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Puerarin reduces endothelial progenitor cells senescence through augmentation of telomerase activity

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ABSTRACT

Endothelial progenitor cells (EPCs) play an important role in both reendothelialization and neovascularization. Ex vivo expansion of EPCs might be useful for potential clinical cell therapy of ischemic diseases. However, ex vivo cultivation of EPCs leads to rapid onset of EPCs senescence, thereby severely limiting the proliferative capacity and clonal expansion potential. Therefore, we investigated whether puerarin might be able to prevent senescence of EPCs. EPCs were isolated from peripheral blood and characterized. After ex vivo cultivation, EPCs became senescent as determined by acidic β-galactosidase staining. Puerarin dose dependently prevented the onset of EPCs senescence in culture. Moreover, puerarin increased proliferation of EPCs as assessed by BrdU incorporation assay and colony-forming capacity. To get further insights into the underlying mechanisms of these effects induced by puerarin, we measured telomerase activity and determined the phosphorylation of serine/threonine protein kinase Akt by using western blot. Puerarin significantly increased telomerase activity and phosphorylation of Akt, a downstream effector of phosphoinositide 3-kinase (PI-3K). Moreover, pretreatment with PI-3K blockers, either wortmannin or LY294002, significantly attenuated the puerarin puerarin-induced telomerase activity. Taken together, the results of the present study indicated that puerarin delayed the onset of EPCs senescence, which may be related to the activation of telomerase through the PI-3K/Akt pathway. The inhibition of EPCs senescence by puerarin in vitro may improve the functional activity of EPCs in a way that is important for potential cell therapy.

1. Introduction

Increasing evidence suggests that endothelial progenitor cells (EPCs) play an important role in endothelium maintenance, being implicated in both reendothelialization and neovascularization (Asahara et al., 1997; Takahashi et al., 1999; Kawamoto et al., 2001; Kocher et al., 2001; Walter et al., 2002; Hill et al., 2003). This beneficial property of EPCs is attractive for cell therapy that targets the regeneration of ischemic tissue. Indeed, clinical investigations have suggested that ex vivo expansion of EPCs might be useful for potential clinical cell therapy of ischemic diseases (Tateishi-Yuyama et al., 2002; Strauer et al., 2002; Assmus et al., 2002; Schachinger et al., 2004). More recently, we have demonstrated that intravenous infusion of autologous EPCs appeared to be feasible and safe, and might have beneficial effects on exercise capacity and pulmonary haemodynamics in adults with idiopathic pulmonary arterial hypertension (Wang et al., 2007). Although these studies are providing intriguing and encouraging insight into the potential use of EPCs in the clinical setting, a major limitation to potential cell therapeutic approaches is the limited number of EPCs in the circulating blood (< 0.05% of leukocytes). Thus, ex vivo expansion of EPCs appears to be necessary. However, ex vivo cultivation of EPCs leads to rapid onset of EPC senescence, thereby severely limiting the proliferative capacity and clonal expansion potential (Assmus et al., 2003). Therefore, an important question with respect to potential cell therapeutic approaches is whether the EPCs senescence induced by ex vivo expansion can be delayed with pharmacological agents, cytokines, or even gene therapy.

Interestingly, Murasawa et al. have 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 (Murasawa et al., 2002). Another study has shown that statins delayed the onset of cellular senescence, and phosphoinositide 3-kinase (PI-3K)/Akt pathway played an important role in atorvastatin-mediated prevention of EPCs senescence (Assmus et al., 2003). In addition, Imanishi et al. have demonstrated that 17b-

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estradiol delayed the onset of senescence in EPCs, which might be related to activation of telomerase (Imanishi et al., 2005b). More recently, we have demonstrated that Ginkgo biloba extract delayed the onset of EPCs senescence, which might be related to activation of telomerase through the PI-3K/Akt pathway (Dong et al., 2007). Moreover, the inhibition of EPC senescence by estrogen and Ginkgo biloba extract in vitro may improve the functional activity of EPCs in a way that is important for potential cell therapy (Imanishi et al., 2005b; Dong et al., 2007).

