Aging reduces susceptibility of vascular smooth muscle cells to H_2O_2-induced apoptosis through the down-regulation of Jagged1 expression in endothelial cells

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Abstract. In addition to excessive proliferation, reduced apoptosis of vascular smooth muscle cells (VSMCs) plays a key role in aging-exaggerated neointima formation after vascular injury. Our previous studies have shown that impaired expression of Jagged1 in the endothelium may be a key event that leads to enhanced VSMC proliferation in the elderly. Here, we are the first to investigate whether the expression of Jagged1 in endothelial cells (ECs) may regulate apoptosis of VSMCs. We discovered that VSMCs co-cultured with senescent ECs exhibited decreased susceptibility to H_2O_2-induced apoptosis compared with those co-cultured with young ECs. Senescent ECs also displayed lower Jagged1 expression compared to young ECs, which was more evident after H_2O_2 stimulation. Overexpression of Jagged1 in senescent ECs significantly promoted H_2O_2-induced apoptosis in the co-cultured VSMCs, whereas silencing Jagged1 expression in young ECs reduced H_2O_2-induced apoptosis in the co-cultured VSMCs. Our studies also revealed that Jagged1 expressed in ECs exerted its pro-apoptotic activity by lowering expression of the anti-apoptotic protein Bcl-2. These results demonstrate that aging reduces the susceptibility of co-cultured VSMCs to H_2O_2-induced apoptosis through impaired Jagged1 expression in ECs.

Introduction

Epidemiologic studies and experimental data have shown that aging is an independent risk factor for the development and progression of cardiovascular diseases (1). Furthermore, aging exacerbates neointimal formation after arterial injury (2,3). However, the underlying mechanisms responsible for this phenomenon are undefined. Neointimal hyperplasia is characterized by the uncontrolled growth of vascular smooth muscle cells (VSMCs). Although the senescence of vascular cells contributes to the age-related regulation of the biological properties of VSMCs (4), reciprocal regulations between the vascular endothelial cells (ECs) and the VSMCs play a critical role in maintaining normal vascular function and adaptable responses to various types of stimulation (5). Our previous study showed that VSMCs co-cultured with older ECs exhibit a higher proliferation level than those VSMCs co-cultured with young ECs, which may explain the enhanced neointima formation after vascular injury in the elderly (3). As reported, both excessive VSMC proliferation and reduced VSMC apoptosis play a key role in the development and progression of neointimal hyperplasia (6-8). However, the regulatory effect of vascular ECs on the apoptosis of VSMCs remains unclear.

The Notch signaling pathway is an evolutionarily conserved mechanism that controls various cell fates through local cell-cell interactions during the development and life span of multicellular organisms (9). Notch critically influences cell proliferation, differentiation and apoptosis. In the mammalian vasculature, Notch receptors (Notch1 to 4) and their ligands (Delta-like1, 3 and 4 and Jagged1 and 2) are highly expressed in both ECs and VSMCs in distinct combinations (10). Previously, Linder *et al* reported that high Jagged1 expression was restricted to the regenerating endothelial wound edge, especially in regions adjacent to proliferative VSMCs, suggesting a cell-cell interaction related to Jagged1 (11). In this regard, we recently demonstrated that Jagged1 exerts effects on age-exaggerated neointimal formation after arterial injury, because the reduced expression of Jagged1 in the old ECs induces accelerated VSMC proliferation *in vivo* and *in vitro* (3). However, it is yet to be elucidated whether the decreased expression of Jagged1 in the old ECs affects the apoptosis of VSMCs.

In the present study, using a co-cultured system to recapitulate *in vivo* conditions, we sought to determine whether the impaired expression of Jagged1 in the ECs may regulate the H_2O_2-induced apoptosis of VSMCs.

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Materials and methods

Primary cell culture. Male Sprague-Dawley rats 3 (young) or 22 (old) months of age were purchased from the Experimental Animal Center of the Third Military Medical University, Chongqing, China. All protocols conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). Rat aortic ECs and VSMCs were isolated and subcultured as previously described (3). In brief, the thoracic aorta was removed from Sprague-Dawley rats and placed into a 100-mm culture dish (Corning, NY, USA) filled with serum-free low-glucose Dulbecco's modified Eagle's medium (DMEM-L, Hyclone, Logan, UT) on ice. For EC culture, the adipose tissue and adventitia of the aorta were removed. The aorta was placed intimal side down on a sterile plate containing 0.2% collagenase type I (Sigma, St. Louis, MO) and incubated at 37˚C for 30 min. Detached ECs were collected, cultured in DMEM-L supplemented with 20% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 75 μg/ml endothelial cell growth supplement (Sigma) and placed in a 50-ml culture flask (Corning). For VSMC culture, the adipose tissue, adventitia and endothelium of the aorta were removed, and the aorta was cut laterally into 1-mm^2 explants and placed in a flask. The growth medium for VSMCs contained DMEM-L with 20% dialyzed FBS, 1% antibiotic/antimycotic and 10 ng/ml platelet-derived growth factor (PDGF, Peprotech EC, London, UK). The flask was then placed in a humidified incubator at 37˚C with 5% CO_2. ECs at passage 1 were used to examine the expression of Jagged1. ECs and VSMCs at passages 3-5 were used in other experiments. More than 95% of the ECs and VSMCs in the culture were positive as determined by staining with antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against the von Willebrand factor, which is specifically expressed in ECs, and against α-smooth muscle actin (α-SM-actin), which is specifically expressed in VSMCs.

