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Xiaoxianggou attenuates atherosclerotic plaque formation in endogenous high Ang II ApoE$^{-/-}$ mice via the inhibition of miR-203 on the expression of Ets-2 in endothelial cells

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ABSTRACT

Background: Atherosclerosis is a chronic immune-inflammatory disorder and one of the leading causes responsible for cardiovascular morbidity and mortality. Traditional Chinese medicine treatment with multi-targets has shown prospects for the therapeutic effect on atherosclerosis. Thus, this study aims to investigate whether xiaoxianggou has benefit for reducing the atherosclerotic plaque area in endogenous high Ang II ApoE$^{-/-}$ mice and investigated the underlying mechanisms.
Methods: Endogenous high Ang II ApoE$^{-/-}$ mice model was generated by using two kidney one clip (2K1C). All mice were treated by intragastric administration with xiaoxianggou two times a week for 16 weeks. En face plaque area was analyzed by oil-red O staining. Serum anti-OxLDL antibodies were measured by ELISA assay. Expression of miR-203 and Ets-2 were evaluated using qRT-RCR and western blotting analysis, respectively.
Results: This study revealed that xiaoxianggou treatment dose-dependently reduced the atherosclerotic plaque area and serum autoantibodies against oxLDL, elevated miR-203 expression and reduced Ets-2 expression in endogenous high Ang II ApoE$^{-/-}$ mice. In primary arterial ECs, Xiaoxianggou reverses the reduced miR-203 expression and the elevated Ets-2 expression induced by AngII, which was further recovered by miR-203 inhibitor. Additionally, miR-203 regulated the expression of Ets-2 by targeting Ets-2-3′ UTR. Moreover, miR-203 inhibitor reversed the reduction of atherosclerotic lesion area induced by Xiaoxianggou.
Conclusions: These findings present that xiaoxianggou plays an anti-atherosclerotic role in endogenous high Ang II ApoE$^{-/-}$ mice model, which is partly due to its antioxidant actions against atherosclerosis and the inhibition of miR-203 on the expression of Ets-2 in endothelial cells.

1. Introduction

Atherosclerosis (AS) is a chronic and relatively benign disease, which progresses silently in most middle-aged and asymptomatic individuals. Inflammation with immune competent cells in lesions producing proinflammatory cytokinesis involved in the formation of atherosclerosis [1]. Atherosclerotic lesions progress from the early fatty streaks to the complex vulnerable plaques that are responsible for the acute consequences of the disease. The alterations of proliferation, apoptosis, invasion, chemotaxis and secretion function in mononuclear cells, endothelial cells and vascular smooth muscle cell are considered to be the early characteristic lesions of AS formation. Oxidized low density lipoprotein (ox-LDL), as an important risk factor for AS development, has been believed to induce these alterations of vascular cells [2].

Xiaoxianggou, a common traditional she medicine full of sweet fragrance, is the dry root and stem of Ficus pandurata hance var. angustifolia Cheng, Ficus panduram Hane var.hoiophylla Migo and Ficus erecta Thunb.var. bcecheyana (Hook.et Am.) King. Emerging evidence has been well established that xiaoxianggou is commonly used to the treatment of gouty arthritis, arthralgias, infantile malnutrition, indigestion, diarrhea, hernia and other diseases [3,4].

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The main functional component of xiaoxianggou is flavone [3]. Our previous study confirmed that xiaoxianggou had an anti-inflammatory action. However, whether it benefits the prevention or treatment of AS development and progression remains unknown. The potential mechanism should be investigated.

MicroRNAs (miRNAs) are highly conserved, single-stranded noncoding RNA molecules, ~22 nt in length, that exert post-transcriptional effects on gene expression by promoting the degradation of mRNA target and/or inhibiting mRNA translation [5]. Many miRNAs have been confirmed to participate in the physiopathology process of AS development. Aberrant expression of miR-203 has been found in several human diseases, including cancer [6], psoriasis [7] and rheumatoid arthritis [8]. Recently, miR-203 was predicted to be associated with early AS [9]. *In vitro* experiment has confirmed that miR-203 contributes to the proliferation of vascular smooth muscle cells [10]. Therefore, we proposed that miR-203 might participate in the AS development. The v-ets erythroblastosis virus E26 oncogene homolog 2 (Ets2) transcription factor has been shown to be a potent transactivator of angiogenic regulators and mediate immune-activation of the endothelium in the late phase of AS [11]. We predicted that miR-203 had the complementary sequence of Ets2 using biological information software, and postulated that miR-203 participated in the process of atherosclerotic plaque formation through the regulation Ets2. In the present study, we first confirmed the effect of Xiaoxianggou on atherosclerotic plaque formation in endogenous high Ang II ApoE$^{-/-}$ mouse model. The potential molecular mechanism was then investigated using *in vivo* and *in vitro* experiments.

