Original Paper

Upregulation of microRNA-876 Induces Endothelial Cell Apoptosis by Suppressing Bcl-XI in Development of Atherosclerosis

Kaicheng Xu^a Peng Liu^b Yue Zhao^c^aDepartment of Anesthesiology, ^bDepartment of Orthopaedics, ^cDepartment of Vascular Surgery, China-Japan Union Hospital, Jilin University, Changchun, China**Key Words**

Atherosclerosis • Endothelial cell apoptosis • ApoE (-/-) • High fat diet (HFD) • ox-LDL • Bcl-XI • miR-876

Abstract

Background/Aims: The injury and apoptotic cell death of endothelial cells hallmark the development of atherosclerosis (AS), characterized by dysregulation of lipid homeostasis, immune responses, and formation of coronary plaques. However, the mechanisms underlying the initiation of endothelial cell apoptosis remain ill-defined. Recent evidence suggests a role of microRNAs in the processes of AS-associated endothelial cell apoptosis. Thus, we studied this question in the current study. **Methods:** AS was developed in ApoE (-/-) mice supplied with high-fat diet (HFD), compared to ApoE (-/-) mice supplied with normal diet (ND). Mouse endothelial cells were isolated from the aortic arch using flow cytometry based on their expression of Pecam-1. Oxidized low-density lipoprotein (ox-LDL) were used to treat human aortic endothelial cells (HAECs) as an *in vitro* model for AS. Gene expression was quantified by RT-qPCR and protein levels were analyzed by Western blotting. Apoptosis was evaluated by FITC Annexin V Apoptosis assay and by TUNEL staining. Prediction of the binding between miRNAs and 3'-UTR of mRNA from the target gene was performed by bioinformatics analyses and confirmed by a dual luciferase reporter assay. **Results:** HFD mice, but not ND mice, developed AS in 12 weeks. Significantly reduced endothelial cell marks and significantly increased mesenchymal cell marks were detected in the aortic arch of the HFD mice, compared to the ND mice. The endothelial cell apoptosis was significantly higher in HFD mice, seemingly due to functional suppression of protein translation of anti-apoptotic Bcl-XI protein through upregulation of miR-876. Similar results were obtained from *in vitro* study. Inhibition of miR-876 abolished the effects of ox-LDL-induced apoptotic cell death of HAECs. **Conclusion:** AS-associated endothelial cell apoptosis may partially result from downregulation of Bcl-XI, through upregulation of miR-876 that binds and suppresses translation of Bcl-XI mRNA.

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Yue Zhao

Department of Vascular Surgery, China-Japan Union Hospital, Jilin University, 126 Xiantai street, Changchun 130033 (China)
Tel. +8643184995222, E-Mail zylpxkc@126.com

Introduction

Atherosclerosis (AS) is a leading health issue among aged people worldwide [1-3]. Endothelial cell injury and apoptosis play an essential role in the development and pathogenesis of AS [3-9]. However, the molecular mechanisms underlying endothelial cell apoptosis are poorly understood.

Apolipoprotein E (ApoE) is well-known to be a potent suppressor for AS [10-12]. ApoE-deficient (ApoE $-/-$) mice display enhanced chronic inflammatory responses to diet-induced hypercholesterolemia [10, 11]. Therefore, 12-weeks of high fat diet (HFD) on (ApoE $-/-$) mice have been used to develop AS model for years [13-15].

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally control protein translation through pairing with the 3'-untranslated region (3'-UTR) of the mRNA from their target genes. Recently, miRNAs have been found to regulate the development of AS via a variety of target factors or pathways [16]. Specifically, systemic delivery of miR-181b has been shown to attenuate AS through modulation of NF- κ B signaling in endothelial cells [17]. Moreover, inhibition of miR-92a was found to prevent endothelial dysfunction and development of AS in mice [18]. In addition, MiR-126-5p was defined as a mitotic factor for endothelial cell growth and overexpression of miR-126-5p inhibited AS by suppressing Dlk1 [19]. Furthermore, miR-26a was revealed to be an anti-apoptotic miRNA in which it prevents endothelial cell apoptosis by targeting TRPC6 in the setting of AS [20]. These studies encouraged us to investigate role of miRNAs in the AS-associated apoptotic death of endothelial cells in the current study.

Here, AS was developed in ApoE $-/-$ mice supplied with HFD, compared to ApoE $-/-$ mice supplied with normal diet (ND). We showed evidence that suggests that AS-associated endothelial cell apoptosis may partially result from downregulation of Bcl-Xl, through upregulation of miR-876 that binds and suppresses translation of Bcl-Xl mRNA.

