previously demonstrated that dietary CLA administration (80:20 blend of *cis*-9,*trans*-11:*trans*-10,*cis*-12-CLA; Loders Crocklaan, Holland) induces regression of pre-established atherosclerosis in $apoE^{-/-}$ mice, despite continuing high-cholesterol challenge [4]. Accumulation of macrophages in atherosclerotic plaques of CLA-fed mice was reduced, coincident with decreased expression of inflammatory markers, including MMP-9, implicating the macrophage as a target through which CLA mediates anti-atherosclerotic effects.

Monocyte recruitment and subsequent trans-endothelial migration, critical steps in the initiation of atherosclerosis, are preceded by adhesion of activated platelets to lesion-prone areas [5]. Upon activation, platelets release the contents of their secretory granules, termed the "platelet-releasate". It is likely that certain platelet-releasate components are chemoattractants

* Corresponding author. Tel.: +353 1 7166748; fax: +353 1 7166701.
E-mail address: Orina.belton@ucd.ie (O. Belton).

0021-9150/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2010.02.003

facilitating monocyte recruitment to the vessel wall and differentiation into macrophages. Activated macrophages express pro-inflammatory genes (e.g. COX-2), and produce inflammatory cytokines, prostaglandins (PGs), MCP-1, and MMPs, which degrade extracellular matrix [6].

Several lines of evidence have established CLA as an agonist of the PPAR transcription-factor family. PPARγ agonists inhibit atherogenesis in the low density lipoprotein receptor (LDLR) and apolipoprotein E (apoE) deficient mouse models [7,8], and suppress monocyte/macrophage homing to atherosclerotic plaques [9]. However, unlike CLA, PPARγ agonists do not regress pre-established atherosclerosis, implying additional mechanisms through which CLA mediates its effects, a hypothesis supported by a growing body of evidence [10,11]. One potential mechanism is inhibition of COX-1 and COX-2, which are implicated in atherosclerosis [12]. CLA-isomers compete with arachidonic acid for access to these enzymes, preventing PG generation, and it has been shown that CLA inhibits PGE_2 generation, partly via suppression of COX-2 [13].

Our objective was to investigate the monocyte/macrophage as a possible target for the anti-atherosclerotic effects of CLA. Specifically, we examined the effect of CLA on monocyte migration in response to chemoattractants that may be critically involved in atherosclerosis, investigated whether CLA alters the pro-inflammatory macrophage phenotype, and propose a PPARγ-independent mechanism through which CLA mediates its effects.

2. Methods

2.1. Isolation of human peripheral blood monocytes

Whole blood (30 ml) was drawn from healthy volunteers into heparin-coated vacutainers. Platelet-rich plasma (PRP) was isolated by centrifugation (190 × *g* for 15 min), and mixed 1:1 with PBS before addition to Lymphoprep solution (1:2) (Nycomed, Norway) and centrifuged (500 × *g* for 30 min). The upper layer was discarded, buffy-coat removed and washed with sterile PBS. Cells were re-suspended in 10 ml serum-free RPMI, seeded and incubated for 2 h at 37 °C. Monocytes adhered to the dish and non-adherent cells were removed by washing. Cells were carefully detached from the dish by gentle scraping, prior to migration assays. Migration assays were carried out 24 h following HPBMC isolation. Full details are provided in Supplementary material.

2.2. THP-1 cell culture and cell treatments

Human THP-1 monocytes (0.5×10^6 cells/ml; ECACC) were cultured in RPMI medium supplemented with fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM) (Gibco BRL, UK). For experiments utilizing macrophage cells, THP-1 monocytes (1×10^6) were differentiated to macrophages with 100 nmol/L phorbol 12-myristate 13-acetate (PMA) (Sigma) for 72 h.