2. Materials and methods

2.1. Animals and drug treatment

6 weeks-old male apolipoprotein (apo)E$^{-/-}$ mice with a C57BL/6 background obtained from Peking University Animal Technology Co., Ltd. (Beijing, China) were fed 16 weeks with a high-fat diet. All animals were maintained five per cage at 24 °C on a 12-h light/dark cycle under certified specific pathogen-free facilities. Two kidney one clip (2K1C) was performed to generate endogenous high Ang II ApoE$^{-/-}$ mouse model. Briefly, mice were anesthetized with 2% sodium pentobarbital (40 mg/kg) by intraperitoneal injection. Abdominal skin and subcutaneous tissue of the left ribs were cut out. The kidney was then exposed, externalized, and carefully maintained with an ophthalmic chalazion forceps. For clipping, the left kidney was gently pulled out and the renal artery of the left kidney was individualized over a short segment by blunt dissection. Subsequently, a clip was placed close to the aorta and then the kidney was gently pushed back into the retroperitoneal cavity. Local injection of penicillin 20000 units per kilogram of body weight was performed. Then, the muscle layer was sutured, and the skin incision was closed with surgical staples. Postoperative intraperitoneal injection of penicillin 20000 units per day for 2 days was performed to prevent infection. A sham procedure, which included the entire surgery with the exception of artery clipping, was applied in control mice. Xiaoxianggou was obtained from Zhejiang Province Hospital of Traditional Chinese Medicine (Hangzhou, China) and perform water boiling to obtain decoction. The mice were given with the different doses of Xiaoxianggou decoction (0 g/kg, 10 g/kg, 20 g/kg and 40 g/kg) by Intragastric administration at a frequency of two times one week for 16 weeks. Eight mice were in each group. The animal experiments were conducted in Guide for the Care and Use of Laboratory Animals of the First Affiliated Hospital of Zhejiang University.

2.2. Culture of mouse primary arterial endothelial cells and ECV304 endothelial cells

Primary arterial endothelial cells were isolated from C57BL/6 mice which were killed by euthanasia. Subsequently, aorta was dissected under sterile conditions, promptly excised, washed with phosphate buffered saline (PBS) and 10% fetal bovine serum (FBS, Hyclone, UT, USA) and cut into 1–2 mm^2 pieces to terminate the digestion. Then, obtained the cells after centrifugation followed by soft resuspension. Primary arterial endothelial cells and ECV-304 cells were detached from the plates by trypsinization, seeded onto 24- or 96-well culture plates and cultured for 24 h in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cultures were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C until reaching 80% confluency.

2.3. Quantitive real-time PCR (qRT-PCR)

To monitor miR-203 and Ets-2 mRNA expression, total RNA was isolated from mouse tissues and cultured cells using Trizol reagent (Invitrogen, CA, USA) and reversely transcribed to cDNA using a SuperScript II kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR analysis was performed using Quanti-Tect SYBR Green PCR Master Mix (Qiagen, Valencia, CA) and ABI GeneAmp 7500 Sequence Detection System (Applied Biosystems, CA, USA). Amplification was performed with an initial denaturing step at 95 °C for 15 s., followed by a three-step PCR program consisting of denaturing at 95 °C for 5 s., annealing at 60 °C for 30 and extending at 72 °C for 30 s for 45 cycles. Data were collected and quantitatively analyzed on the ABI 7500 Fast Sequence Detection System and software (Applied Biosystems). The relative RNA levels were normalized to GAPDH expression and calculated by the $2^{(-\triangle\triangle Ct)}$ method. All reactions were run in triplicate.

2.4. Western blotting analysis

Protein expression of Ets-2 was detected by western blotting. Briefly, cells were washed twice with cold PBS buffer and lysed in the 19 RIPA lysis buffer containing of 1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 2 mol/L urea, pH 7.4. Supernatants were collected after centrifugation and stored at −80 °C. Protein concentration of the lysate was determined by the BCA Protein Assay (Pierce Chemicals Co., Rockford, IL, USA). Samples from these cell lysates were denatured and subjected to 10% SDS-PAGE, and then transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked with TBST containing 5% BSA at 4 °C, probed overnight with primary antibody, anti-Ets2 or anti-β-Actin obtained from Santa Cruz Biotech (Santa Cruz, CA, USA), and then incubated with appropriate horseradish peroxidase-coupled secondary antibodies (GE Healthcare, Michigan, USA) diluted 1:2000 in 5% milk/1 × TBST for 1 h. After washing, the results of immunoblotting were detected using the ECL Plus Western blotting system kit (Amersham, Pittsburgh, PA, USA). Total protein levels were normalized to β-actin and each sample was replicated triple.

2.5. Statistical analysis

The experimental data were determined by Dunnett's *t*-test for comparisons of arthritic scores and real-time PCR quantitation. The statistical analyses were conducted using SAS statistical analysis software (SAS Institute, Cary, NC) and data were presented as mean ± SD. *P* values were determined by *t*-test and the statistically significant difference was considered at $P < 0.05$ or $P < 0.01$.