Materials and Methods

Ethics statement

The study was approved by the Animal Care and Use Committee of Jilin University. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health. All experiments were conducted under the supervision of the facility's Institutional Animal Care and Use Committee according to an Institutional Animal Care and Use Committee-approved protocol.

Animal models

ApoE $-/-$ mice of 8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME, USA) or bred in-house and maintained under sterile conditions. The animals were randomly divided into two groups: the normal-diet group (ND) and the high-fat diet (HFD) group. All the animals were maintained for 12 weeks before analysis.

Quantification of atherosclerotic lesions

Mouse aortic arch was excised and fixed with 4% paraformaldehyde for 6 hours, cryo-protected in 30% sucrose for 24 hours, OCT-embedded, and sectioned at 6 μ m thickness. AS lesions were examined by H&E staining and Oil red O staining (Oil red O staining kit, Abcam, Cambridge, MA, USA). The data were calculated from 10 mice from each group.

Cell culture and transfection

Human aortic endothelial cells (HAECs, American Type Culture Collection, Rockville, MD, USA) were cultured in Endothelial Cell Medium supplied with endothelial cell growth factors, 5% fetal bovine serum (FBS, Invitrogen, CA, Carlsbad, USA) and 1% penicillin/streptomycin (Invitrogen) at 37°C with 5% CO₂. HAECs were transiently transfected with miR-876 mimics, antisense for miR-876 (as-miR-876) or null

controls (RiboBio Co., Ltd., Guangzhou, Guangdong, China), using Lipofectamine 2000 reagent (Invitrogen). The transfection efficiency was nearly 100%.

Ox-LDL treatment of the cells

One day after cell transfection, the HAECs were treated with or without 100µg/ml oxidized low-density lipoprotein (ox-LDL, Beijing Xiesheng Bio-Technology Limited, Beijing, China), after which the cells underwent flow cytometry or protein/RNA extraction.

Cell viability by cell counting kit-8 (CCK-8) assay

The CCK-8 detection kit (Sigma-Aldrich, St. Louis, MO, USA) was used to measure cell viability according to the manufacturer's instructions.

Apoptosis assay and flow cytometry

The dissociated tissue or cultured cells were re-suspended at a density of 10^6 cells/ml in PBS. After double staining with FITC-Annexin V and propidium iodide (PI) from a FITC Annexin V Apoptosis Detection Kit I (Becton-Dickinson Biosciences, San Jose, CA, USA), cells were analyzed using FACScan flow cytometer (Becton-Dickinson Biosciences) for determination of Annexin V+ PI- apoptotic cells. For analyses and isolation of Pecam-1+ cells, the dissociated tissue cells were incubated with PE-cy7-Pecam-1 (Becton-Dickinson Biosciences).

TUNEL staining

TUNEL staining was performed using a TUNEL fluorescence FITC kit (Roche, Indianapolis, IN, USA). HAECs were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Then, cells were incubated with TUNEL reaction mixture at 37°C for 1 h, after which the stained tissues and cells were examined.

RT-qPCR

Total RNA was isolated from mouse aortic arch or sorted cells or cultured cells with miRNeasy mini kit (Qiagen, Hilden, Germany). The extracted RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The first-strand cDNA was used for real-time PCR to quantify mRNA expressions using QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using $2^{-\Delta\Delta Ct}$ method for quantification of the relative mRNA expression levels. Values of genes were first normalized against GAPDH, and then compared to the experimental controls.

Western blotting

The cells were lysed with RIPA buffer containing protease and phosphatase inhibitors (cOmplete ULTRA Tablets, Roche, Nutley, NJ, USA). After centrifugation, the supernatant was collected and quantified. The proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk, the membranes were probed with rat-anti-Pecam-1 (Becton-Dickinson Biosciences, San Jose, CA, USA), rabbit-anti-alpha smooth muscle actin (α -SMA), rabbit anti-Vimentin, rabbit-anti-Bcl-XI, rabbit-anti-Bcl-2, rabbit-anti-BAD, rabbit-anti-BAX rabbit-anti-BID and rabbit-anti-GAPDH (all from Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies are HRP-conjugated against rat or rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). The protein levels were first normalized to GAPDH, and then normalized to the experimental controls. Densitometry of Western blots was quantified with NIH ImageJ software.

