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Down-regulation of lipoprotein lipase increases ABCA1-mediated cholesterol efflux in THP-1 macrophages

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ABSTRACT

The ATP-binding cassette transporter A1 (ABCA1) mediates the efflux of excess cholesterol from foam cells to lipid-poor apolipoprotein A-I, in a process called reverse cholesterol transport. Lipoprotein lipase (LPL) is a lipolytic enzyme expressed by macrophages within atherosclerotic lesions. Lentivirus-mediated RNA interference was used to genetically knock-down (KD) the expression of LPL in THP-1 macrophages. Silencing of the LPL gene was confirmed by end-point PCR, real time PCR, and protein analysis. Suppression of LPL expression correlated with a 1.6-fold up-regulation of ABCA1 mRNA levels, and resulted in a 4.5-fold increase in ABCA1-dependent cholesterol efflux. Replenishing LPL by addition of purified bovine LPL to the cell culture media resulted in down-regulation of ABCA1-mediated cholesterol efflux in both wild-type and LPL knockdown cells. These findings suggest an inverse correlation between macrophage LPL levels and ABCA1 cholesterol transport activity.

1. Introduction

Atherosclerosis is initiated when circulating monocytes adhere to the vascular wall, enter the intimal space and ingest copious amounts of lipids to differentiate into large macrophage-derived foam cells [1]. High density lipoproteins (HDL) protect against atherosclerosis by facilitating the removal of excess free cholesterol from vascular cells. Four different mechanisms have been described for the transport of cholesterol from cells [2]: (i) aqueous diffusion down a concentration gradient, (ii) bidirectional flux mediated by scavenger receptor class-B type I (SR-BI), (iii) ATP-binding cassette transporter A1 (ABCA1)-mediated unidirectional efflux of cholesterol and phospholipids to lipid-poor apolipoprotein acceptors such as apolipoprotein A-I (apoA-I) and apolipoprotein E (apoE), and (iv) ABCG1-mediated unidirectional efflux to nascent HDL particles. Both ABCA1 and ABCG1 are highly expressed in macrophages whereas SR-BI is mainly expressed in steroidogenic tissues and liver. Hence, the ABCA1 and ABCG1 pathways are the dominant cholesterol efflux mechanism in macrophages.

ABCA1 is a member of a large family of transporters that have nucleotide binding domains and hydrolyze ATP (are ATPases) for energy to transport various substrates including lipids, ions and metabolites [3]. ABCA1-mediated cholesterol efflux has an absolute requirement for an apolipoprotein acceptor [4]. ABCA1 levels are tightly regulated in order to maintain lipid homeostasis. Cholesterol-loading and cAMP analogues are major positive regulators of ABCA1 expression [5]. The transcription of ABCA1 gene is also highly up-regulated by the activation of nuclear receptors liver X receptor (LXR), retinoid X receptor (RXR), and peroxisome proliferators activated receptor (PPAR) [6].

Macrophages abundantly express another protein, lipoprotein lipase (LPL), a secreted lipolytic enzyme that facilitates the hydrolysis of triglycerides (TG) in chylomicrons to release fatty acids for energy or storage [7,8]. Depletion of TG converts chylomicrons into cholesterol-rich remnant particles which are ligands for macrophage lipoprotein receptors and are instrumental in macrophage cholesterol accumulation. This mechanism may partially explain the contribution of macrophage LPL to atherogenesis [7]. Several studies have demonstrated that LPL is abundantly expressed in macrophage-derived foam cells lodged within atherosclerotic lesions [7]. Animal models with macrophage specific overexpression of human LPL point to a role for LPL in cholesterol accumulation and lesion growth [9–12]. While the positive correlation between macrophage LPL and atherosclerosis is undisputed, the specific cellular events or mechanisms by which

Abbreviations: ABCA1, ATP-binding cassette transporter A1; LPL, lipoprotein lipase; LPL-KD, LPL knock-down; LXR, liver X receptor; PMA, phorbol 12-myristate 13-acetate; PPAR, peroxisome proliferator-activated receptor; RXR, retinoic-X receptor; shRNA, short hairpin RNA; SR-BI, scavenger receptor type BI; WT, wild-type.

