Resveratrol inhibits adventitial fibroblast proliferation and induces cell apoptosis through the SIRT1 pathway

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Abstract. Atherosclerosis is one of the most important causes of cardiovascular disease and studies have showed that adventitial fibroblasts, which are considered to be the most common cell type of the vascular adventitia, are involved in the development of early atherosclerotic plaques. Resveratrol is a plant polyphenolic compound confirmed to have anti-atherosclerotic and cardioprotective effects. The aim of the present study was to investigate the effects of resveratrol on adventitial fibroblasts *in vitro* and to clarify the underlying mechanism. Adventitial fibroblasts were isolated from the thoracic aorta of 8-week-old SPF Sprague-Dawley rats. Following pre-treatment with different concentrations of resveratrol, cell viability, DNA synthesis ability, cell apoptosis and cell migration ability were assessed *in vitro*. Through transfection with small interfering (si)RNA targeting sirtuin 1 (SIRT1), the role of the SIRT1 pathway in these processes was evaluated. Western blot analysis was used to assess the protein expression of SIRT1. It was demonstrated that resveratrol inhibited the cell viability, DNA synthesis and migratory ability of the adventitial fibroblasts, and induced cell apoptosis in a concentration-dependent manner *in vitro*. These effects were partly through the SIRT1 pathways. siRNA targeting SIRT1 successfully reversed the antiproliferative, antimigratory and pro-apoptotic effects of resveratrol on adventitial fibroblasts. In conclusion, the data showed that resveratrol inhibited cell viability, DNA synthesis and cell migration, and induced cell apoptosis in the rat adventitial fibroblasts *in vitro* through the SIRT1 signaling pathway. As the activation and migration of adventitial fibroblasts contributes to the early development of atherosclerosis, this may be a mechanism underlying the anti-atherosclerotic effect of resveratrol.

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Introduction

Atherosclerosis is the most common cause of cardiovascular disease and is a dynamic pathological process (1). Previous studies have focused on the functions of the vascular intima and media, including endothelial cells and smooth muscle cells (2,3). An increasing number of studies are investigating the effect of the vascular adventitia and adventitial fibroblasts, which are considered to be the most common cell type of the adventitia. Studies have shown that adventitial fibroblasts are involved in inflammatory responses, vascular remodeling, restenosis and the development of atherosclerotic plaques (4,5). The activation and migration of adventitial fibroblasts contribute to the early development of atherosclerosis, during which adventitial remodeling precedes intimal and medial remodeling, and adventitial fibroblast are the first to proliferate, differentiate into myofibroblasts, migrate into the intima and contribute to the formation of atherosclerotic plaques (6). These proliferating and migrated adventitial fibroblasts are involved in the synthesis and release of cytokines, and promote intimal and medial inflammation through its effects on endothelial cells, smooth muscle cells and macrophages. Activated adventitial fibroblasts can also transform into myofibroblasts, synthesize collagen, and lead to extracellular matrix remodeling and restructuring (7). Therefore, the adventitia theory is becoming important as a complementary process to the development of atherosclerosis.

Resveratrol is a plant polyphenolic compound present in red wine and grapes, which has a wide range of effects, including antioxidant, immunomodulatory and chemopreventive effects, in biological systems (8). Resveratrol has been confirmed to have antitumor and cardioprotective potential, is able to repress cancer development and is involved in anti-inflammatory and antiproliferative processes (9). These beneficial effects of resveratrol are considered to be associated with the sirtuin 1 (SIRT1) protein (10). Resveratrol is a natural activator of SIRT1, which is an NAD-dependent protein deacetylase. The SIRT1 pathway regulates processes, including cell apoptosis, proliferation and life span, through the modulation of several genes and transcription factors, including p53, the caspase family, nuclear factor (NF)-κB and the forkhead box O (FOXO) family (10-12). There is now a focus on the effect of resveratrol on the cardiovascular system. It has been reported

that resveratrol enhances the expression and activity of endothelial nitric oxide synthase (eNOS) in human endothelial cells, downregulates the expression of cycloxygenase-2 and inducible NOS in macrophages, and inhibits aortic smooth muscle cell proliferation through the NF-κB pathway (13,14). These factors synergistically contribute to the anti-atherosclerotic and cardioprotective effects of resveratrol. However, there are no reports on the effect of resveratrol on adventitial fibroblasts, which are also considered to be an important component in the development of atherosclerosis.