In the present study, we investigated the regulation of senescence and proliferation in EPCs. Because we previously demonstrated that puerarin, a major effective ingredient extracted from the traditional Chinese medicine Ge-gen (Radix Puerriae), dose and time dependently increased the number and activity of EPCs (Zhu et al., 2004), we further elucidated a potential effect of puerarin on EPC senescence. Our data demonstrated that puerarin potently prevented the onset of EPC senescence and promoted proliferation and colony formation ex vivo. The effects of puerarin might be dependent on telomerase activation, and PI-3K/Akt pathway also appeared to play a major role in these processes.

2. Materials and methods

2.1. Isolation, cultivation and characterization of circulating EPCs

EPCs were isolated, cultured and characterized according to previously described techniques (Hill et al., 2003; Zhu et al., 2004, 2006; Wang et al., 2007; Dong et al., 2007). Briefly, mononuclear cells (MNCs) were isolated from peripheral blood of healthy human volunteers by Ficoll density gradient centrifugation and cultured on fibronectin (Chemicon)-coated dishes in Medium 199 (Sigma) supplemented with 10% fetal-calf serum and VEGF (50 ng/mL, Chemicon). EPCs were characterized as adherent cells double positive for DiLDL-uptake and lectin binding under a laser scanning confocal microscope. They were further documented by demonstrating the expression of KDR, CD34 and AC133 by flow cytometry (data not shown).

2.2. Colony assay

After 4 days of culture, adherent cells were gently detached with EDTA. Cells (1×10^5) were seeded in methylcellulose plates (Methocult GF H4434, CellSystems) with 100 ng/mL human recombinant VEGF. Plates were studied under phase contrast microscopy, and colonies were counted after 10days of incubation by two independent investigators (Assmus et al., 2003; Zhu et al., 2006; Dong et al., 2007).

2.3. EPCs proliferation assay

EPCs' proliferation was assessed from the incorporation of 5-bromo-2′-deoxyuridine (BrdU). Cells (1×10^4cells/well) were incubated in 96-well plastic plates. Then, BrdU (10 μM) was added to the medium and the cells were incubated for another 18h. Subsequently, the cells were fixed and BrdU incorporation was determined with a Cell Proliferation ELISA Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions (Zhu et al., 2006; Dong et al., 2007).

2.4. Senescence-associated β-galactosidase activity assay

Senescence-associated β-galactosidase (SA-b-gal) activity was measured with β-Galactosidase Staining Kit (BioVision). The protocol was according to the manufacturer's instructions. Briefly, EPCs were washed in PBS, fixed for 10–15min at room temperature with 0.5 mL of Fixative Solution, washed and incubated overnight at 37 °C with the Staining Solution Mix. Observe cells under a microscope for development of blue color (200× total magnification) (Assmus et al., 2003; Imanishi et al., 2004; Zhu et al., 2006; Dong et al., 2007).

2.5. Telomeric repeat amplification protocol assay

For quantitative analyses of telomerase activity, the telomeric repeat amplification protocol (TRAP) assay, in the which telomerase reaction product is amplified by polymerase chain reaction (PCR) (Kim and Wu, 1997), was performed using the *Telo*TAGGG PCR ELISA^{PLUS} kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

2.6. Western blotting

The EPCs were treated for 24 h with different of puerarin. Cellular proteins were prepared and separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, as described previously (Zhu et al., 2006; Dong et al., 2007). After the separation, proteins were transferred to a nitrocellulose membrane (Amersham). Membranes were blocked by incubation in Tris-buffered saline (pH 7.5), containing 0.1% (v/v) Tween 20 and 5% (v/v) non-fat dry milk for 2 h, followed by a 2 h incubation at room temperature with rabbit polyclonal antiphospho-Akt-Ser^{473} or anti-Akt antibodies (Cell Signalling Technology). The filters were washed extensively in Tris-buffered saline, containing 0.1% (v/v) Tween 20, before incubation for 1 h with a secondary antirabbit antibody conjugated to horseradish peroxidase. Membranes were then washed and developed using the EZ-ECL Detection Kit (Biological Industries).

2.7. Statistical analysis

Data are expressed as mean ± SD from at least 3 independent experiments. Statistical analysis was performed by the two-tailed *t* test or ANOVA for multiple comparisons.

