Atherosclerosis-Associated Endothelial Cell Apoptosis by MiR-429-Mediated Down Regulation of Bcl-2

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Key Words
Atherosclerosis • Endothelial cell apoptosis • ApoE (-/-)• High fat diet (HFD) • Ox-LDL • Bcl-2 • MiR-429

Abstract

Background/Aims: Endothelial cell injury and subsequent apoptosis play a key role in the development and pathogenesis of atherosclerosis, which is hallmarked by dysregulated lipid homeostasis, aberrant immunity and inflammation, and plaque-instability-associated coronary occlusion. Nevertheless, our understanding of the mechanisms underlying endothelial cell apoptosis is still limited. MicroRNA-429 (miR-29) is a known cancer suppressor that promotes cancer cell apoptosis. However, it is unknown whether miR-429 may be involved in the development of atherosclerosis through similar mechanisms. We addressed these questions in the current study.

Methods: We examined the levels of endothelial cell apoptosis in ApoE (-/-) mice suppled with high-fat diet (HFD), a mouse model for atherosclerosis (simplified as HFD mice). We analyzed the levels of anti-apoptotic protein Bcl-2 and the levels of miR-429 in the purified CD31+ endothelial cells from mouse aorta. Prediction of the binding between miR-429 and 3'-UTR of Bcl-2 mRNA was performed by bioinformatics analyses and confirmed by a dual luciferase reporter assay. The effects of miR-429 were further analyzed in an in vitro model using oxidized low-density lipoprotein (ox-LDL)-treated human aortic endothelial cells (HAECs).

Results: HFD mice developed atherosclerosis in 12 weeks, while the control ApoE (-/-) mice that had received normal diet (simplified as NOR mice) did not. HFD mice had significantly lower percentage of endothelial cells and significantly higher percentage of mesenchymal cells in the aorta than NOR mice. Significantly higher levels of endothelial cell apoptosis were detected in HFD mice, resulting from decreases in Bcl-2 protein, but not mRNA. The decreases in Bcl-2 in endothelial cells were due to increased levels of miR-429, which suppressed the translation of Bcl-2 mRNA via 3'-UTR binding. These in vivo findings were reproduced in vitro on ox-LDL-treated HAECs.

Conclusion: Atherosclerosis-associated endothelial cell apoptosis may result from down regulation of Bcl-2, through increased miR-429 that binds and suppresses translation of Bcl-2 mRNA.
Introduction

Atherosclerosis is one of the leading causes of morbidity and mortality in aged people worldwide. Atherosclerosis is characterized with features including hyperlipidemia, macrophage accumulation, formation of foam cells, and development of inflammation. Apolipoprotein E (ApoE) is a 34-kDa secreted protein, which is well defined as a potent suppressor for atherosclerosis [1, 2]. Beyond its pivotal role in lipoprotein cholesterol transport and in cellular lipid regulation, ApoE is a well-known negative regulator for inflammatory [1, 2]. ApoE-deficient (ApoE −/−) mice display enhanced chronic inflammation in response to spontaneous and diet-induced hypercholesterolemia [1, 2]. Moreover, these mice show an enhanced acute immune response when challenged with bacterial lipopolysaccharide (LPS) [1-7]. High fat diet (HFD) on (ApoE −/−) mice is a widely applied atherosclerosis model, in which mice develop proven atherosclerosis in 12 weeks [8-10].

Endothelial cell injury apoptosis plays a key role in the development and pathogenesis of atherosclerosis, in which the endothelia cells fail to regulate proper lipid homeostasis, immunity and inflammation [11-15]. Moreover, endothelial cell injury and apoptosis can break the integrity of endothelium, facilitate the local lipid deposition into atherogenesis [11-15]. Furthermore, endothelial cell apoptosis may cause plaque instability, resulting in acute coronary occlusion and sudden death [11-15]. Nevertheless, our understanding of the mechanisms underlying endothelial cell apoptosis is still limited.

MicroRNAs (miRNAs) are 18-22-nucleotide non-coding RNAs that negatively regulate human genes during physiological and pathophysiological processes, basically through their base-pairing with the 3′-untranslated region (3′-UTR) of target mRNAs [16-22]. Accumulating evidence has implicated miRNAs as essential regulators of atherosclerosis by targeting key factors in the regulatory pathways [3, 23, 24]. Specifically, miR-429 has been reported as a tumor suppressor in various cancers [25-33]. Moreover, the role of miR-429 in carcinogenesis has been associated with apoptosis augmentation [34, 35]. However, it is unknown whether miR-429 may be involved in the development of atherosclerosis through similar mechanisms.