The present study aimed to investigate the effects of resveratrol on rat adventitial fibroblasts *in vitro*. The results showed that resveratrol inhibited cell viability, DNA synthesis and cell migration, and induced the apoptosis of adventitial fibroblasts in a concentration-dependent manner. These effects occurred partially through the SIRT1 pathway. As the activation of adventitial fibroblasts is an important step in the early development of atherosclerotic plaques, the antiproliferative, antimigratory and pro-apoptotic potential of reseveratrol may be a mechanism underlying of its anti-atherosclerotic effect.

Materials and methods

Ethics statement. Animal experiments were approved by the Soochow University Scientific and Animal Ethics Committee (Suzhou, China; approval no. 20120008) and were in compliance with the Chinese national regulations on the use of experimental animals. The procedures for animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (revised in 1996).

Reagents and drugs. SIRT1 antibodies were purchased from Abcam (Cambridge, MA, USA), The BCA Protein Assay kit, AnnexinV/Propidium Iodide (PI) Apoptosis Detection kit and EdU Imaging kit were obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Other materials and chemicals were purchased from commercial resources.

Cell culture of adventitial fibroblasts. The present study used 8-week-old male SPF Sprague-Dawley rats (n=20). All animals were purchased from the Laboratory Animal Center of Soochow University. They were maintained on standard diet and water with a 12 h light/dark cycle at the Animal Center of the First Affiliated Hospital of Soochow University. Rats were sacrificed using CO_2. Adventitial fibroblasts were prepared from rat thoracic aorta. The adventitial fibroblasts were prepared from the rat thoracic aorta. Briefly, the intima and middle layer of the aorta were scraped off, and the adventitia was sliced into 1 mm^3 pieces, which were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 5% CO_2 and 37˚C. After 24 h, fresh complete medium was added and the cells were cultured until adventitial fibroblasts grew out. Fresh complete medium was replaced every 3 days. The cells were stained with α-actin and Von Willebrand factor to exclude vascular smooth muscle cells and vascular endothelial cells. The cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

RNA interference. Rat small interfering RNA (si)RNA and control scrambled siRNA were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. The adventitial fibroblasts at a density of $1x10^4$ were transfected with 100 nmol/l SIRT1 siRNA (forward 5'-GAAGUUGACCUCCUCAUUGUdTdT-3' and reverse 5'-ACAAUGAGGAGGUCAACUUCdTdT-3') using lipofectamine 2000 according to manufacturer's protocol. The transfected cells were incubated for 48 h at 37˚C prior to subsequent assays. The cells were divided into four groups, which comprised adventitial fibroblasts treated with resveratrol (20 or 80 μmol/l) for 24 h at 37˚C with or without transfection with siRNA targeting SIRT1 (20 μmol/l+siRNA or 80 μmol/l+siRNA).

CCK-8 cell proliferation assay. Cell proliferation was assessed using a CCK-8 assay, based on the enzymatic reduction of WST-8 in living cells and the production of a proportional color change. Briefly, $1x10^4$ cells suspended in 150 μl complete medium were seeded in each well of a 96-well culture plate and incubated at 37˚C under 5% CO_2 for 24 h. The WST-8 cell proliferation reagent (50 μl) was added and incubated for 4 h at 5% CO_2 and 37˚C. The negative control comprised WST-8 reagent and complete medium with no cells. The absorbance was measured at 450 nm using a spectrophotometer and the optical density (OD) was calculated as $OD = OD_{sample} - OD_{control}$. Each experiment was performed in triplicate wells and repeated three times.

EdU DNA synthesis assay. The present study used a Click-iT® EdU Imaging kit to assess cell DNA synthesis. Briefly, the cells cultured in 6-well plates were incubated with EdU for 24 h. Following fixation and permeabilization, 0.5 ml Click-iT® reaction cocktail was added and incubated for 30 min at room temperature in the dark. Hoechst 33342 was used to stain the cell nuclei. The cells were observed using a fluorescence microscope, and nuclei undergoing DNA synthesis were stained red. The numbers of proliferating nuclei were counted in 10 randomly selected fields (magnification, x100) and the average was calculated. The rate of DNA synthesis was calculated as the number of proliferating nuclei / 100. Each experiment was performed in triplicate wells and repeated three times.