Construction and transfection of the plasmid and adenoviral vector. We constructed the rat Jagged1-expressing recombinant plasmid vector using pBOS-SN3T (kindly provided by G. Weinmaster, David Geffen School of Medicine at UCLA, CA, USA) and pCMV-IRES-GFP (kindly provided by Zhang Jin-yu, Institute of Immunology, the Third Military Medical University). Briefly, the SN3tag (encoding rat Jagged1) was isolated by *Xba*I digestion from the pBOS-SN3T vector and then inserted into the *Nhe*I site of pCMV-IRES-GFP. The resultant plasmid was named p-rJagged1.

Adenoviral vectors carrying rat Jagged1 siRNA were constructed. All target sequences of rat Jagged1 (rJagged1, GenBank accession no. NM_019147) were selected by the Internet-based small interfering RNA (siRNA) hairpin search engine (www.ambion.com). The constructs were i) starting nucleotide 483, GGTCAATTTGAGCTGGAGA, ii) starting nucleotide 525, GGAGAGTTACAGAATGGGA and iii) starting nucleotide 694, GGGGCAATACCTTCAATCT. The target sequences were chemically synthesized as complementary oligonucleotides (Genesil, Wuhan, China). Annealed oligonucleotides encoding sense and antisense strands linked by the loop sequence were subcloned into Ad-GS5 (Genesil). Adenoviral vectors with the highest RNA interference (RNAi) efficiency were selected in the preliminary experiments. The selected adenoviral vectors were transfected into 293 cells in a 6-well plate using Metafectene™ (Biontex). The virus was harvested 8 days after transfection and transferred into a T-75 flask with $2x10^6$ 293 cells, resulting in 2 ml of viral stock. The Ad-si/rJagged1 virus was titrated using the standard plaque assay. The titer for Ad-si/rJagged1 was $6x10^9$ plaque-forming units (pfu)/ml. The adenovirus expressed GFP under the CMV promoter, allowing for verification of the infection. An NSC (non-silencing control) sequence was generated in the same manner.

The recombinant plasmid p-rJagged1 or its empty vector pCMV-IRES-GFP were transfected into the cultured ECs (at 50% confluence), and rat Bcl-2 overexpression plasmid p-rBcl-2 or its empty vector pCMV-GFP (kindly provided by Dr Peng Wei at the 309 Hospital of PLA, Beijing, China) were transfected into the cultured VSMCs (at 50% confluence) using transfection reagent according to the manufacturer's instructions (Lipofectamine 2000, Invitrogen, CA, USA). Then, all cells were selected with G418 (600 g/l). Ad-si/rJagged1 or its non-silencing control, Ad-NSC, were transfected into the cultured ECs (at 50% confluence), and the adenovirus carrying rat Bcl-2 siRNA, Ad-si/rBcl-2 or its non-silencing control Ad-NSC (kindly provided by Dr Peng Wei) were transfected into the cultured VSMCs (at 50% confluence). Efficiency of transfection, determined by the measurement of GFP-positive cells, was at least 90%.

Co-culture of ECs with VSMCs. A co-culture system was created as previously described (12). Briefly, VSMCs were first seeded onto the outer side of the PET membrane of a Transwell insert (Corning, with pores of 0.4 mm in diameter) at a density of $1x10^6$ cells/insert. After 6 h, the insert was turned over and inserted into 6-well plates containing DMEM-L supplemented with 20% FBS; the inner side of the PET membrane was then plated with ECs at $1x10^7$ cells/insert. ECs and VSMCs could make cell-cell contact and medium communication through the pores of the membrane inserts (12).

Apoptosis assays. After co-culturing VSMCs with ECs for 24 h, hydrogen peroxide (H_2O_2) was added to some of the cultures. Trypsin was added to the lower chamber to harvest the VSMCs on the outer side of the PET membrane. For Annexin V and 7-amino-actinomycin D (7-AAD) assays, Annexin V binding and 7-AAD staining were carried out using the Annexin V-APC/7-AAD apoptosis detection kit (Keygen Biotechnology, Nanjing, China). Cells were washed twice and resuspended at a concentration of $1x10^6$ cells/ml. A volume of 500 μl of cells was mixed with 5 μl of Annexin V-APC and 7-AAD for 10 min at room temperature in the dark. After adding binding buffer (400 μl), cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences). For Hoechst 33342 staining, the ECs on the inner side of the PET membrane was removed with a cytology brush and washed twice with PBS. Then, the PET membrane was fixed with 4% paraformaldehyde for 1 h. The cells were then rinsed, incubated with 0.1% Triton X-100 for 2 min and washed twice. Hoechst 33342 (5 μg/ml in PBS) staining was performed as previously described (13).