MiRNA target prediction and 3'-UTR luciferase-reporter assay

MiRNAs targets were predicted using the algorithms TargetSan. The Bcl-XI 3'-UTR wild-type (wt) reporter and mutate (mut) reporter plasmids were purchased from Creative Biogene (Shirley, NY, USA). Co-transfection of HAECs with miR-876-modification plasmids and Bcl-XI 3'-UTR reporter plasmids were performed using Lipofectamine 2000. Cells were collected 48 hours after transfection for assay using the dual-luciferase reporter assay system gene assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Statistical analyses

The data in this study are shown as the mean $\pm$ S.D. Differences among groups were analyzed using one-away ANOVA with a Bonferroni correction, followed by Fisher's Exact Test for comparison of two groups (GraphPad Prism, GraphPad Software, Inc. La Jolla, CA, USA). $p < 0.05$ was considered significant.

Results

AS is developed in HFD mice

ApoE (-/-) mice were treated with either HFD or ND for 12 weeks, after which the AS lesions were analyzed. H&E-stained aortic sinus displayed a significant increase in aortic lesion size in HFD mice, compared to ND mice (Fig. 1A-B). Moreover, Oil-red-O-stained aortic sinus displayed a significant increase in lipid content (Fig. 1C-D). These data suggest that HFD successfully induced AS in ApoE (-/-) mice, confirming the establishment of the model. The aortic arch was then isolated for analyzing the levels of endothelial marker Pecam-1 and the mesenchymal markers α -SMA and Vimentin. We detected significantly lower levels of Pecam-1 by mRNA (Fig. 1E), and by Western blot (Fig. 1F) in aortic arch from HFD mice, compared to ND mice. Moreover, we detected significantly higher levels of α -SMA and Vimentin by mRNA (Fig. 1E), and by Western blot (Fig. 1F). Together, these data suggest that HFD induces AS in ApoE (-/-) mice.

Increased endothelial cell apoptosis occurs in HFD mice

To specifically study endothelial cells in aortic arch, we isolated Pecam-1+ cells from the dissociated mouse aortic arch by flow cytometry (Fig. 2A). We found that the percentage

Fig. 1. AS is developed in HFD mice. ApoE (-/-) mice were treated with high-fat diet (HFD) or normal diet (ND) as a control. (A-C) After a 12 weeks, H&E-staining on aortic sinus showed a significant increase in aortic lesion size, shown by representative images (A), and by quantification (B). (C-D) Representative images (C) and quantification (D) of lipid content by Oil-red-O-staining on aortic sinus. (E-F) The aortic arch was isolated for analyzing the levels of Pecam-1, α -SMA and Vimentin by RT-qPCR (E), and by Western blotting (F). The numbers near blotting were the ratio of HFD/ND after normalization with GAPDH. * $p < 0.05$. N=10. Scale bars are 50 μ m.

Fig. 2. Increased endothelial cell apoptosis occurs in HFD mice. (A-B) Pecam-1+ cells were isolated from the dissociated mouse aortic arch by flow cytometry, shown by representative flow charts (A), and by quantification (B). (C-D) The apoptosis of isolated Pecam-1+ cells were analyzed by FITC Annexin V Apoptosis assay, shown by representative flow charts (C), and by quantification (D). (E) Western blotting for apoptosis-associated proteins in purified endothelial cells. (F) RT-qPCR for Bcl-XI in purified endothelial cells. * $p < 0.05$. $N = 10$.

Fig. 3. HFD-induced upregulation of miR-876 suppresses protein translation of Bcl-XI. (A) RT-qPCR for miR-876 in Pecam-1+ endothelial cells from HFD or ND mice. (B) Bioinformatics analyses showing the binding site of miR-876 on the wildtype (wt) of 3'-UTR of Bcl-XI mRNA as well as the sequence of the mutate (mut) 3'-UTR of Bcl-XI mRNA. (C) RT-qPCR levels for miR-876 in miR-876-modified human aortic endothelial cells (HAECs), prepared by transfection of the cells with miR-876 mimics, antisense for miR-876 (as-miR-876) or null controls (null). (D) Luciferase activity of the wt 3'-UTR of Bcl-XI mRNA by co-transfection of miR-876-modified plasmids on HAECs. (E) Luciferase activity of the mut 3'-UTR of Bcl-XI mRNA by co-transfection of miR-876-modified plasmids on HAECs. * $p < 0.05$. NS: non-significant. $N = 5$.

of Pecam-1+ endothelial cells in the aortic arch from HFD mice was significantly lower than those in ND mice (Fig. 2B), suggesting that the low Pecam-1 levels in aorta in HFD mice may result from the reduced endothelial cells. In order to understand the mechanism, the isolated Pecam-1+ cells were further subjected to FITC Annexin V Apoptosis assay. We

