Original article

Resveratrol-induced augmentation of telomerase activity delays senescence of endothelial progenitor cells

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Background Previous studies have shown that resveratrol increases endothelial progenitor cell (EPC) numbers and functional activity. Increased EPC numbers and activity are associated with the inhibition of EPC senescence. In this study, we investigated the effect of resveratrol on the senescence of EPCs, leading to potentiation of cellular function.
Methods EPCs were isolated from human peripheral blood and identified immunocytochemically. EPCs were incubated with resveratrol (1, 10, and 50 μmol/L) or control for specified times. After *in vitro* cultivation, acidic β-galactosidase staining revealed the extent of senescence in the cells. To gain further insight into the underlying mechanism of the effect of resveratrol, we measured telomerase activity using a polymerase chain reaction (PCR)-enzyme-linked immunosorbent assay (ELISA) technique. Furthermore, we measured the expression of human telomerase reverse transcriptase (hTERT) and the phosphorylation of Akt by immunoblotting.
Results Resveratrol dose-dependently inhibited the onset of EPC senescence in culture. Resveratrol also significantly increased telomerase activity. Interestingly, quantitative real-time PCR analysis demonstrated that resveratrol dose-dependently increased the expression of the catalytic subunit, hTERT, an effect that was significantly inhibited by pharmacological phosphatidylinositol 3-kinase (PI3-K) blockers (wortmannin). The expression of hTERT is regulated by the PI3-K/Akt pathway; therefore, we examined the effect of resveratrol on Akt activity in EPCs. Immunoblotting analysis revealed that resveratrol led to dose-dependent phosphorylation and activation of Akt in EPCs.
Conclusion Resveratrol delayed EPCs senescence *in vitro*, which may be dependent on telomerase activation.
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Endothelial progenitor cells (EPCs) have been isolated from peripheral blood and shown to play an important role in endothelium maintenance, being implicated in both reendothelialization and in neovascularization.[1,2] In contrast to differentiated endothelial cells, EPCs, when transplanted, successfully enhance vascular development by *in situ* differentiation and proliferation within ischemic organs.[3] There is growing evidence that these precursors enhance angiogenesis and vascular repair, diminish atherosclerosis and increase ventricular function after myocardial infarction.[4] The beneficial properties of EPCs are of therapeutic value for targeted regeneration of ischemic tissue. However, various risk factors for coronary artery disease (CAD), such as diabetes, hypercholesterolemia, hypertension, and smoking, impair the activities and accelerate senescence of EPCs in healthy volunteers and in patients with CAD,[5] suggesting that EPCs are necessary and required in vascular repair after injury.[6]

Recent studies have demonstrated that increased EPCs numbers and activity is associated with EPCs senescence.[7] Cellular aging or senescence is characterized by cell cycle arrest, and can be triggered by different pathways. Loss of telomerase activity has been suggested to constitute the molecular clock that triggers cellular senescence. Interestingly, Murasawa et al[8] demonstrated that the expression of constitutive human telomerase reverse transcriptase (hTERT) in cultured EPCs enhances their mitogenic and migratory activities, delays senescence, and augments neovascularization in murine hind-limb ischemia. Moreover, Assmus et al[9] demonstrated that 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors reduce senescence and increase proliferation of EPCs via regulation of cell cycle regulatory genes.

Estrogens have been shown to reduce EPC senescence through augmentation of telomerase activity.[10] Resveratrol has been shown to bind to and activate gene transcription by the estrogen receptor subtypes α and β in estrogen-sensitive tissues and cell lines.[11] As an important antioxidant in red wine, resveratrol is likely to contribute to the potential of red wine to prevent human cardiovascular disease. Recently, Howitz et al[12] revealed that resveratrol extended the lifespan of *Saccharomyces*

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cerevisiae. In addition, our previous studies showed that resveratrol increased EPC numbers and functional activity.[13] In the present study, we investigated whether resveratrol was able to prevent senescence in EPCs by augmentation of telomerase activity.