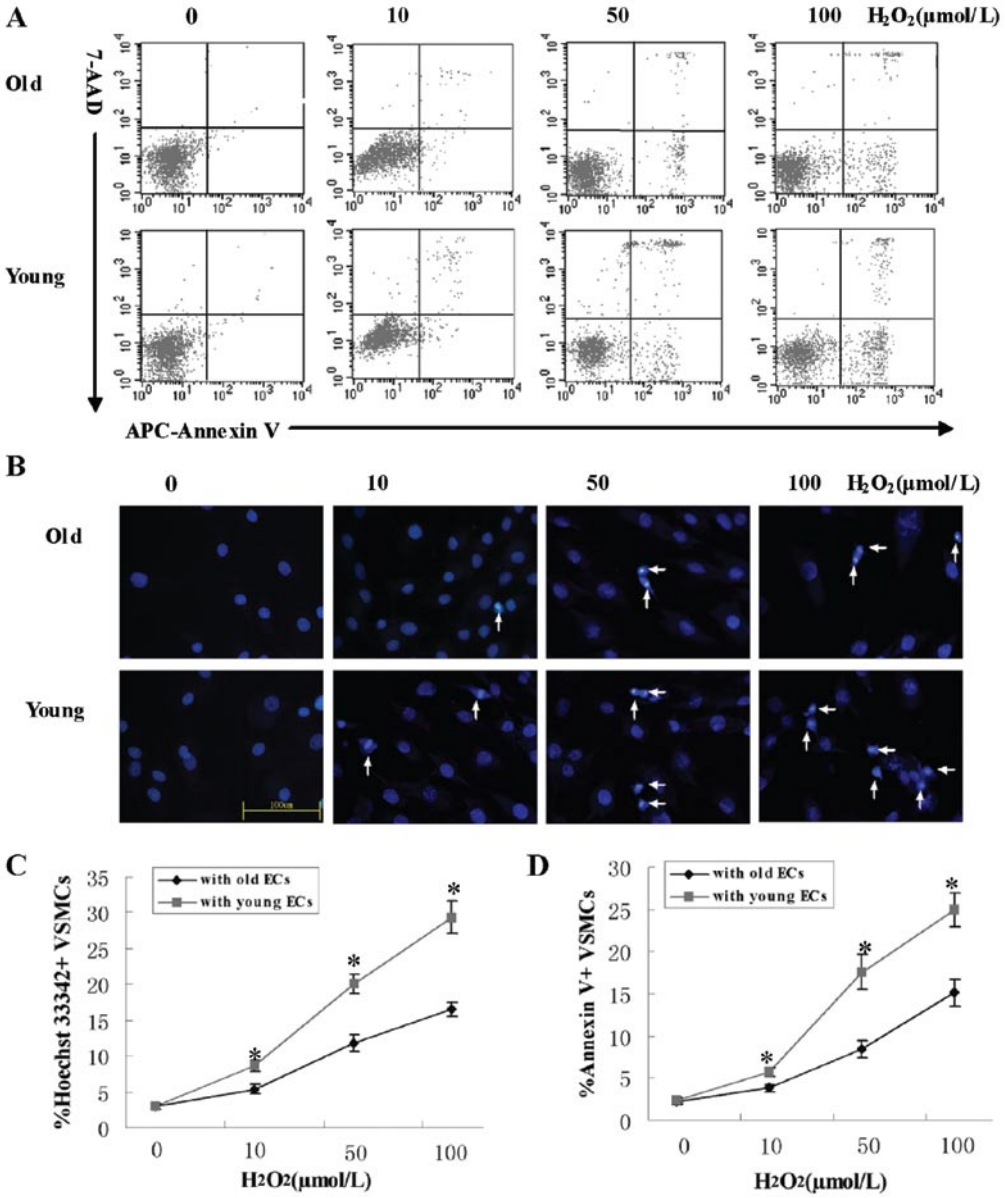

Figure 1. Influence of the co-cultured ECs on H_2O_2-induced apoptosis of the VSMCs. VSMCs were co-cultured with ECs isolated from young or old rats and exposed to H_2O_2 (0, 10, 50 and 100 μmol/l) for 24 h. (A) Representative photographs of apoptosis, as analyzed by FACS. (B) Representative photographs of Hoechst 33342 staining. The nuclei of apoptotic cells are indicated with arrows. (C) Quantification of the Annexin V-positive VSMCs. (D) Quantification of Hoechst-positive VSMCs. *P<0.05 vs. the with old ECs group. Data are presented as the mean ± SEM (n=6).