Fig. 4. Ox-LDL-treated HAECs recapitulate the findings in HFD mice. HAECs were treated with or without 100 μ g/ml ox-LDL to induce cell apoptosis. (A-B) Analyses of cell apoptosis by FITC Annexin V Apoptosis assay, shown by representative flow charts (A), and by quantification (B). (C-D) Analyses of cell apoptosis by TUNEL assay, shown by representative images (C), and by quantification (D). (E) Quantification of viable cell number in an CCK-8 assay. (F) Western blotting for Bcl-Xl. (G) RT-qPCR for miR-876. * p <0.05. N=5. Scale bars are 50 μ m.

detected significantly higher percentage of apoptotic Pecam-1+ endothelial cells in HFD mice, compared to ND mice, shown by representative flow charts (Fig. 2C), and by quantification (Fig. 2D). Thus, HFD induces endothelial cell apoptosis in ApoE (-/-) mice. To figure out the apoptotic factors that may be involved, we examined the major candidates BAD, Bax, Bid, Bcl-Xl and Bcl-2. Although pro-apoptotic proteins were not significantly altered, we detected significantly lower levels of anti-apoptotic proteins Bcl-Xl and Bcl-2 in endothelial cells from HFD mice, compared to ND mice. Moreover, the reduction of Bcl-Xl levels was more pronounced than Bcl-2 (Fig. 2E). Thus, we focused on Bcl-Xl in the current study. To our surprise, the mRNA levels, unlike proteins levels of Bcl-Xl, were not significantly altered in endothelial cells from HFD mice, compared to ND mice (Fig. 2F), suggesting the alteration of Bcl-Xl protein levels may be due to post-transcriptional regulation, e.g. by miRNAs.

HFD-induced upregulation of miR-876 suppresses protein translation of Bcl-Xl

Next, we examined the underlying mechanisms. All Bcl-Xl-targeting miRNAs were then determined by bioinformatics analyses, from which we found that miR-876 levels significantly increased in Pecam-1+ endothelial cells in HFD mice (Fig. 3A). MiR-876 targets 3'-UTR of Bcl-Xl mRNA at one binding site of base pair 85th to 91th (Fig. 3B). MiR-876 mimics, antisense for miR-876 (as-miR-876) and null controls (null) were then prepared to transfect human aortic endothelial cells (HAECs) to confirm the potential of modification of miR-876 levels by these plasmids (Fig. 3C). Next, luciferase reporters containing the 3'-UTR of Bcl-Xl or a mutated 3'-UTR of Bcl-Xl in the miR-876 binding site were constructed and transfected into HAECs (Fig. 3B). We found that miR-876 markedly inhibited the luciferase activity of the vector containing the wild-type binding site, whereas the as-miR-876 increased luciferase activity (Fig. 3D). Moreover, transfection of miR-876 or as-miR-876 did not affect the luciferase activity of the reporter carrying the mutated miR-876 binding site (Fig. 3E). Together, these

Fig. 5. Depletion of miR-876 reduces ox-LDL-induced apoptotic death of HAECs. HAECs were first transfected with null or as-miR-876 plasmids, and then treated with or without 100 µg/ml ox-LDL to induce cell apoptosis. (A-B) Analyses of cell apoptosis by FITC Annexin V Apoptosis assay, shown by representative flow charts (A), and by quantification (B). (C-D) Analyses of cell apoptosis by TUNEL assay, shown by representative images (C), and by quantification (D). (E) Quantification of viable cell number in an CCK-8 assay. (F) Western blotting for Bcl-XI. * $p < 0.05$. $N = 5$. Scale bars are 50 µm.

data suggest that HFD-induced upregulation of miR-876 may suppress protein translation of Bcl-XI.

Ox-LDL-treated HAECs upregulate miR-876 and increase apoptosis

HAECs were treated with 100 µg/ml oxidized low-density lipoprotein (ox-LDL) as an *in vitro* model to study endothelial cells in the setting of AS. First, we confirmed that ox-LDL significantly increased cell apoptosis, by FITC Annexin V Apoptosis assay (Fig. 4A-B), and by TUNEL assay (Fig. 4C-D). Moreover, endothelial cell apoptosis resulted in a significant reduction in viable cell number in an CCK-8 assay (Fig. 4E). Furthermore, ox-LDL significantly decreased Bcl-XI protein (Fig. 4F), and significantly increased miR-876 levels (Fig. 4G).

