MicroRNA let-7c inhibits Bcl-xl expression and regulates ox-LDL-induced endothelial apoptosis

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INTRODUCTION

Atherosclerosis is the lesion primarily underlies coronary artery disease and cerebrovascular disease, two of the most common causes of illness and death worldwide (1). By virtue of their location between the blood and tissues and the multiple functions, the endothelial cells (ECs) play a major role in securing body homeostasis (2). Under pathological conditions, endothelial dysfunction is a driving force in the initiation and development of atherosclerosis (3). Apoptosis is one of the central mechanisms leading to endothelial dysfunction and results in inflammatory cell infiltration, lipid transport and neointima formation. These alterations in endothelial dysfunction and culminates in atherosclerosis. MicroRNAs (miRNAs) are a class of noncoding RNAs that posttranscriptionally regulate the expression of genes involved in diverse cell functions, including differentiation, growth, proliferation, and apoptosis. MiRNA let-7 family is known to be involved in the regulation of cell apoptosis. However, the function of let-7 in ox-LDL induced ECs apoptosis and atherosclerosis is still unknown. Here, we show that let-7c expression was markedly up-regulated in ox-LDL induced apoptotic human umbilical cord vein endothelial cells (HUVECs). Let-7c over-expression enhanced apoptosis in ECs whereas inhibition of let-7c could partly alleviate apoptotic cell death mediated by ox-LDL. Searching for how let-7c affected apoptosis, we discovered that anti-apoptotic protein Bcl-xl was a direct target of let-7c in ECs. Our data suggest that let-7c contributes to endothelial apoptosis through suppression of Bcl-xl. [BMB Reports 2012; 45(8): 464-469]

RESULTS

Let-7c was up-regulated in apoptotic HUVECs induced by ox-LDL

To analyze the kinetics of let-7c expression in ECs apoptosis induced by ox-LDL, HUVECs were exposed to ox-LDL for different
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Fig. 1. Apoptotic/necrotic effects and expression of let-7c in HUVECs exposed to ox-LDL for 0 h, 12 h, 24 h, and 48 h. (A) Flow cytometric analysis of apoptosis and necrosis. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for AV binding (AV−/PI−). The lower right quadrants represent the apoptotic cells; AV positive and PI negative (AV+/PI−). The upper right quadrants contain the necrotic cells, positive for AV binding and for PI uptake (AV+/PI+). (B) Comparison of the apoptotic and necrotic death rate of HUVECs was analyzed using qRT-PCR. Each bar represents the means ± S.D. (n = 3), *P < 0.05 versus 0 h group, **P < 0.01 versus 0 h group.

Fig. 2. Bcl-xl expression in HUVECs after ox-LDL treatment, let-7c mimics or let-7c inhibitor transfection. (A) Manipulation of let-7c in HUVECs. HUVECs were transfected with let-7c mimics or let-7c inhibitors, and let-7c levels were measured by qRT-PCR. (B) HUVECs were exposed to ox-LDL (50 μg/ml) for 48 h, or transfected with let-7c mimics or miR mimics control, or transfected with let-7c inhibitors or miR inhibitors control and further exposed to ox-LDL (50 μg/ml) for an additional 48 h. Bcl-xl protein was measured by western blot, and the β-actin expression was used for protein level normalization. (C) Let-7c does not affect Bcl-xl mRNA. HUVECs were transfected with let-7c mimics or miR mimics control, or let-7c inhibitors or miR inhibitors control, and Bcl-xl mRNA was measured by qRT-PCR. Bcl-xl mRNA expression was not changed by let-7c mimics and let-7c inhibitors. Values are means ± S.D. (n = 3). *P < 0.05 compared with control group, **P < 0.01 compared with control group.

lengths of time. ECs apoptosis was detected by Annexin V-FITC/propidium iodide (PI) double stain and let-7c expression was quantified by qRT-PCR. As shown in Fig. 1A and 1B, incubation of HUVECs with 50 μg/ml ox-LDL for 0 to 48 h resulted in a time-dependent induction of apoptotic cell death. With the increasing apoptotic rate, let-7c was gradually up-regulated after ox-LDL stimulation, and more than 3-fold increase in the let-7c amount was observed at 48 h (Fig. 1C).