Here, we found that ApoE (-/-) mice treated with HFD (simplified as HFD mice) developed atherosclerosis in 12 weeks, while the control ApoE (-/-) mice that had received normal diet (simplified as NOR mice) did not. HFD mice had significantly lower percentage of endothelial cells and significantly higher percentage of mesenchymal cells in the aorta than NOR mice. Significantly higher levels of endothelial cell apoptosis were detected in HFD mice, resulting from decreases in Bcl-2 protein, but not mRNA. The decreases in Bcl-2 in endothelial cells were due to increased levels of miR-429, which suppressed the translation of Bcl-2 mRNA via 3′-UTR binding. These in vivo findings were recapitulated in vitro on ox-LDL-treated HAECS.

Materials and Methods

Ethics statement

The study was approved by the Animal Care and Use Committee of Chinese PLA General Hospital. 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 and quantification of atherosclerotic lesions

Eight-week-old male ApoE−/− mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) or bred in-house and maintained under sterile conditions and standard animal room conditions (temperature, 21 ± 1°C; humidity, 55 – 60%). The animals were randomly divided into two groups: the normal-diet group (NOR) and the high-fat diet (HFD) group. The animals of the HFD group were maintained for 12 weeks.
to induce atherosclerosis, after which the aortas were excised from the mice. The aortic roots along with the basal portion of the heart were fixed with 4% paraformaldehyde for 4 hours, cryo-protected in 30% sucrose for 12 hours, and then embedded in OCT compound. The tissue was cross-sectioned into sections of 6 μm thickness. Atherosclerotic lesions of the aortic root were examined by H&E staining. Oil red O staining was performed according to the manufacturer’s instructions to show the lipid deposition with an Oil red O staining kit (Abcam, Cambridge, MA, USA). Quantification of the images was measured using NIH ImageJ software (Bethesda, MD, USA). The data were calculated from 5 mice for each group. For each mouse, 3 slides that were 20 μm apart from each other were used for quantification.

**Cell culture, treatment and transfection**

Human aortic endothelial cells (HAECs) were purchased from American Type Culture Collection (ATCC PCS-100-011, Rockville, MD, USA) and cultured in Endothelial Cell Medium supplemented 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-429 mimics, antisense for miR-429 (as-miR-429) or null controls (RiboBio Co., Ltd., Guangzhou, Guangdong, China), using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. The sequences for miR-429: 5’- UAAUACUGUCUGGUAAAACCGU-3’; as-miR-429: 5’-ACGGUUUUACCAGACAGUAUUA-3’. The transfection efficiency was nearly 100%. One day after cell transfection, the cells were treated with or without 100 µg / ml oxidized low-density lipoprotein (ox-LDL, Beijing Xiesheng Bio-Technology Limited, Beijing, China). After drug treatment, the cells were used for 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. Briefly, cells were seeded in a 96-well microplate at a density of 5 x 104 / ml. After 24h, cells were treated with resveratrol. Subsequently, CCK-8 solution (20 ml / well) was added and the plate was incubated at 37°C for 2 hours. The viable cells were counted by absorbance measurements with a monochromator microplate reader at a wavelength of 450 nm. The optical density value was reported as the percentage of cell viability in relation to the control group (set as 100%).

**Apoptosis assay and flow cytometry**

For analysis of cell proliferation, the dissociated tissue cells or cultured cells were re-suspended at a density of 106 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) equipped with Cell Quest software (Becton-Dickinson Biosciences) for determination of Annexin V+ PI- apoptotic cells. For analyses and isolation of CD31+ cells, the dissociated tissue cells were incubated with PE-cy7-CD31 (Becton-Dickinson Biosciences) and then subjected to flow cytometry.