METHODS

Isolation of mononuclear and cultivation of EPCs

EPCs were cultured according to previously described techniques.[3,5] Briefly, total mononuclear cells (MNC) were isolated from the peripheral blood of healthy human volunteers by Ficoll density gradient centrifugation (1.077; Sigma, USA). A total of 10^6 MNC/cm^2 were plated on 24-well plates coated with human fibronectin (2 μg/cm^2; Chemicon International, USA) and maintained in Medium 199 (M199; Sigma) supplemented with 20% fetal calf serum (HyClone, USA), vascular endothelial growth factor (VEGF; 10 μg/L; CytoLab/Peprotech Asia, Israel), bovine brain extract (12 μg/ml; Sigma), penicillin (100 U/ml), and streptomycin (100 μg/ml). After 4 days in culture, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), new medium was applied, and the culture was maintained for 7 days. Volunteers had no risk factors for CAD, including hypertension, diabetes, smoking, a positive family history of premature CAD and hypercholesterolemia, and were free of wounds, ulcers, retinopathy, recent surgery, inflammation, malignant diseases, and medications that may influence EPCs kinetics. Informed consent was obtained from all volunteers (aged between 25 and 35) and all procedures were performed in accordance with national and international laws and policies.

Human EPCs identification

Fluorescent chemical detection of EPCs was performed on attached MNC after 7 days in culture. Direct fluorescent staining was used to detect dual binding of fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin (UEA-1; Vector Laboratories, Burlingame, CA, USA) and dioctadecyl-3,3,3',3'-tetrametylindocarbocyanine (DiI)-labeled acetylated low-density lipoprotein (acLDL; Molecular Probes, Eugene, OR, USA) (DiLDL). Briefly, attached cells were first incubated with acLDL (2.4 μg/ml) at 37°C for 1 hour and then fixed with 2% paraformaldehyde for 10 minutes. After washing, cells were reacted with UEA-1 (10 μg/ml) for 1 hour. Following staining, samples were viewed with an inverted fluorescent microscope (Leica, Wetzlar, Germany), and initial observations were further confirmed by laser scanning confocal microscopy (LSCM; Leica). Cells that were doubly fluorescent were identified as differentiating EPCs.[3] Two independent investigators evaluated the number of EPCs per well by counting 15 randomly selected high-power fields (×200), under an inverted fluorescent microscope.

Attached MNC were further identified by flow cytometry analysis. The MNC were detached with 1 mmol/L EDTA, followed by repeated gentle flushing through a pipette tip. Cells (2×10^5) were incubated for 30 minutes at 4°C with phycoerythrin-conjugated anti-AC133 (Miltenyi Biotech, Germany), phycoerythrin-conjugated anti-human CD34 (Immunotech, France), and FITC-conjugated anti-VEGFR (vascular endothelial growth factor receptor)-2 (Abcam, Cambridge, USA). Isotype-identical antibodies served as negative controls. After treatment, cells were fixed in 1% paraformaldehyde. Quantitative fluorescence-activated cell sorting (FACS) was performed on a FACS Vantage SE flow cytometer (Becton Dickinson, USA).

RNA isolation and quantitative real-time polymerase chain reaction

To investigate the effect of resveratrol on the expression of hTERT mRNA, EPCs at day 7 were treated with resveratrol at 1, 10 or 50 μmol/L for 4 hours, with or without pretreatment with wortmannin (10 nmol/L) for 30 minutes. Total RNA was extracted using the RNeasy RNA extraction kit (Qiagen). Briefly, cells were lysed in guanidinium isothiocyanate buffer, and RNA was purified according to the manufacturer's instructions. The purified RNA was suspended in diethyl pyrocarbonate-treated water. To generate cDNA, 1 mg total RNA was treated with DNaseI (Ambion, Austin, Texas, USA) to remove any contaminating genomic cDNA. The DNaseI-treated RNA (100 ng) was then converted into cDNA using murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies). The transcribed cDNA was then used for polymerase chain reaction (PCR) amplification to estimate the expression of hTERT. Two specific primers matching the published sequences were used to identify and amplify hTERT (sense primer: 5'-CAG ATT CGC CAT TGT TCA CCC-3'; antisense primer: 5'-TTT ACT CCC ACA GCA CCT CCC-3'). The PCR product was 198 bp in length. The reaction mixtures were heated at 94°C for 60 seconds, followed by 28 cycles of 94°C for 15 seconds, 60°C for 15 seconds and 72°C for 30 seconds. Subsequently, PCR products were visualized on agarose gels stained with 1.5% ethidium bromide. β-Actin was amplified as a control.