3. Results

3.1. Puerarin prevented EPCs senescence

EPCs were generated from peripheral blood mononuclear cells as previously described (Hill et al.s, 2003; Zhu et al., 2004, 2006; Wang et al., 2007; Dong et al., 2007). To assess the onset of senescence, acidic β-galactosidase was detected as a biochemical marker for acidification typical for the onset of cellular senescence (Mathon and Lloyd, 2001; Dimri et al., 1995). Cultivation of EPCs resulted in an increase in SA-β-gal-positive cells after prolonged cultivation. Co-incubation with puerarin significantly inhibited the increase in SA-β-gal-positive cells. The inhibition of EPCs senescence occurred dose dependently, with a plateau at 1.0 mmol/L puerarin (Fig. 1).

3.2. Effects of puerarin on proliferation of EPCs

Having demonstrated that puerarin prevented the onset of senescence, we examined whether that translates into an increase of proliferation. The BrdU incorporation assay demonstrated that the mitogenic potential of EPCs treated with puerarin exceeded that in untreated (control) EPCs — an effect that was dose dependent (Fig. 2).

3.3. Effects of puerarin on clonal expansion in EPCs

To investigate the clonal expansion potential of the cultivated EPCs, we performed an outgrowth assay. For this purpose, EPCs were cultivated for 4 days. Then, cells were detached, and 1×10^5 10^5EPCs were seeded in methylcellulose plates in the presence or absence of puerarin. As shown in Fig. 3, the number of colonies was significantly higher in EPCs that had been pretreated with puerarin.

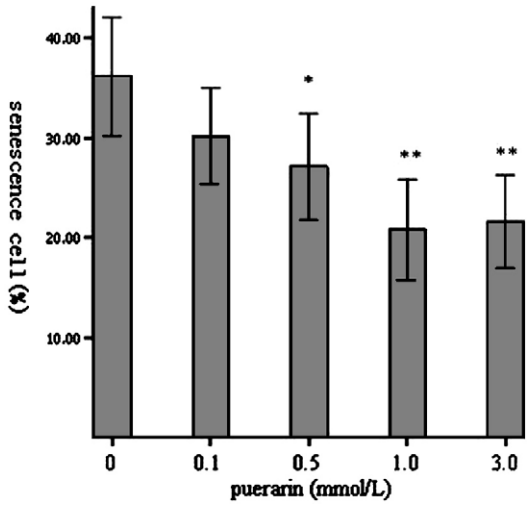

Fig. 1. Puerarin prevented EPCs' senescence. Freshly isolated mononuclear cells were cultivated in Medium 199 supplemented with 10% fetal-calf serum and VEGF. At day 4, cells were seeded in either indicated doses of puerarin in methylcellulose plates for 7 days. The number of blue cells was counted manually from a total of 200 cells. Data are mean±SD, $n=6$; *$P<0.05$, **$P<0.01$ vs control.

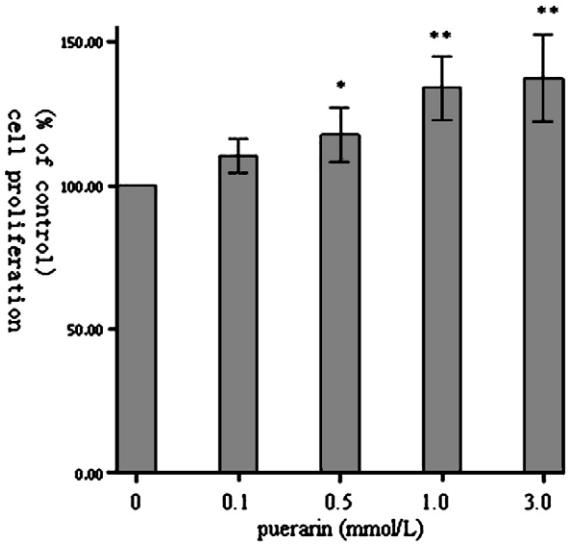

Fig. 3. Effects of puerarin on clonal expansion in EPCs. Mononuclear cells were cultivated in Medium 199 supplemented with 10% fetal-calf serum and VEGF. At day 4, cells were seeded in either indicated doses of puerarin in methylcellulose plates, and colonies were counted after additional 10 days of cultivation. Data are mean±SD, $n=6$; *$P<0.01$ vs control.