**Real-time RT-PCR**

Aorta intimal RNA was isolated from mouse aortas. After cleaning with ice-cold PBS, mouse aortas were flushed with TRIzol reagent (Invitrogen) using an insulin syringe and the eluate was collected in a 1.5 ml tube and prepared for RNA extraction. Total RNA and miRNAs were extracted from tissue or cultured cells with miRNeasy mini kit or RNeasy kit (Qiagen, Hilden, Germany), respectively. Complementary DNA (cDNA) was randomly primed from 2μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT-qPCR was subsequently performed in triplicate with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using 2-△△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 anti-CD31 (Becton-Dickinson Biosciences, San
Jose, CA, USA), rabbit-anti-alpha smooth muscle actin (α-SMA, Abcam), rabbit anti-Vimentin, rabbit-anti-Bcl-2 and rabbit-anti-GAPDH (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 as has been described before, using the algorithms TargetSan (https://www.targetscan.org) [36]. The Bcl-2 3'-UTR reporter plasmid (pRL-Bcl-2) was purchased from Creative Biogene (Shirley, NY, USA). Mir-429-modified HAECs were transfected with 0.5 μg pRL-Bcl-2 by Lipofectamine 2000 (5 × 10^4 cells per well). 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 ± S.D. Differences among groups were analyzed using one-away ANOVA with a Bonferoni 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**

**HFD induces atherosclerosis in ApoE (-/-) mice**

ApoE (-/-) mice treated with high-fat diet (HFD; simplified as HFD mice) have been widely used as an atherosclerosis model. Here, we used this model for analyzing endothelial cell apoptosis. After a 12-week HFD treatment, atherosclerotic lesions were analyzed in HFD mice, or ApoE (-/-) mice that had received normal diet (NOR) as a control. Analysis of H&E-stained histological sections of the aortic sinus showed a significant increase in aortic lesion size (Fig. 1A-B). Analysis of Oil-red-O-stained histological sections of the aortic sinus showed a significant increase in lipid content (Fig. 1C), suggesting that HFD successfully induced atherosclerosis in ApoE (-/-) mice. The aortas were isolated for analyzing the levels of CD31, an endothelial cell marker, and the levels of α-SMA, a mesenchymal cell marker. We found that HFD significantly decreased CD31 levels by mRNA (Fig. 1D), and by Western blot (Fig. 1E). HFD significantly increased α-SMA and Vimentin levels by mRNA (Fig. 1F), and by Western blot (Fig. 1E). Together, these data suggest that HFD induces atherosclerosis in ApoE (-/-) mice.

**HFD induces endothelial cell apoptosis in ApoE (-/-) mice**

To understand the bases of the low endothelial cell signals (CD31) in HFD mice, we isolated CD31+ cells from the dissociated mouse aorta by flow cytometry. HFD mice had significantly lower percentage of CD31+ endothelial cells in the aorta, by representative flow charts (Fig. 2A), and by quantification (Fig. 2B). These data suggest that the low CD31 levels in aorta in HFD mice result from the decreased endothelial cell number in HFD mice. Next, these isolated CD31+ cells were subjected to flow cytometry-based apoptosis assay, showing that HFD mice had significantly higher percentage of apoptotic CD31+ endothelial cells, by representative flow charts (Fig. 2C), and by quantification (Fig. 2D). Thus, HFD induces endothelial cell apoptosis in ApoE (-/-) mice.

**Endothelial cell apoptosis is attributable to the decreases in Bcl-2 by HFD-induced miR-429, which suppresses the translation of Bcl-2 mRNA via 3'-UTR binding**

Next, we examined the underlying mechanisms. First, we screened major apoptosis-associated proteins by Western blot in CD31+ endothelial cells from HFD and NOR mice. Specifically, we found that the levels of Bcl-2, a key anti-apoptosis protein, were significantly decreased in CD31+ endothelial cells from HFD mice by Western blot (Fig. 3A). Interestingly,
Fig. 1. HFD induces atherosclerosis in ApoE (-/-) mice. We used ApoE (-/-) mice treated with high-fat diet (HFD; simplified as HFD mice) for analyzing endothelial cell apoptosis. ApoE (-/-) mice that had received normal diet (NOR) were used as a control. (A-C) After a 12-week HFD treatment, analysis of H&E-stained histological sections of the aortic sinus showed a significant increase in aortic lesion size (A), shown by quantification (A), and by representative images (B). Analysis of Oil-red-O-stained histological sections of the aortic sinus showed a significant increase in lipid content (C). (D-F) The aortas were isolated for analyzing the levels of CD31, an endothelial cell marker, and the levels of α-SMA and Vimentin, two mesenchymal cell markers. (C) RT-qPCR for CD31. (D) Western blot for CD31, Vimentin and α-SMA. (E) RT-qPCR for α-SMA and Vimentin. * p < 0.05, N=5.

