FEBS Letters xxx (2015) xxx–xxx

journal homepage: www.FEBSLetters.org

The lncRNA MALAT1 protects the endothelium against ox-LDL-induced dysfunction via upregulating the expression of the miR-22-3p target genes CXCR2 and AKT

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ARTICLE INFO

Article history:
Received 12 August 2015
Accepted 23 August 2015
Available online xxxx

Edited by Tamas Dalmay

Keywords:
CXCR2
miRNA-22-3p
MALAT1
Endothelial cells
ox-LDL

ABSTRACT

CXCR2 plays a key role in protecting the integrity of the endothelium. Emerging evidence has demonstrated that the long ncRNAs (lncRNA) Human metastasis associated lung adenocarcinoma transcript 1 (MALAT1) participates in the regulation of the pathophysiological processes. However, whether there is crosstalk between CXCR2 and MALAT1 remains unknown. In this study, we demonstrated that MALAT1 was upregulated in patients with unstable angina. MALAT1 silencing significantly downregulated the expression of the miR-22-3p target gene CXCR2 via reversing the effect of the miR-22-3p, resulting in the aggravation of Oxidized low-density lipoprotein (ox-LDL)-induced endothelial injury; this process was associated with the AKT pathway. Thus, MALAT1 protects the endothelium from ox-LDL-induced endothelial dysfunction partly through competing with miR-22-3p for endogenous RNA.

1. Introduction

Oxidized low-density lipoprotein (ox-LDL) has been widely demonstrated to be involved in the development of atherosclerosis by causing an oxidative chain reaction and inducing endothelial dysfunction [1,2]. After binding to the lectin-like ox-LDL receptor-1 on the endothelial cell surface, ox-LDL and its lipid constituents induce reactive oxygen species (ROS) accumulation and nitric oxide (NO) inhibition [2]. These factors contribute to the destruction of the integrity of the endothelium and cause a series of atherosclerosis-related diseases [3]. Moreover, ox-LDL induces high expression of interleukin-8 in endothelial cells [4–6]; interleukin-8 is the ligand of the chemokine receptor CXCR2. Numerous studies have demonstrated that CXCR2 has the ability to promote cell proliferation, diminish cell apoptosis and enhance angiogenesis [7,8], indicating that CXCR2 plays a key role in protecting the endothelium from ox-LDL-induced injury. However, the regulation of the CXCR2-mediated protective effect on endothelial cells remains elusive.

Genome-sequencing projects have demonstrated that more than ninety percent of the genome is transcribed as non-coding RNAs (ncRNAs). Only 2% of the human genome contains protein-coding genes [9], indicating the need for a redefinition of the concept of ncRNAs. ncRNAs include microRNAs (miRNAs), which consist of 18–24 nucleotides, and long ncRNAs (lncRNAs), which are longer than 200 nucleotides in length. Recently, accumulating evidence has strongly implied that ncRNAs play a key role in regulating pathophysiological processes [10–12]. Human metastasis associated lung adenocarcinoma transcript 1 (MALAT1; also known as NEAT2), is an 8.7 kb lncRNA that maps to chromosome 11q13 and has been demonstrated to be overexpressed in several cancers [13]. A previous study reported that MALAT1 promoted aggressive renal cell carcinoma through Ezh2 [14]. MALAT1 silencing significantly suppresses the proliferation of esophageal squamous cell carcinoma through the cell cycle at G2/M [15]. Furthermore, the high expression levels of conserved MALAT1 are involved in the physiological progress of endothelial cells [16]

Author contributions: Y.T., Y.Z., Y.L. and C.S. designed the experiments. Y.T., X.J., Y.X. and Y.C. performed the experiments. Y.T. and Y.Z. wrote the main manuscript text and prepared the figures.

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http://dx.doi.org/10.1016/j.febslet.2015.08.046
0014-5793/

and are associated with microvascular complications [17]. In this study, we also found a marked increase in the expression of MALAT1 in endothelial cells following 24 h of ox-LDL treatment. However, little is known about the contribution of MALAT1 to endothelial cell dysfunction.

lncRNAs may function as miRNAs "sponges" by interacting through common response elements, resulting in the modulation of the miRNA activity [18]. This crosstalk between lncRNAs and miRNAs can be called the 'competitive endogenous RNA (ceRNA)' network. The ceRNA network is associated with lots of biological processes, and the intervention of the interaction between lncRNAs and miRNAs can be critical for biological development [10,14,15]. Because both the coding RNA CXCR2 and the non-coding RNA MALAT1 have an effect on the integrity of the endothelium, we hypothesed that MALAT1 post-transcriptionally regulated CXCR2 via ceRNA in response to ox-LDL-induced endothelial dysfunction. Recent examples demonstrating that MALAT1"sponges" miRNAs to regulate specific target genes support this hypothesis [14,15]. Here, we evaluated the possibility of crosstalk between CXCR2 and MALAT1 and investigating whether the regulatory mechanism was associated with the ceRNA network.

2. Materials and methods

The experiments were conducted in accordance with the Declaration of Helsinki and this study was approved by the ethics committee of the Xinhua Hospital School of Medicine, Shanghai Jiaotong University.