Bcl-xl is one of the target genes for let-7c in HUVECs

Recently, the let-7 family of miRNAs was demonstrated to potentiate apoptosis in human hepatocellular carcinoma by inhibiting Bcl-xl (24). Bcl-xl is one of the anti-apoptotic members of the Bcl-2 family and decreased in ECs apoptosis mediated by various stimuli (25). In order to elucidate the relationship between let-7c and Bcl-xl in HUVECs, we manipulated let-7c level in HUVECs by transfecting with let-7c mimics or let-7c inhibitor. When 50 nM let-7c mimics was transfected into HUVECs, the level of let-7c increased up to 11.5-fold. In contrast, transfection of let-7c inhibitor decreased let-7c level by 64% (Fig. 2A). These data suggest that transfection of let-7c mimics or let-7c inhibitor can significantly alter let-7c level in HUVECs. Ox-LDL treatment decreased Bcl-xl level by 39% compared to control (Fig. 2B left). Furthermore, over-expression of
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Fig. 4. Apoptosis in HUVECs subjected to ox-LDL treatment. let-7c mimics or let-7c inhibitor transfection. HUVECs were exposed to ox-LDL (50 μg/ml) for 48 h, or transfected with let-7c mimics or miR mimics control, or transfected with let-7c inhibitor or miR inhibitor control and further exposed to ox-LDL (50 μg/ml) for an additional 48 h. (A) The activity of Caspase-3 was determined by luminescent substrate assays for Caspase-3. (B) Quantitative measurement of apoptosis and necrosis by FCM after AV/PI double stain. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for AV binding (AV−/PI−). The lower right quadrants represent the apoptotic cells; AV positive and PI negative (AV+/PI−). The upper right quadrants contain the necrotic cells, positive for AV binding and for PI uptake (AV+/PI+). (C) Comparison of the apoptotic and necrotic death rate of HUVECs. Values are means ± S.D. (n = 3), and **P < 0.01 compared with control group.

Fig. 3. Sequence-specific suppression of Bcl-xl gene expression by let-7c. (A) The putative target site of Bcl-xl mRNA 3'UTR determined by computational predictions. The target sequence was cloned into pMIR-REPORT vector (pMIR-Bcl-xl-3'UTR). pMIR-Bcl-xl-3'UTR mutant was also generated with a single mutation (indicated by an italic character) in the target site. (B) HUVECs were transfected with pMIR-Bcl-xl-3'UTR or pMIR-Bcl-xl-3'UTR mutant together with let-7c inhibitor or miR inhibitor control, and luciferase activity was measured and normalized to β-galactosidase expression levels. Let-7c inhibitor elevated expression of luciferase containing a wild-type let-7c binding site (left) but not a mutant binding site (right). Values are means ± S.D. (n = 3), and **P < 0.01 compared with the miR inhibitor control group.

Let-7c modulates apoptosis in HUVECs

To investigate the role of let-7c in ECs apoptosis, we transfected HUVECs with let-7c miRNAs and then subjected them to ox-LDL treatment. Caspase-3 activity assay and flow cytometry (FCM)
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were used to analyze changes in apoptosis. As a result, ox-LDL stimulation increased Caspase-3 activity by nearly 6.5 fold in HUVECs (Fig. 4A left), whereas the let-7c inhibitor transfection significantly decreased the Caspase-3 activity (Fig. 4A right). Moreover, the activation of Caspase-3 was more intense in let-7c mimics-transfected HUVECs than in miRNA mimics control-transfected HUVECs (Fig. 4A middle). Double stain of Annexin V-FITC/PI showed apoptotic rate was up-regulated in the presence of ox-LDL (Fig. 4B and 4C left). But this effect was attenuated when introducing let-7c inhibitor into ox-LDL-stimulated HUVECs (Fig. 4B and 4C right). On the other hand, let-7c mimics transfection increased apoptosis of HUVECs by nearly two-fold, compared to miRNA mimics control (Fig. 4B and 4C middle). Together, the let-7c over-expression and inhibition data suggest that let-7c contributes to apoptotic effect of ox-LDL in HUVECs.