Immunoblotting

After 7 days of culture, EPCs were treated with different concentrations of resveratrol (1, 10 and 50 μmol/L) for 4 hours to detect Akt activity; EPCs were treated with different concentrations of resveratrol (1, 10 and 50 μmol/L) for 24 hours to detect hTERT. Cellular proteins were prepared and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane (Millipore), as described previously.[14] Membranes were blocked by incubation for 2 hours in Tris-buffered saline (10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl) containing 0.1% (v/v) Tween 20 and 5% (v/v) non-fat dry milk, followed by a 2-hour incubation at room temperature with anti-hTERT Ab (Calbiochem), rabbit polyclonal anti-phospho-Akt-Ser473, or anti-Akt

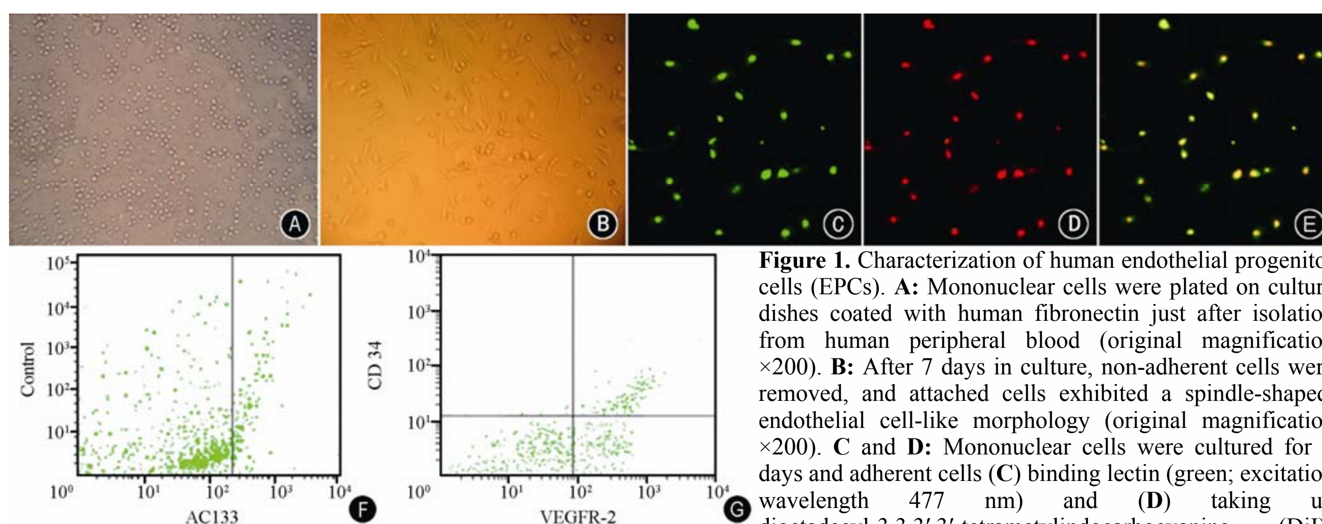

Figure 1. Characterization of human endothelial progenitor cells (EPCs). **A:** Mononuclear cells were plated on culture dishes coated with human fibronectin just after isolation from human peripheral blood (original magnification ×200). **B:** After 7 days in culture, non-adherent cells were removed, and attached cells exhibited a spindle-shaped, endothelial cell-like morphology (original magnification ×200). **C** and **D:** Mononuclear cells were cultured for 7 days and adherent cells (**C**) binding lectin (green; excitation wavelength 477 nm) and (**D**) taking up dioctadecyl-3,3,3′,3′-tetrametylindocarbocyanine (DiI)-labeled acetylated low-density lipoprotein (DiLDL; red; excitation wavelength 543 nm) were assessed under a laser scanning confocal microscope (original magnification ×200). **E:** Double positive cells for DiLDL-uptake and lectin (yellow). **F** and **G:** Adherent cells positive for AC133 (**F**) and CD34/vascular endothelial growth factor receptor (VEGFR)-2 (**G**) by flow cytometry analysis.

antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA). The filters were washed extensively in Tris-buffered saline containing 0.1% (v/v) Tween 20, before incubation for 1 hour with a secondary anti-rabbit antibody conjugated to horseradish peroxidase. Membranes were then washed and developed using enhanced chemiluminescence substrate (Amersham Pharmacia Biotech). The profile of each band was plotted using NIH Image, and the densitometric intensity corresponding to each band was measured as an intensity value.

Senescence-associated β-galactosidase activity assay