2.1. Patient population and blood collection

A lot of 23 patients diagnosed with unstable angina (UA) by our Division of Cardiology and 23 healthy subjects were recruited. Their diagnoses were based on a history of chest pain, coronary angiography results and characteristic ECG changes. The baseline characteristics of the two groups were compared. A 10 ml sample of peripheral blood was collected in an EDTA-containing vacutainer tube from each individual for further analysis.

2.2. Cell culture

HUVECs were isolated from fresh human umbilical cords following the methods of a previous study [19]. Briefly, the separated cord was placed in cord buffer containing NaCl (0.14 M), KCl (0.004 M), phosphate buffer (0.001 M) and glucose (0.011 M). We washed the blood out of the cord and added an appropriate amount of 0.2% collagenase type II (Sigma) into the umbilical vein for 15 min at 37 °C. After incubation, the solution containing HUVECs was rinsed into a centrifuge tube. After discarding the buffer, the cells were cultured in DMEM high glucose medium containing 20% fetal bovine serum at 37 °C in a 5% CO_2 incubator. The entire process was performed under aseptic conditions within 3 h. Cells from passages three to five were used for this study.

2.3. Cell transfection

miR-22-3p mimics and inhibitors (Dharmacon, 50 nM) were used to upregulate or downregulate the miR-22-3p expression. Cells were transfected with the CXCR2-siRNA (Ribobio), MALAT1-shRNA (Hanbio), or miR-22-3p mimics, inhibitors or controls (Dharmacon) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Changes in RNA expression were determined by qRT-PCR 24 h after transfection, and changes in protein expression were measured by Western blotting 48 h after transfection.

2.4. RNA preparation, RT and qPCR

Total RNA was extracted using the Trizol reagent (Invitrogen) following the manufacturer's protocol. Next, the Primer-Script™ one step RT-PCR kit (TaKaRa) was used for the reverse transcription of mRNAs and lncRNAs, and the miRcute miRNA cDNA kit (Tiangen) was used for the reverse transcription of miRNAs. The SYBR kit (TaKaRa) was used for detection in the ABI7500 system (Applied Biosystems). β-Actin was used as the endogenous control for mRNAs and lncRNAs, and U6 was used as the endogenous control for miRNAs. The relative expression levels among groups were calculated by the 2^{-DDCt} method. The following primers were used:

CXCR2 F: 5′-CCGTTTTCTCCTTCCTGGGT-3′, and R: 5′-GCTGTGA CCTGCTGTTATTGG-3′; MALAT1: F: 5′-ATGCGAGTTGTTCTCCGTCT-3′, and R: 5′-TATCTGCGGTTTCCTCAAGC-3′; β-actin: F: 5′-AGAGCC TCGCCTTTGCCGAT-3′, and R: 5′-TGCCAGATTTTCTCCATGTCGT-3′; miR-22-3p: 5′-AAGCTGCCAGTTGAAGAACTGT-3′; U6: 5′- CGCTTC GGCAGCACATATACTAAAATTGGAAC-3′.

2.5. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay Kits (Elabscience) were used to detect interleukin-1or interleukin-8 in cell culture supernatants following 24 h of ox-LDL treatment.

2.6. RNA immunoprecipitation assay (RIP)

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) was used according to the manufacturer's protocol to analyze whether MALAT1 was associated with the RISC complex. IgG (Millipore) was used as the negative control and anti-snRNP70 as the positive control (Millipore).

2.7. Western blotting (WB)

Primary antibodies targeting Ago2 (1:1000 dilution; Millipore), CXCR2 (1:1000 dilution; Cell Signaling), p-AKT, AKT (1:1000 dilution; Cell Signaling), and cleaved Casp3 (1:1000 dilution; Cell Signaling) were used. The antibody for β-actin (1:1000 dilution; Beyotime) was used as the endogenous control. The assay was performed as follows. HUVECs were lysed for 1.5 h on ice by a cell lysis solution containing 1% phenylmethanesulfonyl fluoride buffer. Then 12% SDS–PAGE gels (Millipore) were used for equal protein loading (50 μg) electrophoresis. The gels were run under the same experimental conditions.

2.8. Flow cytometric analysis

The apoptosis of HUVECs induced by ox-LDL was assessed using a FITC Annexin V Apoptosis Detection Kit I (BD) following the manufacturer's protocol. In brief, the cells were washed with cold PBS and resuspended at a density of 1×10^6 cells/ml using 1× Binding Buffer. Then, 5 μl of FITC Annexin V and 5 μl propidium iodide were added to the resuspended cells for 15 min at room temperature in the dark. The cells were analyzed with a Beckman Coulter FC500 (Beckman) within 1 h.

2.9. Capillary-like structure formation assay

An in vitro endothelial tube formation assay was performed to study the modulation of angiogenesis by miR-22-3p, CXCR2 or MALAT1 as described previously [20]. Briefly, 10^4 endothelial cells pretreated with miR-22-3p, CXCR2-siRNA or MALAT1-shRNA for 24 h were seeded into a Matrigel-coated 96-well plate. Following 6 h of incubation at 37 °C, pictures were taken using a fluorescent microscope. Each assay was repeated five times.

Please cite this article in press as: Tang, Y., et al. The lncRNA MALAT1 protects the endothelium against ox-LDL-induced dysfunction via upregulating the expression of the miR-22-3p target genes CXCR2 and AKT. FEBS Lett. (2015), http://dx.doi.org/10.1016/j.febslet.2015.08.046