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MicroRNA-29a regulates pro-inflammatory cytokine secretion and scavenger receptor expression by targeting LPL in oxLDL-stimulated dendritic cells

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ABSTRACT

There is increasing evidence that microRNAs (miRNAs) play important roles in cell proliferation, apoptosis and differentiation that accompany inflammatory responses. However, whether microRNAs are associated with DC immuno-inflammatory responses with oxidized low density lipoprotein (oxLDL) stimulation is not yet known. Our study aims to explore the link of miRNAs with lipid-overload and immuno-inflammatory mechanism for atherosclerosis. In DCs transfected with microRNA-29a mimics or inhibitors, we showed that microRNA-29a plays an important role in proinflammatory cytokine secretion and scavenger receptor expression upon oxLDL-treatment. Furthermore, we suggest an additional explanation for the mechanism of microRNA-29a regulation of its functional target, lipoprotein lipase. We conclude that microRNA-29a could regulate pro-inflammatory cytokine secretion and scavenger receptor expression by targeting lipoprotein lipase in oxLDL-stimulated dendritic cells.

1. Introduction

Atherosclerosis is currently the leading cause of human morbidity and mortality in developed countries, and has been established as a chronic inflammatory disease [1]. Oxidized low-density lipoproteins (oxLDLs) remain the most important risk factor for atherosclerosis. Recently, the immuno-inflammatory mechanisms of atherosclerosis have gained tremendous interest and the process has been recognized as a complex interplay between modified lipids, different cells of the immune system, endothelial cells, and smooth muscle cells [2]. Aside from the well-known cell types such as monocytes, T cells, and B cells, the unexpected roles of dendritic cells (DCs) in atherosclerosis have recently been discovered [3]. DCs, specialized antigen-presenting cells, are central to the regulation of immunity because they are at the interface of the innate and adaptive immune systems. DCs also play an important role in this activation and affect the initiation and progression of atherosclerosis, either pro-atherogenically or anti-atherogenically [4]. However, despite ongoing research regarding the immune-inflammatory mechanisms in atherosclerosis, the intracellular molecular events that induce and regulate the activation have yet to be elucidated.

MicroRNAs (miRNAs), a novel class of short (~22 nucleotides) non-coding RNAs, have been identified as important post-transcriptional inhibitors of gene expression by base pairing with the 3′ untranslated regions (UTRs) of mRNAs and promoting mRNA stability [5,6]. miRNAs are implicated in various physiologic and pathologic processes [7,8], including cardiogenesis, hematopoietic lineage differentiation, and oncogenesis.

Accumulating evidence suggest that miRNAs also play an important role in cardiovascular disease [9–15]. The MiR-29 family, predicted to function as inhibitors of numerous mRNAs involved in extracellular matrix (ECM) production and fibrosis, are pathologically associated with tumor and cardiac fibrosis through the regulation of collagens [16,17]. The expression of miR-29a is downregulated after myocardial infarction [18]. Holmstrom et al. [19] revealed the differential expression of miR-29a in mature DCs relative to immature DCs. In this study, the increased expression of miR-29a in oxLDL-stimulated DCs is first established, in

Abbreviations: DC, dendritc cell; miRNA, microRNA; OxLDL, oxidized low density lipoprotein; TNF-α, tumor necrosis factor-α; IL, interleukin; LPL, lipoprotein lipase; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; UTR, untranslated regions; SRA, scavenger receptor A

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addition, the interaction between miR-29a and lipoprotein lipase (LPL) and their functional roles in the lipid uptake and pro-inflammatory cytokine secretion by oxLDL-stimulated DCs were determined. Furthermore, miR-29a is shown to decrease the pro-inflammatory cytokine secretion and enhance scavenger receptors expression efficiently by targeting LPL, evidence that regulation of LPL is associated with miR-29a in DCs is also provided.

2. Materials and methods

The investigation conformed with the principles outlined in the Declaration of Helsinki for the use of human blood and was approved by the Ethics Committee of Experimental Research of Zhejiang University.

2.1. Human primary peripheral blood DC culture

Peripheral human blood was obtained from healthy donors. Mononuclear cells were isolated by centrifugation through a Ficoll-isopaque (Sigma) density gradient [20]. To obtain the monocytes, the mononuclear cells were allowed to adhere to 6-wells plates with 5% autologous serum for 2 h at 37 °C, in a 5% CO_2 incubator. Non-adherent cells were removed and the adherent cells (monocytes) were co-cultured with with 1000 units/ml granulocyte GM-CSF and 1000 units/ml interleukin-4 for 5–7 days to obtain the immature DCs, monocytes were cultured in a complete medium (RPMI 1640 with 10% fetal calf serum). OxLDL (30 ug/ml) was added at day 6 and cells were harvested at day 7.