Depletion of miR-876 reduces ox-LDL-induced apoptotic death of HAECs

Finally, we examined whether depletion of miR-876 may inhibit or attenuate the apoptotic cell death of HAECs by ox-LDL. HAECs were thus transfected with null or as-miR-876, and then exposed to ox-LDL. We found that depletion of miR-876 significantly decreased ox-LDL-induced cell apoptosis, by FITC Annexin V Apoptosis assay (Fig. 5A-B), and by TUNEL assay (Fig. 5C-D), resulted in increases in viable endothelial cell number by ox-LDL treatment in an CCK-8 assay (Fig. 5E), seemingly through increases in Bcl-XI protein (Fig. 5F). Thus, depletion of miR-876 reduces ox-LDL-induced apoptotic death of HAECs.

Discussion

During vascular homeostasis, the endothelium contribute to formation of vasodilation, suppression of smooth muscle cell outgrowth, and inhibition of aberrant inflammatory

responses [4-8]. Many evidence has highlighted endothelial dysfunction as an early characteristic for AS, since presence of endothelial cell dysfunction and injury before structural alterations to the vessel wall [4-8]. On the other hand, many risk factors predisposed to AS have been demonstrated to induce endothelial dysfunction, including upregulation of nitric oxide production or activity and reduction of oxidative stress [4-8].

Mechanistically, interactions among endothelial cells and other cell types like T lymphocytes, macrophages and smooth muscle cells coordinates the pathogenesis of AS. It is likelihood that pro-atherosclerotic factors are produced and secreted to induce the apoptosis of endothelial cells [4-8]. However, the detailed molecular signaling remains ill-defined.

Aberrantly expressed miRNAs have recently been reported to play a role in the regulation of endothelial cell survival/death in the setting of AS. For example, miR-26a was revealed to be an anti-apoptotic miRNA in which it prevents endothelial cell apoptosis in the development of AS [20]. In this study, the authors figured out that miR-26a is a novel anti-apoptotic miRNA, directly targets TRPC6 in vascular endothelial cells, but is dysregulated in AS. In a pathway involved miR-26a, TRPC6, intracellular calcium, cytochrome c and caspase 3, AS-related endothelial cell apoptosis is regulated [20]. Moreover, AS-associated endothelial cell apoptosis has also been attributed to the down-regulation of Bcl-2, through increased miR-429 that binds and suppresses translation of Bcl-2 mRNA [9]. However, targets other than TRPC6 and Bcl-2 that are associated with AS-related endothelial cell apoptosis have not been reported.

Here, we found that ApoE (-/-) mice treated with 12 weeks of HFD developed AS, while the control ApoE (-/-) mice fed with ND did not, demonstrated by the features of that the endothelial cells started to express mesenchymal markers to undergo epithelial-mesenchymal transition [21]. When we analyzed major apoptosis-associated proteins by Western blot in Pecam-1+ endothelial cells from those mice, we found that Bcl-2 and Bcl-Xl, 2 key anti-apoptosis proteins, were suppressed in Pecam-1+ endothelial cells from HFD mice. The data for Bcl-2 are consistent with a previous report [9]. However, we found that the decreases in Bcl-Xl were more pronounced, which has not been shown before.

Since the mRNA of Bcl-Xl appeared not changed, we proposed that Bcl-Xl might be regulated by a miRNA. All Bcl-Xl-targeting miRNAs were then determined by bioinformatics analyses, and we screened all these miRNAs and found that miR-876 levels significantly increased in Pecam-1+ endothelial cells from HFD mice. We thus performed *in vitro* analysis on the role of miR-876 in an experimental model for AS, and confirmed this regulation loop in the control of AS-associated endothelial cell apoptosis by a loss-of-function approach. MiR-876 is a miRNA, the function of which is rarely understood. First of all, haploinsufficiency of the miR-876 appears to be associated with craniofacial abnormalities [22]. Moreover, the elevated expression of circulating miR-876 has been shown as a specific response to severe EV71 infections [23]. Our study should provide additional information to understand the biological function of miR-876.

The current study was based on mouse model for AS. Despite many advances in animal models for AS, we still do not have specific animal models for specific human conditions [24]. Since each animal model has its own advantages and disadvantages, future work may address to extrapolate the current findings into clinical studies.

To summarize, our study identified a new role for miR-876 in the regulation of endothelial cell apoptosis during AS, and sheds new insight into development of innovative AS therapy.

Disclosure Statement

The authors have declared that no competing interests exist.

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