After the addition of several concentrations of resveratrol into EPCs every 48 hours, starting at day 4, EPCs at day 14 were harvested and senescence-associated β-galactosidase (SA-β-gal) activity was measured with a β-Galactosidase Staining Kit (Bio Vision) as described previously.[15] Briefly, EPCs were washed in PBS, fixed for 3 minutes (room temperature) in 2% paraformaldehyde, washed, and incubated for 24 hours at 37°C (no CO2) with fresh SA-β-gal staining solution. This comprised 1 mg/ml 5-bromo-4-chloro-3-indyl β-D-galactopyranoside (X-gal), 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L NaCl, 2 mmol/L $MgCl_2$, 0.01% sodium deoxycholate and 0.02% Nonidet-40. EPCs were counterstained with 4′,6-diamino-phenylindole (0.2 μg/ml in 10 mmol/L Tris-HCl, pH 7.0, 10 mmol/L EDTA, 100 mmol/L NaCl) for 10 minutes to determine total cell number.

Telomeric repeat amplification protocol assay

For quantitative analyses of telomerase activity, the telomeric repeat amplification protocol assay, in which the telomerase reaction product is amplified by PCR, was performed using the TeloTAGGG PCR ELISAPLUS kit (Roche Molecular Biochemicals), according to the manufacturer's procedure, as described previously.[16]

Statistical analysis

All data are presented as the mean ± standard error (SE) of at least five independent experiments. Statistical analysis was performed by an unpaired Student's *t* test for single comparisons or by one-way analysis of variance (ANOVA) for multiple test by SPSS 10.0 (SPSS, Chicago, USA). Probability values were considered statistically significant at $P<0.05$.

RESULTS

Characterization of human EPCs

Total MNCs isolated and cultured for 7 days resulted in a spindle-shaped, endothelial cell-like morphology (Figure 1). EPCs were characterized as adherent cells positive for DiLDL uptake and lectin binding using LSCM (Figure 1). Cells were further identified by demonstrating the expression of AC133 ((21.6±5.3)%), CD34 ((29.2±5.9)%), and VEGFR-2 ((59.7±8.8)%; Figure 1) as characterized by others.[6]

Resveratrol stimulates the expression of hTERT mRNA and hTERT protein in human EPCs

To investigate the effect of resveratrol on the expression of hTERT mRNA, EPCs were treated with different concentrations of resveratrol (1, 10 and 50 μmol/L) for 4 hours, and reverse transcriptase-PCR analysis was performed. We found that resveratrol stimulated the expression of hTERT mRNA in a dose-dependent manner, with a maximal increase at 50 μmol/L (Figure 2). Interestingly, pretreatment with the PI3-K blocker wortmannin, significantly attenuated the increase in hTERT mRNA induced by resveratrol.