Western blot analysis. The expressions of Jagged1 and Bcl-2 were analyzed by Western blotting. Briefly, total cell proteins was separated by SDS-PAGE (10% polyacrylamide gel) and transferred to a PVDF membrane (Roche, Basel, Switzerland) by electroblotting for 3 h at 150 mA. The membrane was immunoblotted with antibodies against Jagged1 or Bcl-2 (both from Santa Cruz Biotechnology) at 4˚C overnight. Immunoreactivity was detected using the enhanced chemiluminescence reaction system (Amersham Pharmacia Biotech, NJ, USA) according to the manufacturer's directions. GADPH was used as a loading control. The expression of each protein was quantified by scanning densitometry and normalized against GAPDH. Data were expressed as a relative optical density value.

Statistical analysis. Data are presented as the mean ± SEM. The SPSS v.13.0 software was used for the statistical analyses. Differences among the groups were evaluated either by the unpaired Student's t-test or by one-way ANOVA followed by a post-hoc test. Values of P<0.05 denote statistical significance.

Results

Decreased H_2O_2-induced apoptosis in VSMCs co-cultured with senescent ECs compared to VSMCs co-cultured with young ECs. To investigate whether VSMC apoptosis may be different between young and old ECs, VSMCs were co-cultured with both EC types. Flow cytometry and Hoechst 33342 staining revealed similar apoptosis rates in VSMCs co-cultured with senescent and young ECs without H_2O_2 treatment (Fig. 1). Following the addition of the apoptosis inducer H_2O_2 (10, 50 and 100 μmol/l) into the co-culture system for 24 h (14), H_2O_2 increased the apoptosis rates of the co-cultured smooth

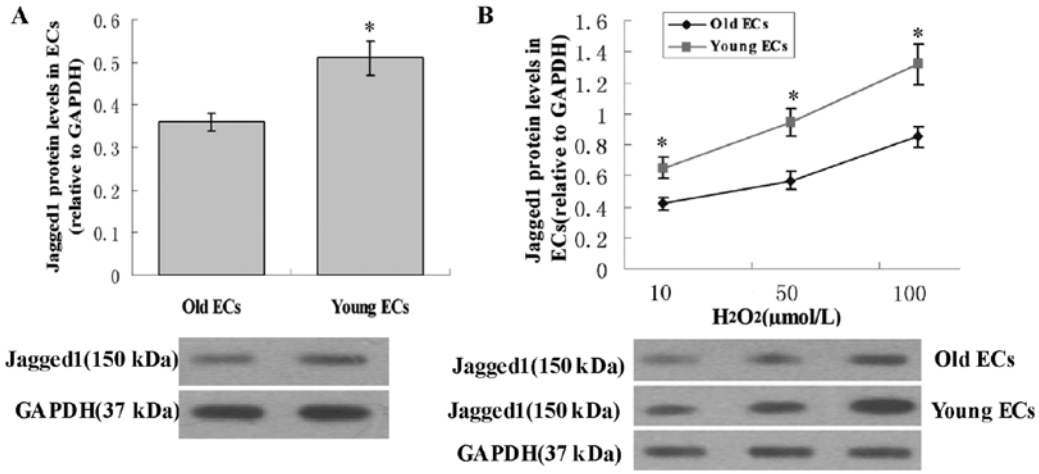

Figure 2. Jagged1 expression in cultured ECs isolated from old and young rats. The photographs are representative of 6 independent experiments. rJagged1 protein expression levels are normalized against the expression levels of the loading control, GAPDH. The results are expressed as mean ± SEM (n=6). (A) Western blotting assay and quantitative analysis of Jagged1 protein abundance without H_2O_2 stimulation. (B) Western blotting assay and quantitative analysis of Jagged1 protein abundance after stimulation with H_2O_2 (10, 50 and 100 μmol/l for 24 h). *P<0.05 vs. with old ECs group.