Cell apoptosis assay. A cell apoptosis assay was performed using an apoptosis detection kit. The cells were cultured in 6-well plates to 70% confluence at a density of $1x10^5$ cells/well. Following digestion with trypsin and washing twice with PBS, the cells were labeled with 5 μl Annexin V-fluorescein isothiocyanate (FITC) and 5 μl PI for 5 min each at room temperature in the dark. The cell suspensions were analyzed by fluorescence-activated cell sorting with CellQuest version 3.3 (BD Biosciences, San Jose, CA, USA) software.

Cell migration assay. A Transwell assay was performed to assess cell migration. Briefly, freshly isolated cells were pre-washed twice with serum-free DMEM and $5x10^4$ cells were seeded into the upper chamber of a Transwell plate. The lower chamber contained complete medium to induce cell migration. Following incubation for 24 h at 5% CO_2 and 37˚C, the non-migrated cells were scraped off with a cotton swab, and the migrated cells were stained purple with 0.1%

hexamethylpararosaniline and counted in 10 randomly selected fields (magnification, x100) using an inverted microscope. The migration rate was calculated as follows: Migration rate = migration number$_{(Treated)}$ / migration number$_{(Control)}$. Each assay was performed in triplicate wells and repeated three times.

Western blot analysis. The harvested cells were lysed with RIPA lysis buffer and protease inhibitor cocktail. Protein concentrations were quantified using a BCA protein assay kit. The proteins (30 μg per lane) were separated using 10% SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. The membrane was blocked with Tris-buffered saline-Tween 20 (TBS-T) and 5% nonfat dried milk for 2 h, and incubated with SIRT1 (1:500; cat. no. 19A7AB4) primary antibody overnight at 4˚C. Following washing twice with TBS-T and incubation with peroxidase-conjugated secondary antibodies (1:1,000; A996702 Amyjet Scientific, Co., Ltd., Wuhan, China) for 1 h at room temperature, the bands were detected using an chemiluminescence detection system (Invitrogen; Thermo Fisher Scientific, Inc.). The band intensities were quantified using the Photo-Image System (Siemens AG, Erfurt, Germany).

Statistical analysis. All data are presented as the mean ± standard deviation of at least three independent experiments. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Multiple comparisons were performed using one-way analysis of variance with Scheffe's post-hoc test when the distributions were normal, or with the non-parametric Kruskal-Wallis test with a Dunn post-hoc test when the distributions were not normal. P<0.05 (two-tailed) was considered to indicate a statistically significant difference.

Results

Resveratrol inhibits adventitial fibroblast viability and DNA synthesis. The present study used a CCK-8 assay to detect alterations in cell viability. The CCK-8 results are expressed as OD and cell viability was positively correlated with the OD index. As shown in Fig. 1, resveratrol inhibited the cell viability of the adventitial fibroblasts in a concentration-dependent manner. The 20 μmol/l resveratrol group (1.18±0.15) showed a decreased OD, compared with the control group (1.56±0.10; P<0.05), and in the 80 μmol/l resveratrol group, cell viability compared was inhibited further, compared with the control (0.68±0.16; P<0.05). The cells transfected with siRNA targeting SIRT1 showed a reversal in cell viability, compared with the non-transfected cells in the 80 μmol/l+siRNA (0.82±0.02) group (P<0.05). In the Edu DNA synthesis assay, the proliferating nuclei were stained red and normal nuclei were blue. As shown in Fig. 2A, with the increased concentration of resveratrol, the DNA synthesis of the adventitial fibroblast was inhibited. The synthesis rates were 33.34 ± 2.85% in the control, 23.48 ± 1.34% in the 20 μmol/l resveratrol group and 7.04 ± 0.98% in the 80 μmol/l resveratrol group (P<0.05; Fig.2B). When transfected with siRNA, the DNA synthesis inhibition was rescued, with synthesis rates of 31.28 ± 2.02% in the 20 μmol/l resveratrol + siRNA group and 14.6 ± 1.57% in the 80 μmol/l resveratrol + siRNA group, which were higher, compared with that in the non-transfected group (P<0.05).