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LPL precipitates fatty lesions are not well understood. Since ABCA1 is a key player in cellular cholesterol homeostasis in macrophages, we hypothesized that macrophage LPL may regulate ABCA1 expression and function. Short interfering RNA (siRNA) technology was used to silence the *LPL* gene in THP-1 cells, a human acute monocytic leukemia cell line. Our data confirm that LPL levels inversely correlate with ABCA1 expression and cholesterol efflux in THP-1 macrophages.

2. Methods

2.1. Cell culture and differentiation

THP-1 monocytes were obtained from ATCC and maintained in growth media (RPMI 1640 supplemented with 10% FBS (Atlanta Biologicals), 50 units/mL penicillin, 50 μg/mL streptomycin, 10 mM HEPES, pH 7.4, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol) at 37 °C and 5% CO_2. Monocytes were differentiated to macrophages in differentiation media (growth media without FBS, supplemented with 1 mg/mL BSA and 200 nM phorbol-12-myristate-13-acetate (PMA)) within 48–72 h as evidenced by their adherence to the culture plate.

2.2. Silencing the LPL gene

Wild-type (WT) THP-1 monocytes were seeded into two T 25 cm^2 tissue culture flasks in growth medium at a density of 1×10^5 cells/mL. The next day, the cells were resuspended in 5 mL of growth media supplemented with 5 μg/mL polybrene. LPL shRNA Lentivirus (Santa Cruz Biotechnology, 0.5×10^6 infectious units of virus in 100 μL) was added, cells were chilled for 15 min, and transferred to 37 °C. The control flask was handled identically with the omission of Lentivirus. After 48 h, the viral load was removed by centrifugation, cells were washed with PBS, and cultured in growth media. Cells transfected successfully (designated LPL-KD THP-1 cells) were selected by treatment with 10 μg/mL puromycin until all cells in the control flask were confirmed dead.

2.3. RNA isolation and RT-PCR

RNA was isolated using TRI reagent (Sigma) and Direct-zol™ RNA miniprep kit (Zymo Research) according to the manufacturers' protocols. RNA was quantified by spectrophotometry at 260 nm and 4 μg of RNA was used to synthesize cDNA by reverse transcription using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), dNTPs, and oligodT primers (Promega).

End-point PCR was performed using cDNA and primer pairs shown (Table 1). The PCR amplicons were resolved by 2% agarose gel and the DNA bands were quantified by ImageJ (NIH) analysis. The cDNA was also subjected to real time quantitative PCR using a Smart cycler (Cepheid Inc), RealMasterMix (5PRIME), and primer pairs shown (Table 1). A melting temperature (T_m) of 85 °C or higher was obtained, confirming primer-specific amplification. β-Actin was used as the house-keeping gene control for both conventional and quantitative PCR. The threshold cycle (C_T) values were used to calculate fold change in transcript levels using the $2^{-\Delta\Delta C_T}$ method [13] as follows: fold change $= 2^{-\left(C_{T\ \text{target}} - C_{T\beta\text{-actin}}\right)_{\text{siRNA}} - \left(C_{T\ \text{target}} - C_{T\beta\text{-actin}}\right)_{\text{control}}}$.

Table 1
Primer sequences for end-point and real-time PCR.