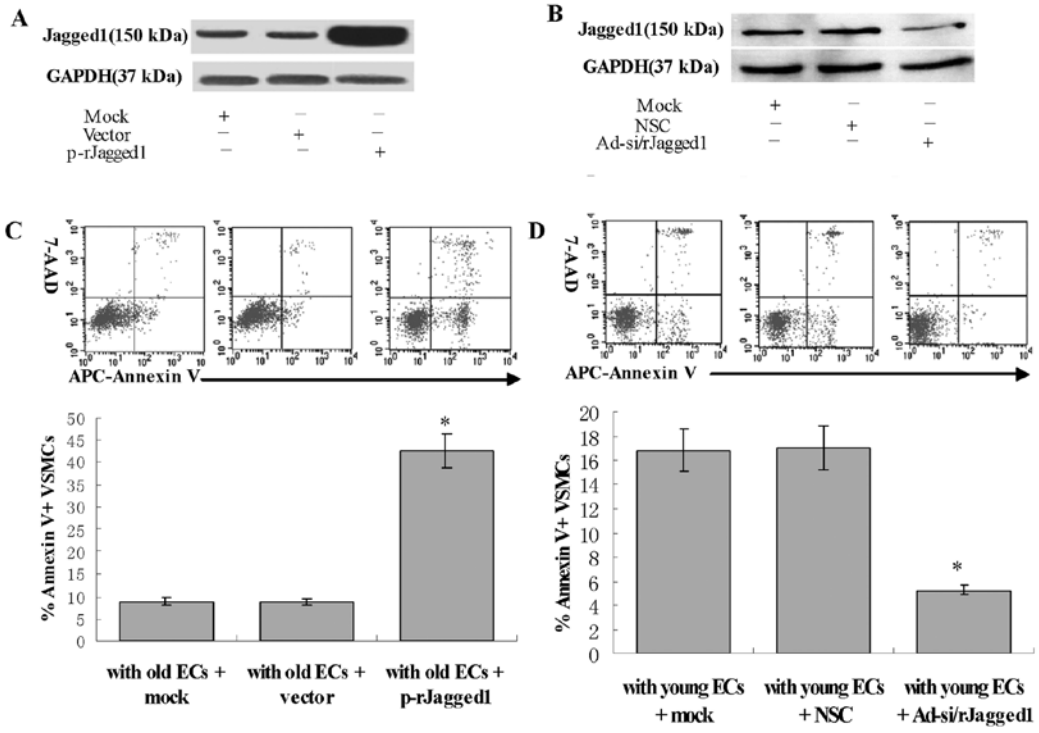

Figure 3. Effect of Jagged1 overexpression in senescent ECs or Jagged1 knockdown in young ECs on VSMC apoptosis. Cells were exposed to H_2O_2 (50 μmol/l) for 24 h. The photographs are representative of 6 independent experiments. rJagged1 protein expression levels are normalized against the expression levels of the loading control, GAPDH. The results are expressed as mean ± SEM (n=6). (A) Western blotting assay of Jagged1 expression in old ECs transfected with p-rJagged1, mock (without transfection) or vector. *P<0.05 vs. mock and vector. (B) Western blotting assay of Jagged1 expression of young ECs transfected with Ad-si/rJagged1, mock or non-silencing control (NSC). *P<0.05 vs. mock and NSC. (C) FACS analysis and quantification of the Annexin V-positive VSMCs that were co-cultured with old ECs transfected with p-rJagged1, mock or vector. *P<0.05 vs. mock and vector. (D) FACS analysis and quantification of the Annexin V-positive VSMCs that were co-cultured with young ECs transfected with Ad-si/rJagged1, mock or NSC. *P<0.05 vs. mock and NSC.

muscle cells in a dose-dependent manner. More interestingly, the VSMCs co-cultured with senescent ECs displayed lower apoptosis rates at each H_2O_2 concentration than those co-cultured with young ECs, and the differences between the two groups of co-cultured VSMCs increased with increases in the H_2O_2 concentration (Fig. 1). These results demonstrated that the VSMCs co-cultured with senescent ECs showed lower susceptibility to H_2O_2-induced apoptosis compared to those co-cultured with young ECs.

Senescent endothelial cells express less Jagged1 protein than young ECs, with the differences more evident after H_2O_2 stimulation. Using flow cytometry, our previous studies revealed a lower Jagged1-positive percentage in senescent

ECs than in young ECs under normal conditions (3). In the present study, Western blotting results showed that senescent ECs exhibited a lower Jagged1 protein level than young ECs under normal conditions (Fig. 2A). Interestingly, Western blotting showed that a 24-h treatment with H_2O_2 (10, 50 and 100 μmol/l) dose-dependently increased the protein level of Jagged1 in ECs. Moreover, senescent ECs showed lower Jagged1 protein levels at each H_2O_2 concentration than young ECs, and the differences between the two increased with increasing H_2O_2 concentration (Fig. 2B), suggesting that ECs elevate Jagged1 expression in response to oxidative stress. However, this response is impaired in aged individuals.

Role of endothelial Jagged1 in the H_2O_2-induced apoptosis of VSMCs. To further investigate whether age can regulate VSMC apoptosis via endothelial Jagged1, we first co-cultured VSMCs with senescent ECs overexpressing rJagged1. Senescent ECs were transfected with 2 μg of p-rJagged1 or the control empty vector plasmid and then co-cultured with VSMCs treated with 50 μmol/l H_2O_2 for 24 h. Western blotting results confirmed that p-rJagged1 transfection significantly increased Jagged1 levels in the senescent ECs compared with no transfection (mock) and vector transfection (2.76±0.18 vs. 0.55±0.05 vs. 0.58±0.06, respectively, n=6, P<0.05) (Fig. 3A). In addition, H_2O_2-induced apoptosis of VSMCs co-cultured with p-rJagged1-transfected senescent ECs was significantly increased compared to VSMCs in the mock or the vector-transfected groups (Fig. 3C).