Fig. 2. HFD induces endothelial cell apoptosis in ApoE (-/-) mice. (A-B) We isolated CD31+ cells from the dissociated mouse aorta by flow cytometry. HFD mice had significantly lower percentage of CD31+ endothelial cells in the aorta, by representative flow charts (A), and by quantification (B). (C-D) The isolated CD31+ cells were subjected to flow cytometry-based apoptosis assay, shown by representative flow charts (C), and by quantification (D). * p < 0.05, N=5.
the transcripts of Bcl-2 in endothelial cells did not differ between HFD and NOR mice (Fig. 3B), suggesting that Bcl-2 may be regulated by HFD at post-transcriptional level, e.g. by miRNAs. All Bcl-2-targeting miRNAs were then determined by bioinformatics analyses, and we screened all these miRNAs and found that miR-429 levels significantly increased in CD31+ endothelial cells from HFD mice (Fig. 3C). MiR-429 targets 3'-UTR of Bcl-2 mRNA at one binding site of base pair 2820th to 2826th (Fig. 3D). Hence, we modified miR-429 levels in human aortic endothelial cells (HAECs), prepared by transfection of the cells with miR-429 mimics, antisense for miR-429 (as-miR-429) or null controls (null). First, modification of miR-429 levels in HAECs was confirmed by RT-qPCR (Fig. 3E). Next, the intact 3'UTR of Bcl-2 mRNA was cloned into a luciferase reporter plasmid, and used for transfection of miR-429-modified HAECs. The luciferase activities were quantified. * p < 0.05, N=5.

Fig. 3. Endothelial cell apoptosis is attributable to the decreases in Bcl-2 by HFD-induced miR-429, which suppresses the translation of Bcl-2 mRNA via 3'-UTR binding. (A-B) The levels of Bcl-2 were examined in CD31+ endothelial cells from HFD or NOR mice by Western blot (A), and by RT-qPCR (B). (C) The levels of miR-429 in CD31+ endothelial cells from HFD or NOR mice. (D) Bioinformatics analyses showing that miR-429 targets 3'-UTR of Bcl-2 mRNA at one binding site of base pair 2820th to 2826th. (E) MiR-429 levels in miR-429-modified human aortic endothelial cells (HAECs), prepared by transfection of the cells with miR-429 mimics, antisense for miR-429 (as-miR-429) or null controls (null). (F) The intact 3'UTR of Bcl-2 mRNA was cloned into a luciferase reporter plasmid, and used for transfection of miR-429-modified HAECs. The luciferase activities were quantified. * p < 0.05, N=5.
UTR reporter for Bcl-2, while as-miR-429 significantly increased the luciferase activity of 3' UTR reporter for Bcl-2, compared to null control (Fig. 3F). Together, these data suggest that endothelial cell apoptosis is attributable to the decreases in Bcl-2 by HFD-induced miR-429, which suppresses the translation of Bcl-2 mRNA via 3'-UTR binding.

Ox-LDL-treated HAECs recapitulate the findings in HFD mice

To confirm the findings in HFD mice, we used an in vitro model of atherosclerosis, in which HAECs were treated with or without 100µg/ml oxidized low-density lipoprotein (ox-LDL) to induce cell apoptosis similar to in vivo model. (A-B) The analyses of cell apoptosis by representative flow charts (A) and by quantification (B). (C) Quantification of viable cell number in a CCK-8 assay. (D-F) Ox-LDL significantly increased the levels of miR-429 (D) and significantly decreased the protein levels of Bcl-2 (E), without altering Bcl-2 mRNA (F). * p < 0.05, N=5.

UTR reporter for Bcl-2, while as-miR-429 significantly increased the luciferase activity of 3' UTR reporter for Bcl-2, compared to null control (Fig. 3F). Together, these data suggest that endothelial cell apoptosis is attributable to the decreases in Bcl-2 by HFD-induced miR-429, which suppresses the translation of Bcl-2 mRNA via 3'-UTR binding.