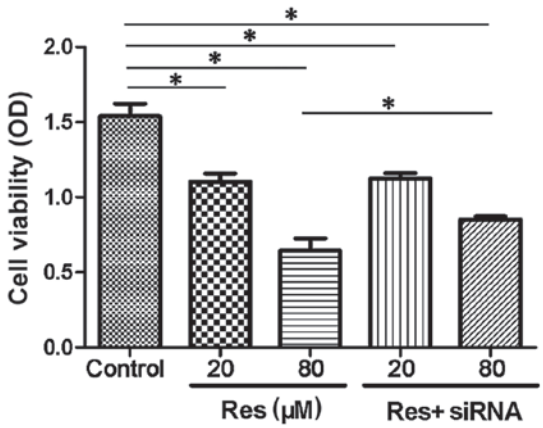

Figure 1. Resveratrol inhibits adventitial fibroblasts viability. The 20 μmol/l resveratrol group showed decreased cell viability, compared with the control group (*P<0.05). The 80 μmol/l group further inhibited cell viability, compared with the control group (*P<0.05). Transfection with siRNA targeting SIRT1 reversed the inhibition in the 80 μmol/l+siRNA group (*P<0.05, vs. 80 μmol/l group). Res, resveratrol; siRNA, small interfering RNA; SIRT1, sirtuin 1; OD, optical density.

High-dose resveratrol induces adventitial fibroblast apoptosis. The present study analyzed cell apoptosis using an AnnexinV/PI immunofluorescent cytometry assay. The proportions of apoptotic cells are expressed as AnnexinV (FITC)-positive and PI (phycoerythrin)-negative. As shown in Fig. 3A and B, resveratrol induced cell apoptosis in a concentration-dependent manner, and this pro-apoptotic effect was also reversed by siRNA transfection. The levels of cell apoptosis were 4.7 ± 0.55% in the control, 7.9 ± 0.17% in the 20 μmol/l resveratrol group and 32.73 ± 1.68% in the 80 μmol/l resveratrol group. There was a significant difference between the 80 μmol/l resveratrol group and the control (P<0.05). Following siRNA transfection, the cell apoptotic rate showed a marginal decrease (6.2 ± 0.5%) in the 20 μmol/l resveratrol + siRNA group. A more marked decrease (22.4 ± 1.36%) was found in the 80 μmol/l resveratrol+siRNA group, compared with the corresponding 80 μmol/l resveratrol group (P<0.05). This suggested that resveratrol induced cell apoptosis through activation of the SIRT1 pathway.

High-dose resveratrol inhibits adventitial fibroblast migration. The present study assessed cell migration *in vitro* using a Transwell assay and the migrated adventitial fibroblasts were stained purple (Fig. 4A). By counting the number of migrated cells, it was found that the migratory ability of the adventitial fibroblasts was inhibited with the increase of resveratrol concentration. The migration index was 1.0±0.12 in the control, 0.88±0.13 in the 20 μmol/l resveratrol treatment group and 0.18±0.02 in the 80 μmol/l resveratrol treatment group. There was a significant difference between the 80 μmol/l resveratrol treatment group and the control group (P<0.05). Following inhibition of the SIRT1 pathway with siRNA, the inhibitory effect of resveratrol on adventitial fibroblast migration was rescued, and the migration rates were 0.90±0.11 in the 20 μmol/l resveratrol + siRNA group