We next co-cultured VSMCs with young ECs with silenced rJagged1 expression. Young ECs were first transfected with 20 pfu/cell of Ad-si/rJagged1 or the NSC and then co-cultured with VSMCs treated with 50 μmol/l H_2O_2 for 24 h. Western blotting results confirmed that transfection with Ad-si/rJagged1 significantly decreased the Jagged1 level in young ECs compared with the mock and the NSC-transfected young ECs (0.28±0.03 vs. 0.96±0.11 vs. 0.92±0.09, respectively, n=6, P<0.05) (Fig. 3B). In addition, the level of H_2O_2-induced apoptosis of VSMCs co-cultured with Ad-si/rJagged1-transfected young ECs was significantly lower than that of the VSMCs in the mock group or those co-cultured with the NSC-transfected young ECs (Fig. 3D). These results suggest that Jagged1 expressed in ECs is crucial for H_2O_2-induced apoptosis of VSMCs.

Endothelial Jagged1 exerts its pro-apoptotic function by reducing levels of Bcl-2 protein. Bcl-2 family proteins play a critical role in the regulation of apoptosis (14). To further study the mechanism by which Jagged1 expressed in ECs promotes VSMC apoptosis, we examined the potential role of Jagged1 in regulating the mitochondrial Bcl-2 family member. We first examined the anti-apoptotic protein Bcl-2 in VSMCs, and we found that VSMCs co-cultured with senescent ECs showed increased Bcl-2 protein levels, 24 h after treatment with H_2O_2 at 50 μmol/l compared with VSMCs co-cultured with young ECs (0.21±0.02 vs. 0.11±0.01, n=6, P<0.05).

To further demonstrate whether the Bcl-2 protein levels of VSMCs may be altered with respect to Jagged1 levels in ECs, we transfected senescent ECs with 2 μg p-rJagged1 or vector and co-cultured them with VSMCs. After H_2O_2 treatment at 50 μmol/l for 24 h, the VSMCs co-cultured with senescent ECs overexpressing Jagged1 showed decreased Bcl-2 protein

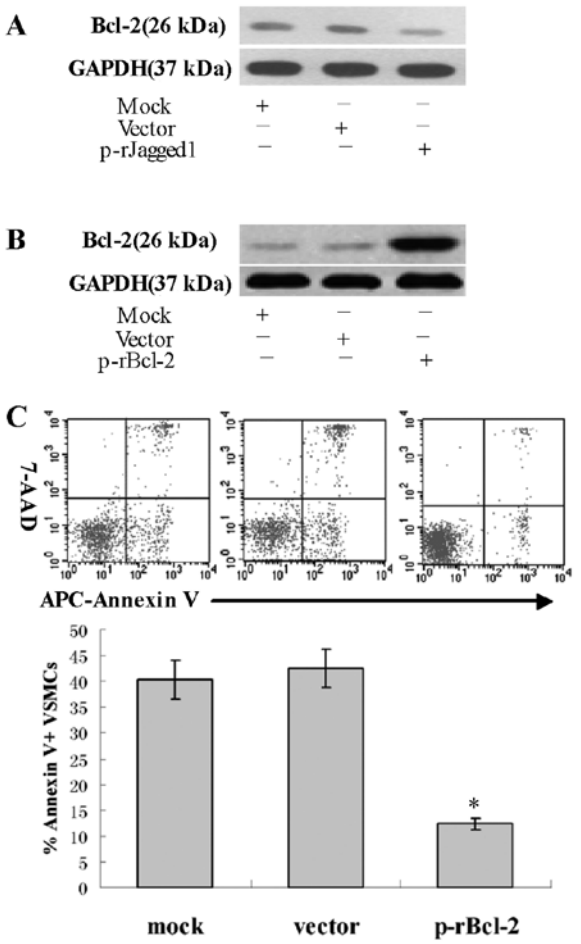

Figure 4. Endothelial Jagged1 exerts its pro-apoptotic function by lowering levels of Bcl-2 protein in VSMCs. Cells were exposed to H_2O_2 (50 μmol/l) for 24 h. The photographs are representative of 6 independent experiments. Bcl-2 protein expression levels are normalized against the expression levels of the loading control, GAPDH. The results are expressed as mean ± SEM (n=6). (A) Western blotting of Bcl-2 expression in VSMC co-cultured with old ECs transfected with p-rJagged1, mock and vector. *P<0.05 vs. mock and vector. (B) Western blotting of Bcl-2 expression in VSMCs transfected with p-rBcl-2, mock or vector that were co-cultured with old ECs transfected with p-rJagged1. *P<0.05 vs. mock and vector. (C) FACS analysis for apoptosis in VSMCs transfected with p-rBcl-2, mock or vector that were co-cultured with old ECs transfected with p-rJagged1. *P<0.05 vs. mock and vector.

levels compared with the VSMCs in the mock and vector-transfected groups (0.07±0.01 vs. 0.20±0.02 vs. 0.22±0.03, respectively, n=6, P<0.05) (Fig. 4A). Next, we overexpressed Bcl-2 in the VSMCs of the co-culture system using a plasmid and found that overexpression of Bcl-2 in VSMCs abolished both the decrease in the Bcl-2 protein levels (Fig. 4B) and the increase in VSMC apoptosis caused by the overexpression of Jagged1 in the co-cultured ECs (Fig. 4C).