**Ox-LDL-treated HAECs recapitulate the findings in HFD mice**

To confirm the findings in HFD mice, we used an in vitro model of atherosclerosis, in which HAECs were treated with or without 100µg/ml oxidized low-density lipoprotein (ox-LDL) to induce cell apoptosis similar to in vivo model. First, we confirmed that ox-LDL significantly increased cell apoptosis, by representative flow charts (Fig. 4A), and by quantification (Fig. 4B). Endothelial cell apoptosis resulted in a significant reduction in viable cell number in a CCK-8 assay (Fig. 4C). Moreover, ox-LDL significantly increased the levels of miR-429 (Fig.
4D) and significantly decreased the protein levels of Bcl-2 (Fig. 4E), without altering Bcl-2 mRNA (Fig. 4F). Together, these data suggest that ox-LDL-treated HAECs recapitulate our findings in HFD on mice. Thus, our study suggest that atherosclerosis-associated endothelial cell apoptosis may result from down regulation of Bcl-2, by augmented miR-429 that binds and suppresses translation of Bcl-2 mRNA (Fig. 5).

Discussion

Atherosclerosis is a complex immune-inflammatory disease of arteries of medium-to-large size. Crosstalk among endothelial cells, macrophages and smooth muscle cells coordinates the development of atherosclerosis. First of all, injury of the vascular endothelium is a proven initial step in the pathogenesis of atherosclerosis. Pro-atherosclerotic factors include high glucose, angiotensin II and oxygen reactive species that are all promote the apoptosis of endothelial cells. Specially, ox-LDL is able to facilitate endothelial cell apoptosis through ill-defined pathways.

Aberrantly expressed miRNAs have been shown to be involved in the regulation of endothelial cell survival/death, supported by many recent reports. Specifically, miR-429 has been reported as a tumor suppressor in various cancers [25-33]. Moreover, the role of miR-429 in carcinogenesis has been associated with apoptosis augmentation. For example, the expression levels of miR-429 in esophageal carcinoma tissues were found to be lower than those in adjacent non-neoplastic tissues. This relatively low expression was found to be significantly associated with the occurrence of lymph node metastases. Apoptosis and migration rates were found to be significantly higher in esophageal carcinoma cells with miR-429 depletion [35]. In another study, miR-429 was found to obviously downregulate much more in hepatitis B virus (HBV)-related hepatocellular carcinoma. MiR-429 was found to directly target NOTCH1 and reduced both mRNA and protein levels of NOTCH1 which stimulated proliferation and suppressed apoptosis in HCC cells [34]. These studies encouraged us to examine a possible role of miRNAs in the development of atherosclerosis through modulation of apoptosis-related proteins.

Here, we found that ApoE (-/-) mice treated with HFD (simplified as HFD mice) developed atherosclerosis in 12 weeks, while the control ApoE (-/-) mice that had received normal diet (simplified as NOR mice) did not. HFD mice had significantly lower percentage of endothelial cells and significantly higher percentage of mesenchymal cells in the aorta than NOR mice. These data confirm that we had used a qualified model for atherosclerosis. Importantly, we screened major apoptosis-associated proteins by Western blot in CD31+ endothelial cells from HFD and NOR mice. Specifically, we found that the levels of Bcl-2, a key anti-apoptosis protein, were significantly decreased in CD31+ endothelial cells from HFD mice by Western blot, but not by mRNA. These findings not only highlight Bcl-2 as a key player in development of atherosclerosis in HFD mice, but also indicate that HFD may modulate Bcl-2 at post-transcriptional level. Although the post-transcriptional regulation of a protein may involve translation control by miRNAs and degradation control by phosphorylation, acetylation, ubiquitination, SUMOylation, etc, in the case of Bcl-2, it is
more likely that miRNAs are involved. All Bcl-2-targeting miRNAs were then determined by bioinformatics analyses, and we screened all these miRNAs and found that miR-429 levels significantly increased in CD31+ endothelial cells from HFD mice, which is consistent with a previous report in esophageal carcinoma research [35]. In addition, we used an in vitro atherosclerosis model to confirm this model in endothelial cell apoptosis.

Together, our study identified a new role for miR-429 in the regulation of endothelial cell apoptosis during atherosclerosis, and shed new insight into development of innovative therapy by targeting miR-429 in atherosclerosis treatment.

**Disclosure Statement**

The authors have declared that no competing interests exist.

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