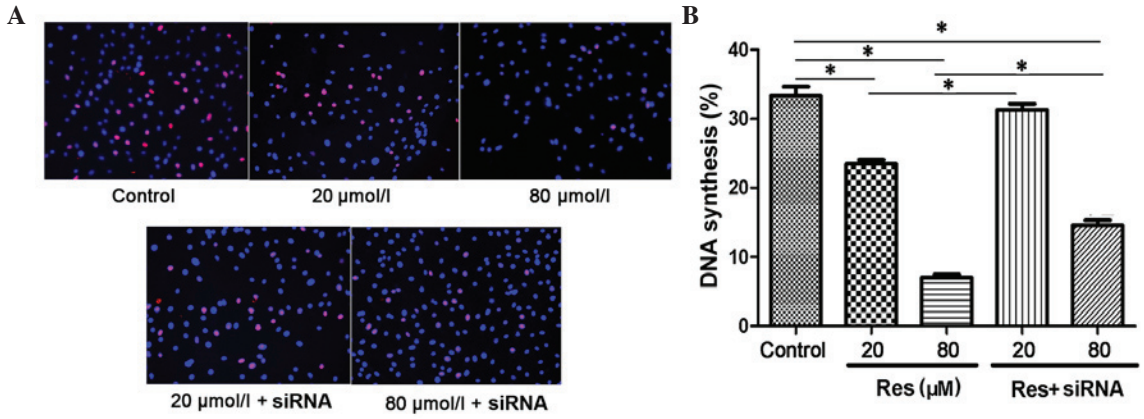

Figure 2. Resveratrol inhibits DNA synthesis of adventitial fibroblasts. (A) DNA synthesis observed under an immunofluorescent microscope (magnification, x100). Nuclei in DNA synthesis are stained red. Normal nuclei are stained blue. (B) Statistical analysis. Resveratrol inhibited DNA synthesis of the adventitial fibroblasts in a concentration-dependent manner (*P<0.05, vs. control). siRNA transfection reversed this inhibition in the 20 μmol/l+siRNA group (*P<0.05, vs. 20 μmol/l group) and 80 μmol/l+siRNA group (*P<0.05, vs. 80 μmol/l group). Res, resveratrol; siRNA, small interfering RNA; SIRT1, sirtuin 1.

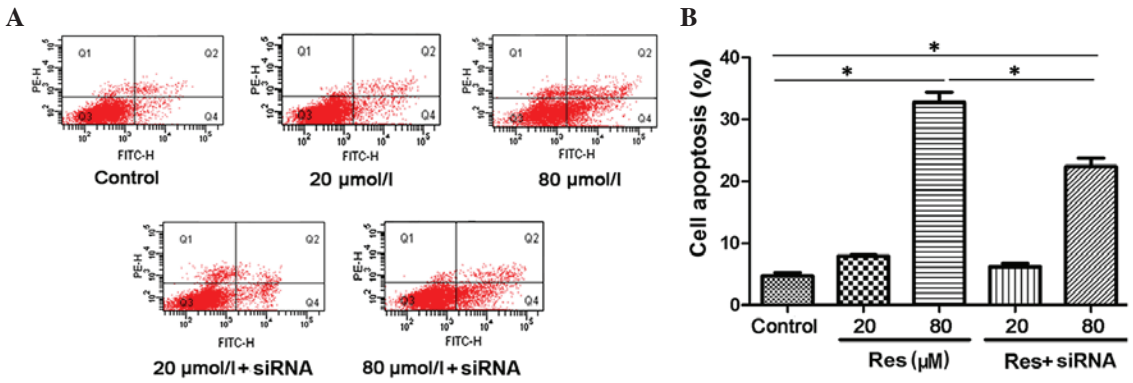

Figure 3. Resveratrol induces adventitial fibroblast apoptosis. (A) Flow cytometric analysis of cell apoptosis following resveratrol treatment. Q4 represents the ratio of apoptotic cells. (B) Statistical analysis of the results. High-dose resveratrol induced cell apoptosis of adventitial fibroblasts (control, vs. 80 μmol/l group; *P<0.05). This pro-apoptotic effect was partially reversed by siRNA transfection. The apoptotic ratio was significantly decreased in the 80 μmol/l+siRNA group, compared with the 80 μmol/l resveratrol group (*P<0.05). There was a significant difference between the 80 μmol/l+siRNA group, compared with the control group (*P<0.05). Res, resveratrol; siRNA, small interfering RNA; SIRT1, sirtuin 1; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