**DISCUSSION**

Accumulating evidence suggests that some specific miRNAs play an important role in vascular diseases through regulating ECs behaviors. MiR-222 and miR-17-92 cluster have been identified as pro-angiogenic miRNAs (26, 27). In contrast, miR-221 and miR-222 inhibited ECs migration, proliferation, and angiogenesis (17, 28). The contribution of miR-155, miR-31, and miR-126 to vascular inflammation and diseases has also been documented (29). Thus, the identification of miRNAs and their respective targets may offer new therapeutic strategies to treat vascular diseases including atherosclerosis. In the present study, we found that let-7c increasing during ox-LDL induced ECs apoptosis regulated levels of anti-apoptotic protein Bcl-xL.

Endothelial apoptosis induced by ox-LDL is an initiating stage of atherosclerosis. We previously utilized a miRNA microarray to analyze the miRNA expression profile in ox-LDL-induced apoptotic HUVECs. We found that 4 miRNAs (miR-142-3p, miR-1207-3p, miR-365 and let-7c) were up-regulated after ox-LDL treatment, and inhibition of miR-365 could partly alleviate apoptotic cell death induced by ox-LDL via regulating the expression of Bcl-2 (23). The preliminary results gave an important indication of miRNAs associated with atherosclerosis, and changes in ECs expression induced by miR-365 were just one part of the mechanism responsible for ox-LDL-induced changes in ECs apoptosis. So we continue to assess other miRNAs involved in modulating ECs apoptosis. Among these up-regulated miRNAs, let-7c is known to be involved in the regulation of apoptosis (30). The ubiquitously expressed let-7 family was one of the first mammalian miRNAs to be identified. The let-7 family is comprised of 12 family members (let-7a1, a2, a3, b, c, d, e, f1, f2, g, i and miR-98) located on 8 different chromosomes (31). In studies of various human cancers including lung, colon, ovarian, gastric cancer, leiomyoma and melanoma, let-7 family members have been described to be down-regulated and exert pro-apoptotic effect during cancer progression. Further mechanism researches reveal that let-7 targets include cell cycle regulators such as CDC25A and CDK6, promoters of growth including RAS and c-myc and a number of early embryonic genes including HMGA2, Mlin-41 and IMP-1 (32). Shimizu et al. very recently showed that let-7 miRNAs down-regulated in human hepatoma cells and tissues directly target anti-apoptotic protein Bcl-xl and enhance apoptosis in cooperation with an anti-cancer drug targeting Mcl-1 in hepatocellular carcinoma (24). In this study, we also investigated the expression of let-7c in ox-LDL-stimulated HUVECs. Consistent with the microarray result, our qRT-PCR assay revealed that let-7c expression was significantly up-regulated during HUVECs apoptosis, indicating that let-7c acts as a pro-apoptotic miRNA in this process.