To investigate the effect of resveratrol on the expression of hTERT protein, EPCs were treated with different

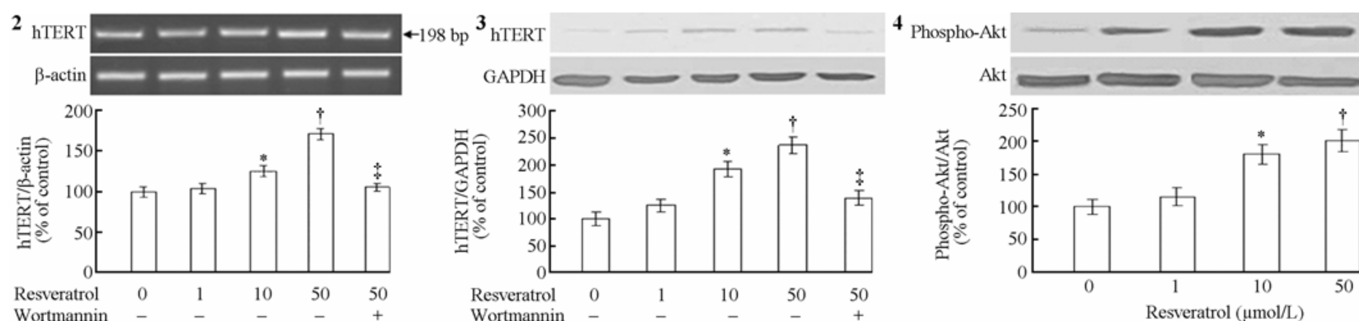

Figure 2. Induction of human telomerase reverse transcriptase (hTERT) mRNA in EPCs by resveratrol. EPCs at day 7 were treated with the indicated concentrations of resveratrol for 4 hours, and total RNA was isolated from these cells. Representative images from five independent experiments are shown (top). The bar graph shows hTERT mRNA normalized for β-actin (bottom). Data are expressed as a ratio of the test value to the value for unstimulated cells. $^*P$ <0.05, $^†P$ <0.01 compared with control. $^‡P$ <0.01 compared with resveratrol (50 μmol/L).

Figure 3. Effect of resveratrol on hTERT protein expression. Endothelial progenitor cells (EPCs) at day 7 were stimulated for 24 hours with the indicated doses of resveratrol. The expression of hTERT was analyzed by immunoblotting with anti-hTERT Ab. Representative images from five independent experiments are shown (top). The bar graph shows hTERT normalized for GAPDH (bottom). Data are expressed as a ratio of the test value to the value for unstimulated cells. $^*P$ <0.05 and $^†P$ <0.01 compared with control. $^‡P$ <0.01 compared with resveratrol (50 μmol/L).

Figure 4. Effect of resveratrol on Akt phosphorylation. Endothelial progenitor cells (EPCs) at day 7 were stimulated for 4 hours with the indicated doses of resveratrol, and phosphorylation of Akt was determined with a phospho-specific Akt antibody. Representative blots from five independent experiments are shown (top). The bar graphs show phospho-Akt-normalized Akt (bottom). Data are expressed as a ratio of the test value to the value for unstimulated cells (set at 100%). $^*P$ <0.05 and $^†P$ <0.01 compared with the control.

concentrations of resveratrol (1, 10 and 50 μmol/L) for 24 hours before immunoblotting analysis was performed. We found that resveratrol stimulated the expression of hTERT protein in a dose-dependent manner, with a maximal increase at 50 μmol/L (Figure 3).

The expression of hTERT mRNA is regulated by the PI3-K/Akt pathway;[17] therefore, we examined the effect of resveratrol on Akt activity in EPCs. EPCs were stimulated with several different doses of resveratrol for 24 hours and immunoblots were performed with a phosphospecific Akt antibody directed at the Ser473 phosphorylation site. As illustrated in Figure 4, stimulation with resveratrol led to dose-dependent phosphorylation of Akt.