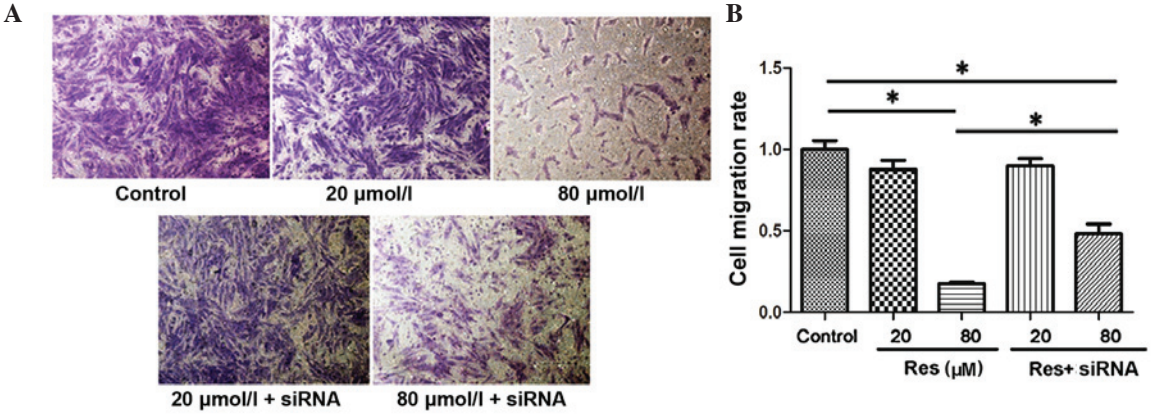

Figure 4. Resveratrol inhibits adventitial fibroblast migration. (A) Cell migration was measured using a Transwell assay. Migrated cells are stained purple (magnification, x100). (B) Statistical analysis of the results of cell migration. High-dose (80 μmol/l) resveratrol inhibited adventitial fibroblasts migration, compared with the control (*P<0.05). The migratory ability was partially rescued following siRNA transfection in the 80 μmol/l+siRNA group (*P<0.05, vs. 80 μmol/l group). Res, resveratrol; siRNA, small interfering RNA; SIRT1, sirtuin 1.

and 0.48±0.14 in the 80 μmol/l resveratrol + siRNA group (80 μmol/l resveratrol, vs. 80 μmol/l resveratrol+siRNA; P<0.05; Fig. 4B).

Resveratrol upregulates the expression of SIRT1. The present study used western blot analysis to detect alterations in the level of SIRT1. As shown in Fig. 5, the protein expression of

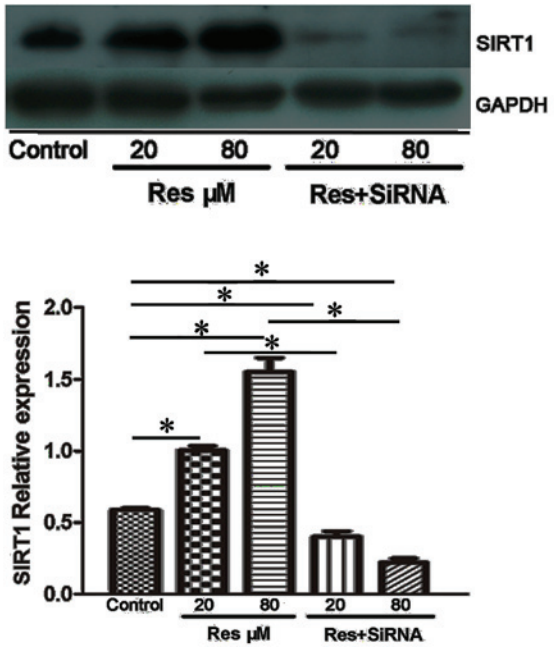

Figure 5. Resveratrol upregulates the protein expression of SIRT1. The results of the western blot analysis showed that the protein expression levels of SIRT1 were elevated in the 20 and 80 μmol/l resveratrol treatment groups, compared with control (*P<0.05). Protein expression of SIRT1 was down-regulated in the 20 μmol/l+siRNA group (*P<0.05, vs. 20 μmol/l group) and the 80 μmol/l+siRNA group (*P<0.05, vs. 80 μmol/l group). Res, resveratrol; siRNA, small interfering RNA; SIRT1, sirtuin 1.

SIRT1 was increased following resveratrol treatment. The adjusted protein expression levels of SIRT1 were 0.59±0.01 in the control, 1.00±0.03 in the 20 μmol/l resveratrol group and 1.55±0.09 in the 80 μmol/l resveratrol group. Following treatment with SIRT1 siRNA, the protein expression levels were decreased in the 20 μmol/l + siRNA group (0.41±0.03), and was significantly different, compared with that in the 20 μmol/l group (P<0.05). A further decrease in the protein expression of SIRT1 was found in the 80 μmol/l + siRNA group, with an expression of 0.22±0.02 (P<0.05, vs. 80 μmol/l + siRNA group).

Discussion

Increasing evidence has shown that the aorta adventitia is involved in the development of atherosclerosis and the process of plaque formation. The adventitia is no longer determined as a supportive tissue, but is actively involved in the formation and progression of atherosclerosis (5,15). Adventitial fibroblasts are the major cell type in the adventitia, and studies have confirmed that these cells are active in the early stage of atherosclerosis, proliferating first in plaque formation. Following proliferation, they differentiate into myofibroblasts and secrete several inflammatory factors. They also migrate into the inner layers of the artery wall and affect the kinetics of smooth muscle cells in the media or endothelial cells in the intima of the artery wall (16,17). The inflammatory factors secreted by adventitia fibroblasts inhibit the release of nitric oxide from endothelial cells, increase the transition of smooth muscle cells and promote the pathological process of atherosclerosis (18). Therefore, agents or drugs able to inhibit the proliferation and migration of adventitia fibroblasts may contribute to the prevention or delay of atherosclerosis formation and progression.