Prior to migration assays, monocytes were incubated overnight in serum-free medium (SFM). THP-1 monocytes (undifferentiated) and HPBMC cells were treated with *cis*-9,*trans*-11-CLA (50 μM and 10 μM, respectively), *trans*-10,*cis*-12-CLA (50 μM and 10 μM, respectively), linoleic acid (LA) (50 μM and 10 μM, respectively), oleic acid (OA) (50 μM and 10 μM, respectively), troglitazone (TRO, 10 μM), COX-2-inhibitor (NS-398, 10 μM) or DMSO for 18 h. CLA, OA and LA were from Cayman Chemicals (MI, USA), troglitazone, NS-398 and LPS and were from Sigma–Aldrich (Dublin, Ireland). All migration experiments were carried out independently a minimum of three times, with three replicates per treatment in each experiment.

To isolate macrophage-conditioned-media, THP-1 macrophages were treated for 6 or 18 h with 50 μM *cis*-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, LA, OA or DMSO and conditioned-media was removed. LPS-treatment (30 ng/ml for 6 h) induced an inflammatory phenotype where required. All compounds were added in SFM and three replicates were used for each treatment. Experiments were performed 3 times. CLA concentration was selected following optimization of migration conditions (Supplemental Figs. 1 and 2), while concentrations of troglitazone and NS-398 were based on published studies [14,15].

2.3. Isolation of platelet-releasate

Whole blood (100 ml) was drawn from ten healthy, non-smoking, male volunteers aged 25–30 years and free from medication for at least 10 days into 0.15% (v/v) acid-citrate-dextrose (ACD) (38 mM anhydrous citric acid, 75 mM sodium citrate, 124 mM D-glucose). PRP was isolated as above and acidified to pH 6.5. PGE_1 (1 μM) was added. Platelets were pelleted by centrifugation (720 × *g* for 10 min), re-suspended in a modified HEPES buffer, termed "JNL" (130 mM NaCl, 10 mM trisodium citrate, 9 mM NaHCO3, 6 mM dextrose, 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, 10 mM Tris pH 7.4) and supplemented with 1.8 mM $CaCl_2$. Platelet concentration was determined using a Sysmex^{tm} Haematology Analyser (TOA Medical Electronics, Japan). 10^8 platelets were incubated at 37 °C in an aggregometer under constant stirring (1100 rpm) and stimulated with thrombin-receptor-activating-peptide (TRAP), or left unstimulated (resting control). Intact platelets were removed by centrifugation (750 × *g*, 10 min), harvesting the supernatant and repeating the centrifugation. The resulting supernatant was the "platelet-releasate" in JNL buffer. Proteins released from thrombin-stimulated platelets were identified by electrospray tandem mass spectrometry following separation by strong cation exchange, as described previously [16].

2.4. Monocyte migration assay

Undifferentiated THP-1 monocytes and HPBMCs were treated with CLA, LA, OA, TRO, NS-398 and DMSO as above, and applied to 24-well transwell migration plates (Invitrogen, USA), consisting of upper and lower chambers, separated by membranes punctuated with pores 5 μm (THP-1) or 2 μm (HPBMCs) in diameter. 1×10^5 monocytes were seeded in a final volume of 125 μl SFM into the upper chamber. Known (MCP-1, 25 mg/ml) or putative (resting/activated platelet-releasate, 10–500 μl) chemoattractants were added to the lower chamber in a final volume of 1 ml. Plates were incubated for 2 h at 37 °C. Upper wells were placed into 2.5% glyceraldehyde for 15 min, to fix cells adherent to the underside of the membrane. Membranes were washed with PBS, and upper chambers immersed in 0.1% crystal-violet for 45 min. Following two PBS washes, adherent cells were visualized by light microscopy. Average number of cells in ten 40× fields of view were counted, and the average value of three replicates per treatment were expressed as a percentage of vehicle (DMSO) control (100%), to allow for inter-individual variability in the chemoattractant potency of platelet-releasate from different donors. Migration experiments were repeated three times.

2.5. Gene expression analysis

COX-2, $cPLA_2$ and CCR-2 mRNA levels were determined using semi-quantitative real time PCR. Total RNA was isolated from THP-1 macrophage lysates using the RNeasy kit (Qiagen, UK), and reverse-transcribed using MMLV reverse transcriptase (Promega, USA), according to manufacturers' instructions. COX-2 primers (sense-TTGTACCCGGACAGGATTCTATG-, antisense-