2.2. Small RNA transfection by electroporation treatment

Pre-miR™ precursor molecules for miR-29a, anti-miR™ inhibitor for miR-29a were obtained from Dharmacon. SiLPL and siNS (not significant) duplex were synthesized by Dharmacon RNAi Technologies. DCs were harvested and resuspended in Opti-MEM (Invitrogen Life Technologies) at a concentration of 4×10^7 cells/ml. Next, 7.5 ug miRNA inhibitor/mimic and siRNA (Dharmacon) were transferred to a 4-mm cuvette (Peqlab Biotechnologie) and filled to a final volume of 100 ul with Opti-MEM. Cell suspension was added and immediately pulsed in a Gene Pulser II apparatus (Bio-Rad) at 300 V, 150 uF, and 100 ohms. To confirm the efficiency of transfection, the same amount of Cy3-labeled negative control was also transfected and the efficiency of miR-29a overexpression and inhibition were at least 90%.

2.3. HPLC analysis of lipid levels

DCs were transfected with miRNA mimic and inhibitor, oxLDL was added 6 h after transfection, and cells were further incubated for 24 h. The sterol analyses were performed using an HPLC system (model 2790, controlled with Empower Pro software; Waters Corp., Milford, MA). Sterols were detected using a photodiode array detector equipped with a 4-μL cell (model 996; Waters Corp.). Analysis of cholesterol and cholesteryl esters was performed after elution with acetonitrile-isopropanol 30:70 (v/v) [21] and detection by absorbance at 210 nm.

2.4. ELISA assays of inflammatory markers and LPL expression

DCs that have undergone 24 h incubation with oxLDL were used for ELISA. Culture supernatants were analyzed to determine the presence of tumor necrosis factor-α (TNF-α), IL-6, LPL using Sandwich Enzyme Immunoassay kits (RD) according to the manufacturer's instructions.

2.5. Cloning of 3′ UTR of LPL mRNA and reporter gene assay

The 293T were cotransfected with p-LPL UTR miRNA luciferase reporter vector and miR-29a mimic/inhibitor using lipofectamine 2000 (Invitrogen). Cells were also transformed with the PGL3-control vector, which is useful for monitoring transfection efficiency. MiRNA mimic/inhibitor negative, an miRNA non-homologous to the human genome, was used as a control. All cells were also transfected with pRL-TK (Promega) for normalization control. After 24 h, firefly luciferase activities were determined using the dual-luciferase reporter assay system. Relative reporter activity was obtained by normalization to the Renilla control. The ratio of the luciferase activity of each construct was calculated using a luminometer.

2.6. mRNA real time quantitative PCR

This step was done after 24-h incubation of DCs with oxLDL. The mRNA levels were analyzed using the SYBR-GREEN reagent kits with gene specific primers on the Applied Biosystems 7000 real time PCR system according to the manufacturer's instruction. Specific fragments were amplified and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified to serve as internal standard, the primer sequences are listed in Supplementary Table 1.

2.7. Western blot analysis

Protein extracts were denatured and the solubilized proteins (20 μg) were subjected to electrophoresis on 10% polyacryl amide SDS gels, This was followed by probing with antibodies (Abcom) for rabbit anti-human-LOX1, scavenger receptor A (SRA), CD36 (diluted 1:1000 in TBST), or rabbit anti-actin (diluted 1:5000 in TBST), and then by goat anti-rabbit secondary antibody labeled with far-red-fluorescent Alexa Fluor 680 dye. All signals were detected by Odyssey (Li-cor, USA). Densitometric analysis was performed using Quantity One (Bio-Rad) to scan the signals.

2.8. Statistical analysis

Data is presented as mean ± S.D. of at least three independent experiments and were compared using the two-tailed paired Student's *t*-test or one-way ANOVA, a difference with a $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Increased expression of miR-29a in oxLDL-treated DCs

Considering that oxLDL is a major pro-inflammatory factor and auto-antigen in the development of atherosclerosis, oxLDL-stimulated human primary peripheral blood DCs were chosen as the cell model for determining the miRNAs associated with atherosclerosis. In this study, miR-29a was found to be aberrantly expressed in oxLDL-stimulated DCs for the first time using TaqMan Real-time PCR. After stimulation with oxLDL, the miR-29a levels in DCs were increased threefold (Supplementary Fig. 1A), suggesting the role of miR-29a in the immuno-inflammatory response to oxLDLs. The miR-29a levels in DCs were then modulated. Transfection of miRNA-29a mimic resulted in an apparent increase in miR-29a levels (Supplementary Fig. 1B) compared with miR-29a inhibitor and miRNA negative transfection.