Furthermore, we co-cultured VSMCs with young ECs transfected with 20 pfu/cell of Ad-si/rJagged1 or NSC. After a 24-h H_2O_2 treatment at 50 μmol/l, the VSMCs co-cultured with the young ECs with silenced Jagged1 exhibited increased Bcl-2 protein levels compared with the VSMCs in the mock and NSC-transfected groups (0.32±0.02 vs. 0.11±0.01 vs. 0.12±0.01, respectively, n=6, P<0.05) (Fig. 5A). We next used RNA interference technology to knock down Bcl-2 expression in the VSMCs in the above co-culture system, and we found that Bcl-2 knockdown in the VSMCs abolished both the

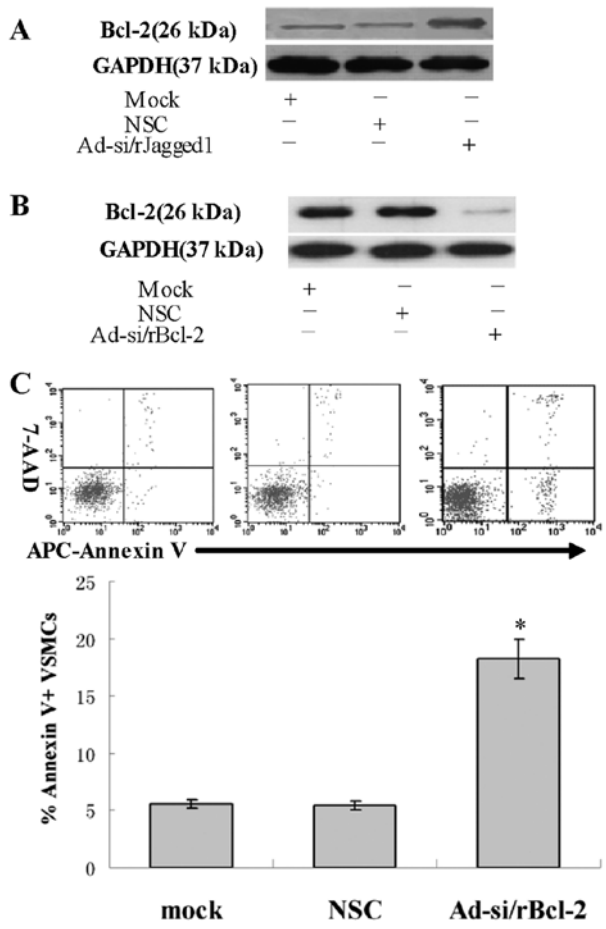

Figure 5. Endothelial Jagged1 exerts its pro-apoptotic function by lowering levels of Bcl-2 protein in VSMCs. Cells were exposed to H_2O_2 (50 μmol/l) for 24 h. The photographs are representative of 6 independent experiments. Bcl-2 protein expression levels are normalized against the expression levels of the loading control, GAPDH. The results are expressed as mean ± SEM (n=6). (A) Western blotting of Bcl-2 in VSMCs co-cultured with young ECs transfected with Ad-si/rJagged1, mock or NSC. *P<0.05 vs. mock and NSC. (B) Western blotting of Bcl-2 in VSMCs transfected with Ad-si/rBcl-2, mock or NSC that were co-cultured with young ECs transfected with Ad-si/rJagged1. *P<0.05 vs. mock and NSC. (C) FACS analysis for apoptosis in VSMCs transfected with Ad-si/rBcl-2, mock or NSC that were co-cultured with young ECs transfected with Ad-si/rJagged1. *P<0.05 vs. mock and NSC.

increase of the Bcl-2 protein levels (Fig. 5B) and the decrease of VSMC apoptosis (Fig. 5C) caused by Jagged1 knockdown in the co-cultured ECs. These results suggest that endothelial Jagged1 can promote apoptosis by reducing the Bcl-2 protein levels in VSMCs.

Discussion

The major finding of the present study is that old ECs reduce the susceptibility of VSMCs to H_2O_2-induced apoptosis compared to young ECs via down-regulating Jagged1 expression. We arrived at this conclusion based on several lines of evidence. First, smooth muscle cells co-cultured with senescent ECs exhibited a decreased susceptibility to H_2O_2-induced apoptosis when compared with those VSMCs co-cultured with young ECs. Second, senescent ECs displayed lower Jagged1 expression levels than young ECs, which was more evident after H_2O_2 stimulation. Third, H_2O_2-induced apoptosis in VSMCs was enhanced by the overexpression of Jagged1 in ECs, whereas it was decreased by silencing Jagged1 expression in ECs. Fourth, Jagged1 expressed in ECs decreased the Bcl-2 protein levels in VSMCs. The elevated apoptosis caused by endothelial Jagged1 overexpression was abolished by raising the Bcl-2 protein levels in smooth muscle cells, which was achieved by overexpressing the rBcl-2 plasmid. On the contrary, lowering the Bcl-2 protein levels in VSMCs through an adenovirus carrying rBcl-2 siRNA abolished the reduction in apoptosis induced by silencing endothelial Jagged1 expression. These findings suggest that the reduced expression of Jagged1 in old ECs attenuates the apoptosis of co-cultured VSMCs, which may account for aging-exaggerated neointima formation.