Resveratrol is a flavor found in grapes, which has been confirmed to be beneficial to the cardiovascular system as an anti-atherosclerotic agent (8,9). Evidence shows that resveratrol is able to inhibit the progression of atherosclerotic plaques through acting on endothelial cells, smooth muscle cells or inflammatory cytokines. It weakens the adherent effect of monocytes to the endothelium and inhibits the chemotaxis or migration of several inflammatory cells. It can also inhibit smooth muscle cell proliferation and activation. These effects may be mediated by activating the AMP-activated protein kinase and Akt pathways (18,19). As the adventitia is becoming increasingly recognized as an important component in the progression of atherosclerosis, the present study investigated the effect of resveratrol on adventitial fibroblasts. The results demonstrated that resveratrol inhibited the proliferation and migration of adventitial fibroblasts, and induced cell apoptosis in a dose-dependent manner. The EdU assay showed that resveratrol reduced the DNA synthesis of adventitial fibroblasts *in vitro*. As the adventitia and adventitial fibroblasts are essential in the early stage of atherosclerosis prior to activation of endothelial cells or smooth muscle cells in the intima and medial layer of the aorta (2), the results of the present study suggested that the anti-atherosclerotic effect of resveratrol may be medicated, at least in part, through the direct inhibition of cell viability, proliferation and migration, and the induction of cell apoptosis on adventitial fibroblasts in the adventitia.

The results of the present study showed that the apparent inhibition of cell survival and induction of cell apoptosis by resveratrol on adventitial fibroblasts were mediated by the SIRT1 pathway. Inhibition of the SIRT1 pathway by siRNA successfully reversed the effects of resveratrol. SIRT1 is an NAD-dependent protein deacetylase, and the SIRT1 pathway is essential in the viability, proliferation and differentiation of various types of cells, including vascular smooth muscle cells and colon cancer cells (11,19). SIRT1 is able to modulate several downstream molecules, including the caspase and FOXO families (13,20). The interaction or balance among these pathways finally decides the fate of cells. It has been shown that the activation of SIRT1 in different cells may act in two ways; one is to protect cells from the apoptosis induced by various conditions through regulating the expression and activity of the FOXO family; the other is to induce the activation of the caspase family and promote cell apoptosis (21,22). It has been shown that SIRT1 inhibits angiotensin II-induced vascular smooth muscle cell hypertrophy through modulating nicotinamide adenine dinucleotide phosphate oxidase 1 and GATA binding protein 6 (23). Pretreatment with resveratrol inhibits adipocyte proliferation by inducing the overexpression of SIRT1 and subsequently reducing the expression of peroxisome proliferator-activated receptor-γ (24). The present study showed that resveratrol treatment upregulated the protein expression of SIRT1. As resveratrol is a natural activator of SIRT1 and the overexpression of SIRT1 may further activate caspase 9 (25), the upregulation of SIRT1 was in correlation with the increased cell apoptosis, decreased cell viability and altered cell proliferation observed.

This present study focused on alterations in the cell kinetics of adventitial fibroblasts following resveratrol treatment *in vitro*. However, atherosclerosis is a complicated process and several cell types are involved in this process, including endothelial cells, smooth muscle cells, adventitial fibroblasts and monocytes. The interactions among these cells lead to final plaque formation (3). A previous study showed that resveratrol has an overall anti-atherosclerotic effect on these various cell types *in vivo* (26), therefore, further investigations aim to investigate the *in vitro* effect of resveratrol on the interactions of these cells involved in atherosclerosis.

In conclusion, the present study demonstrated that resveratrol inhibited cell viability, DNA synthesis and migration, and induced apoptosis of the adventitial fibroblasts through activation of the SIRT1 pathway. As adventitial fibroblasts are important in the development of atherosclerosis, this may be a mechanism underlying the anti-atherosclerotic effect of resveratrol.

Acknowledgements

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