Resveratrol prevents EPCs senescence

To assess the onset of senescence, acidic β-galactosidase was detected as a biochemical marker of the acidification typical for the onset of cellular senescence. Prolonged cultivation of EPCs resulted in an increase in SA-β-gal-positive cells (Figure 5). Co-incubation with resveratrol significantly inhibited the increase in SA-β-gal-positive cells (Figure 5). The inhibition of EPCs senescence occurred dose-dependently, with a maximal effect achieved at 50 μmol/L. By day 14, the proportion of SA-β-gal positive cells in 50 μmol/L resveratrol-treated EPCs was markedly decreased compared with that in controls (*P* <0.01) (Figure 5).

Effects of resveratrol on telomerase activity in EPCs

Cellular senescence is critically influenced by telomerase, which elongates telomeres, thereby counteracting the reduction in telomere length induced by each cell division. Therefore, we measured telomerase activity using the TeloTAGGG Telomerase PCR ELISA kit. As demonstrated in Figure 6, resveratrol dose-dependently increased telomerase activity.

DISCUSSION

Recent studies have demonstrated that atherosclerotic risk factors inversely correlate with the number of EPCs and impair their function to varying degrees. They also increases the rate of EPCs senescence.[5,18,19] Endothelial dysfunction and cell loss are prominent features in cardiovascular disease.[20] Given that EPCs play a role in postnatal neovascularization, an improved understanding of the mechanisms that regulate the biological activity of EPCs may provide new insights into the pathogenesis of vasculogenesis. We previously demonstrated that resveratrol significantly induces EPC proliferation and migration, stimulates G1/S transition, and promotes angiogenesis *in vitro*. The mechanisms involved in the increase in the number and activity of EPCs are not clear. In the present study, we have shown, for the first time, that resveratrol prevents the onset of EPCs senescence, which may be related to the activation of telomerase, leading to the increment of cellular function.

The mechanisms by which resveratrol delays the onset of senescence in EPCs remain unclear. Telomerase is a cellular reverse transcriptase that catalyzes the synthesis and extension of telomeric DNA, which delays the development of senescence.[21,22] Telomerase activity is expressed in a highly regulated manner in certain somatic cell populations, such as lymphocytes and hematopoietic stem cells. There is a good correlation between the expression of hTERT mRNA and the presence of telomerase activity in extracts of tissue culture cells, and normal and cancer tissues.[23] In the present study, we showed that telomerase activity in EPCs is upregulated by