The decreased susceptibility to apoptosis is one of the phenotypic changes in VSMCs during neointimal formation, which contributes to the increased neointima seen in aging animals in response to injury (2). Furthermore, retarded EC recovery may contribute to enhanced intimal hyperplasia (15). Thus, it is necessary to investigate the effects of ECs on the apoptosis of VSMCs. Using a co-culture system to recapitulate *in vivo* conditions in this study, we observed no difference in the apoptosis of smooth muscle cells co-cultured with senescent and young ECs under basal conditions. However, when exposed to H_2O_2, which is increased in vascular injury and can induce apoptosis of VSMC (13), the increase in the amount of apoptosis was greater in the VSMCs co-cultured with the young ECs than in those VSMCs co-cultured with the old ECs. Because the ECs and VSMCs are both in a proliferative state in the medium containing fetal bovine serum and PDGF, they behave like vascular cells *in vivo* after arterial injury. These data collectively suggest that aging-attenuated H_2O_2-induced apoptosis of VSMCs may account for exaggerated neointima formation after arterial injury in aging animals.

Notch signaling is an evolutionarily conserved mechanism to control cell fates, including differentiation, proliferation and apoptosis through close-range cell interactions. Notch signaling is significantly involved in the responses of the vasculature to injury and growth stimulation (16). Jagged1, a ligand for Notch receptors, is abundantly present in ECs. Jagged1 in ECs can regulate the differentiation of mural cells (VSMCs, pericytes or fibroblasts) (17) and the proliferation of VSMCs (3). However, its effect on apoptosis, another cell-fate decision, in VSMCs remains unknown. Consistent with our previous reports, we observed that expression of Jagged1 in old ECs was impaired compared to young ECs. Interestingly, the present study showed that the increase in Jagged1 expression in senescent ECs was significantly lower than that in young ECs following H_2O_2 stimulation. Overexpression of Jagged1 in senescent ECs overtly enhanced H_2O_2-induced apoptosis in the co-cultured smooth muscle cells, whereas silencing Jagged1 in young ECs reduced H_2O_2-induced apoptosis in the co-cultured smooth muscle cells, suggesting that Jagged1 plays a pro-apoptotic role in the EC-VSMC interaction and that reduced VSMC apoptosis in the elderly is related to the down-regulation of Jagged1 in ECs *in vitro*. Several reports have stated that Notch activation plays a pro-apoptotic role in some cells, including mammalian B-lymphoid cells (18), neural progenitor cells (19), erythroleukemia cells (20) and

ovarian cancer cells (21), while others have shown that Notch plays an anti-apoptotic role in the HeLa cell line (22), breast epithelial cells (23) and T cell acute lymphoblastic leukemia cells (24). The highly cell type-specific effect of Notch activation on apoptotic cell death may account for the controversy.

Mitochondrial apoptotic signaling plays an essential role in programmed cell death, which is controlled by pro-apoptotic and anti-apoptotic members of the Bcl-2 protein family. Our data show that the smooth muscle cells co-cultured with senescent ECs exhibited higher Bcl-2 protein levels than those VSMCs co-cultured with young ECs. Overexpression of Jagged1 in senescent ECs markedly decreased the Bcl-2 protein levels. The elevated apoptosis caused by endothelial Jagged1 overexpression was abolished by raising the Bcl-2 protein levels by overexpressing the rBcl-2 plasmid. Conversely, lowering the Bcl-2 protein levels in VSMCs via an adenovirus carrying rBcl-2 siRNA abolished the reduction in apoptosis induced by silencing endothelial Jagged1 expression. Increasing the Bcl-2 protein expression protects cells from apoptosis (14). However, the down-regulation of Bcl-2 was observed in Notch1-overexpressed cells (25). Because endothelial-expressed Jagged1 can activate Notch in adjacent VSMC (17), we therefore infer that suppressing Jagged1 in the ECs may prevent Notch activation in adjacent VSMCs and cause up-regulation of Bcl-2 expression.

In summary, our results demonstrate that aging reduces the susceptibility of co-cultured VSMCs to H_2O_2-induced apoptosis by impairing Jagged1 expression in ECs. Mitochondrial apoptotic signaling may be involved in endothelial Jagged1-induced VSMC apoptosis, suggesting that Jagged1 in the endothelium may act as an apoptosis activator to co-cultured VSMCs. These findings will enable us to gain a greater understanding of the mechanisms of aging-exaggerated neointima formation after arterial injury.

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