Gene & amplicon size (bp)	Primer type	Primer sequence
huLPL (308)	Sense 2	5′-GGAATGTATGAGAGTTGGGT-3′
	Antisense 2	5′-GGGCTTCTGCATACTCAAAG-3′
huABCA1 (157)	Sense	5′-AACAGTTTGTGGCCCTTTTG-3′
	Antisense	5′-AGTTCCAGGCTGGGGTACTT-3′
huABCG1 (317)	Sense	5′-GGTTCTTCGTCAGCTTCGAC-3′
	Antisense	5′-GTTTCCTGGCATTCAGGTGT-3′
huSR-BI (216)	Sense	5′-CTGTGGGTGAGATCATGTGG-3′
	Antisense	5′-GCCAGAAGTCAACCTTGCTC-3′
β-Actin (285)	Sense	5′-TCATGAAGTGTGACGTTGACATCCGT-3′
	Antisense	5′-CTTAGAAGCATTTGCGGTGCACGATG-3′

2.4. Analysis of de novo LPL protein synthesis

The level of LPL protein translation was compared in WT and LPL-KD THP-1 macrophages by pulse-chase labeling. This protocol tags only *de novo* synthesized metabolites during biosynthesis. WT and LPL-KD THP-1 monocytes were plated at 2.5×10^6 cells per well on a 12-well tissue culture plate and differentiated as above. The cells were depleted of methionine by incubation in methionine-free minimum essential medium (MEM) for 30 min, and then incubated with 200 μCi/mL of ^{35}S-labeled methionine (radioactive label) in MEM for 4 h. Any incompletely synthesized proteins were chased to completion using 100 μM cold methionine (Sigma) supplemented with 100 units/mL heparin for 30 min. Heparin was added to allow the dissociation of LPL from the cell surface proteoglycans. The medium was collected and cleared of cellular debris while the cellular monolayer was solubilized using 150 μL/well of lysis buffer (0.1% Triton-X-100 in 50 mM Tris·HCl, pH 8.0). Both media and lysates were adjusted to 10% glycerol and 0.05% Triton X-100.

A chicken anti-LPL IgY (2 μg/mL) immobilized on goat anti-chicken IgY-agarose was used to immunoprecipitate LPL from 1 mL media or 100 μL of lysates, after ascertaining equal radioactivity in WT and LPL-KD samples. Equal volumes of immunoprecipitated samples were resolved by 10% SDS–PAGE. The gel was then dried between cellophane sheets and LPL protein bands were visualized by autoradiography.

2.5. Cholesterol efflux assay

Wild-type or LPL knock down (LPL-KD) THP-1 cells were plated at 2.5×10^6 cells/mL per well. They were differentiated as above for 48 h. The differentiated macrophage monolayer was rinsed with PBS, and incubated with ^3H-cholesterol labeled oxidized LDL (final 0.25 μCi/mL/well and 50 μg oxLDL/mL/well) for 48 h. The labeled oxidized LDL was prepared by drying 3 μCi of [1,2-^3H(N)]-cholesterol in toluene (Perkin Elmer) under nitrogen gas and re-suspending it in 500 μL of efflux media (growth media without FBS, supplemented with 1 mg/mL BSA) by sonication in a 23 °C water bath for 8 min. To this, 600 μg of ox-LDL was added and incubated on ice for 20 min. (Oxidized LDL was obtained by prolonged exposure of LDL isolated from human plasma by sequential density gradient centrifugations [14] to the atmosphere.) This solution was transferred to 37 °C for 20 min, returned to RT, before mixing it with efflux media for a final volume of 12 mL. Each well of differentiated macrophages received 1 mL of this ^3H-cholesterol labeled oxidized LDL. After 2 days the cell monolayer was rinsed with PBS + 1 mg/mL BSA. Next, cellular cholesterol pools were equilibrated by incubation for 48 h in efflux media supplemented with unlabeled 20 μg/mL cholesterol. After washing with PBS + 1 mg/mL BSA, cholesterol efflux was initiated by incubation in efflux media in the presence or absence of 32.7 μg/mL apolipoprotein-AI. After 12–14 h, the media was collected and cellular debris was removed by centrifugation. The macrophage monolayer was washed twice with PBS/BSA, the cells were