The Bcl-2 protein family was discovered by analysis of the t(14;18) chromosome translocation breakpoint in B-cell follicular lymphoma. Bcl-xl, one of the anti-apoptotic members of the Bcl-2 family, is critically involved in the mitochondrial pathway of apoptosis (33). Bcl-xl blocks diverse apoptotic stimuli via inhibiting cytochrome-c release, maintaining mitochondrial membrane potential, inhibiting the production of reactive oxygen species, heterodimerizing with pro-apoptotic molecules and attenuating pro-apoptotic Bcl-2 (34). Previous studies demonstrated that Bcl-xl was down-regulated in ECs apoptosis induced by Ang II (11), endo-statin (35), hypoxia (36) and hypoxia/reoxygenation (25) . To the contrary, over-expression of Bcl-xl was able to protect ECs from apoptosis mediated by DNA damaging agent and pro-inflammatory cytokines (37). Identifying the factors that regulate Bcl-xl levels is of paramount importance for better maintaining ECs viability and developing more effective therapies for atherosclerosis. In this study, we found let-7c over-expression induced the down-modulation of Bcl-xl protein but not of Bcl-xl mRNA, and let-7c blocking by let-7c inhibitor prevented Bcl-xl down-modulation induced by ox-LDL. Further luciferase reporter assay analysis demonstrated the Bcl-xl gene is one of the direct targets of let-7c in ECs. As Bcl-xl is closely related to cell apoptosis, we therefore explore the effect of let-7c on ox-LDL-mediated ECs apoptosis. As a result, let-7c over-expression enhanced ECs apoptosis whereas inhibition of let-7c could partly alleviate apoptotic cell death induced by ox-LDL, indicating let-7c promotes apoptosis in ox-LDL-treated HUVECs through attenuating Bcl-xl expression. These results suggest that let-7c high expression in ox-LDL-induced endothelial apoptosis may become a new biomarker in atherosclerosis. However, we must consider the discrepancy between in vitro and in vivo performance of let-7c. Multiple parameters, such as changes in expression in tissue, secretion of miRNA by cells, stability of miRNA molecule and interaction with other miRNAs may influence let-7c behavior in vivo. So, studies utilizing animal models of atherosclerosis and human atherosclerotic plaque samples are required to elucidate the in vivo effect of let-7c on endothelial apoptosis and atherosclerosis. Moreover, we only tested let-7c expression and its effect on apoptosis in HUVECs. We should further test other cell-lines which are not related with atherosclerotic process to know whether let-7c activation is specific in atherosclerosis.

In conclusion, we found the miRNA let-7c negatively regulates anti-apoptotic gene Bcl-xl expression in HUVECs. Transfection of let-7c inhibitor partially protects HUVECs from ox-LDL-induced
apoptotic death. We believe that once the functional roles of miRNAs have been further clarified, they will become suitable therapeutic targets in endothelial apoptosis and atherosclerosis.

MATERIALS AND METHODS

Cell culture
HUVECs were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and 100 μg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA).

Ox-LDL preparation
Human ox-LDL was isolated from fresh human plasma by sequential ultracentrifugation, as described previously (40).

FCM analysis of apoptosis
HUVECs were cultured in 6-well plates and exposed to ox-LDL (50 μg/ml) for 0, 12, 24, and 48 h. Cells were harvested and stained with the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Jiangsu, China). The number of cells undergoing apoptosis was determined by FCM.

MiRNA quantitative real-time PCR (qRT-PCR)
To quantify the expression of let-7c, we synthesized cDNA from 10 ng of RNA sample using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Quantitative PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems, CA, USA) specific for let-7c in a 7900HT RT-PCR machine (Applied Biosystems, CA, USA) according to the manufacturer’s protocol.

Transfections with miRNAs
The let-7c mimics and let-7c inhibitor (Ribo Bio, Guangzhou, China) were transfected into HUVECs at a final concentration of 50 nM using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for an additional 48 h. After that, protein extracts were prepared and Caspase-3 activity was measured using Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) according to the manufacturer’s instructions. After transfection and ox-LDL treatment, HUVECs were collected and a quantitative measurement of apoptosis was determined by FCM as described previously.

Statistical analysis
Data are presented as mean ± S.D. and evaluated by analysis of variance (ANOVA) or student t-test when appropriate. Significance was established at a level of P < 0.05.

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REFERENCES