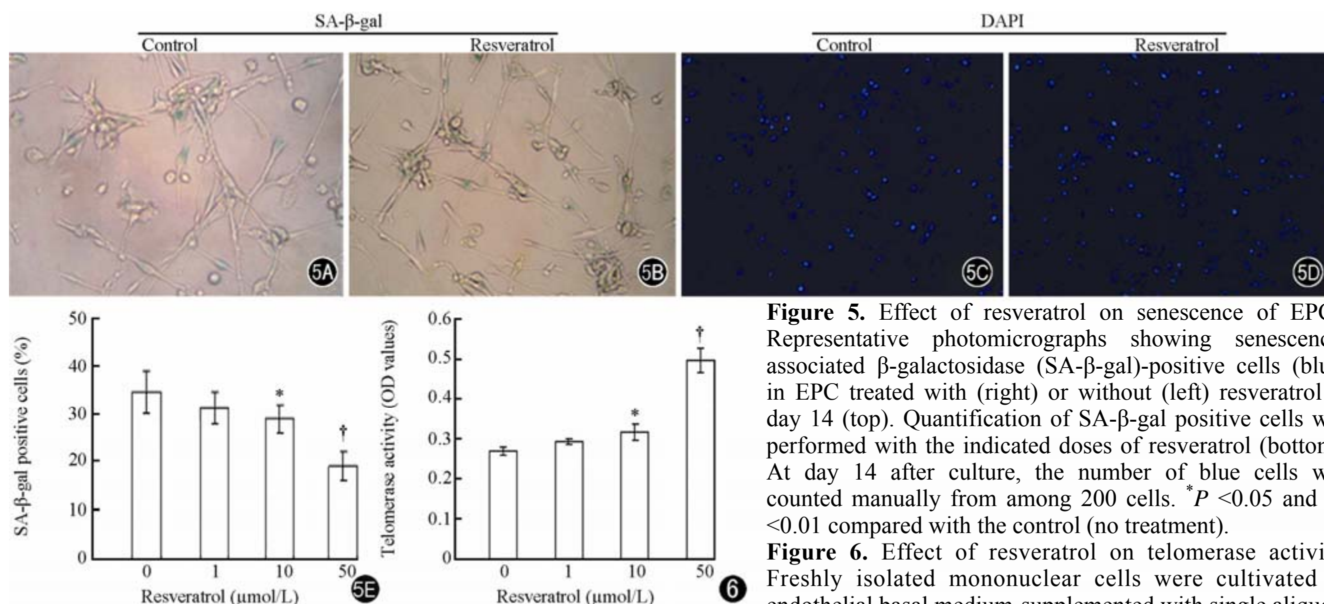

Figure 5. Effect of resveratrol on senescence of EPCs. Representative photomicrographs showing senescence-associated β-galactosidase (SA-β-gal)-positive cells (blue) in EPC treated with (right) or without (left) resveratrol at day 14 (top). Quantification of SA-β-gal positive cells was performed with the indicated doses of resveratrol (bottom). At day 14 after culture, the number of blue cells was counted manually from among 200 cells. *P <0.05 and †P <0.01 compared with the control (no treatment).

Figure 6. Effect of resveratrol on telomerase activity. Freshly isolated mononuclear cells were cultivated in endothelial basal medium-supplemented with single aliquots of EGM-2MV. The six-well plates were replated at day 4. After an additional 3 days of cultivation, resveratrol was added for 24 hours and telomerase activity was measured. *P <0.05 and †P <0.01 compared with control (no treatment).

treatment with resveratrol. Furthermore, we demonstrated that this activation accompanied by upregulation of the hTERT mRNA and increment of the hTERT protein. Thus, we speculate that resveratrol delays the onset of EPCs senescence, most likely through telomerase activation. However, with regard to senescence, the structure of the telomere appears at least as important as its absolute length, in relation to telomere function. In addition, we cannot rule out the possibility that a telomere-independent mechanism regulates replicative senescence. Further studies are required to elucidate the mechanisms underlying the inhibitory effects of resveratrol on senescence in EPCs.

The molecular mechanisms by which resveratrol activated telomerase activity remain determined. Recent studies have demonstrated that the serine/threonine kinase Akt, also named protein kinase B, enhanced telomerase activity through phosphorylation of TERT in human umbilical cord endothelial cells and melanoma cells.[24] Besides the direct phosphorylation of TERT, Akt might also act by increasing the activity of the endothelial nitric oxide synthase, as nitric oxide has been demonstrated to activate telomerase and delay endothelial cell senescence.[25] Klinge et al[26] found that resveratrol stimulated nitric oxide production by increasing estrogen receptor α-Src-caveolin-1 interaction and phosphorylation in human umbilical vein endothelial cells. In this study, resveratrol significantly increased Akt phosphorylation. Taken together, our data indicate that resveratrol increased Akt phosphorylation in EPCs, which might lead to increased phosphorylation of TERT. The augment of phosphorylation of TERT might increase telomerase activity and thereby delay the onset of EPCs senescence. Further studies are required to validate this hypothesis.

In conclusion, this study demonstrated that resveratrol delays the onset of senescence in EPCs, which may be related to activation of telomerase and Akt phosphorylation. The inhibition of EPC senescence by resveratrol, *in vitro*, may improve the functional activity of EPCs for potential cell therapy.

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