3.4. Effects of puerarin on telomerase activity in EPCs

Cellular senescence is critically influenced by the telomerase, which elongates telomeres, thereby counteracting telomere length reduction induced by each cell division. Therefore, we measured telomerase activity in EPCs using the *Telo*TAGGG PCR ELISA^{PLUS} kit. As demonstrated in Fig. 4, puerarin dose dependently increased telomerase activity.

3.5. Puerarin induces telomerase activity via the PI3K/Akt pathway

Recent studies have demonstrated that Akt plays an essential role in regulating cell senescence and telomerase activity (Kang et al., 1999; Breitschopf et al., 2001). Moreover, PI-3K/Akt pathway has been shown to play an important role in atorvastatin-mediated prevention of EPCs senescence (Assmus et al., 2003). Thus, we examined whether the PI3K/Akt pathway was involved in the puerarin-induced telomerase activation. EPCs were pretreated with or without phosphatidylinositol 3-kinase (PI-3K) blockers, either wortmannin (20 nmol/L) or LY294002 (μmol/L), before incubation with puerarin and were then subjected to assess the telomerase activity. Interestingly, either wortmannin or LY294002 clearly attenuated the increase in telomerase activity by puerarin (Fig. 4).

We next investigated whether puerarin would induce the phosphorylation of Akt, a downstream effector of PI-3K. EPCs were stimulated with different puerarin for 24 h, and immunoblots were performed with anti-phospho-Akt (Ser^{473}) or anti-Akt antibody. As shown in Fig. 5, stimulation with puerarin led to dose-dependent

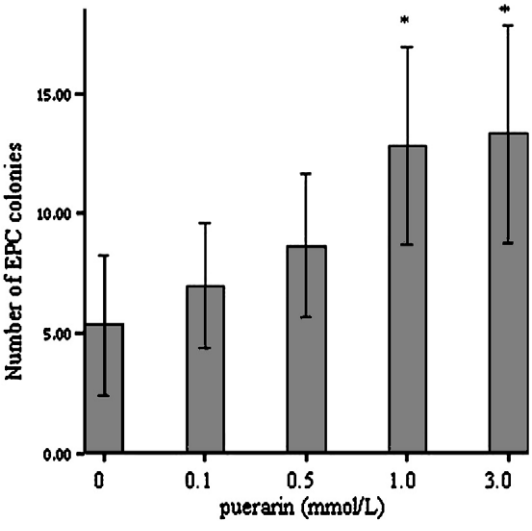

Fig. 2. Effects of puerarin on proliferation in EPCs. EPCs were treated with indicated concentrations of puerarin. Cells were harvested 7 days after culture. Cell proliferation was detected as described in the Materials and methods. Data are mean±SD, $n=5$; *$P<0.05$, **$P<0.01$ vs control.

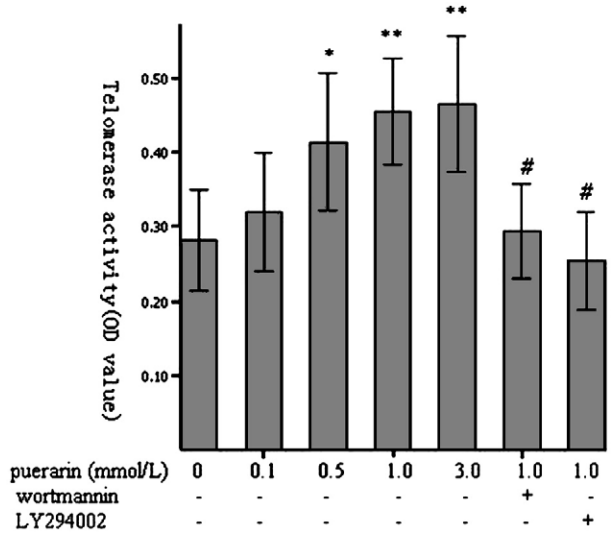

Fig. 4. Effects of puerarin on telomerase activity in EPCs. Freshly isolated mononuclear cells were cultivated in Medium 199 supplemented with 20% fetal-calf serum and VEGF. After 4 days of cultivation, EPCs were incubated with either indicated doses of puerarin in the presence or absence of LY294002 (10 μmol/L), wortmannin (20 nmol/L) for 24 h, and telomerase activity was measured by the TRAP assay. Data are mean±SD, $n=6$; *$P<0.05$, **$P<0.01$ vs control, #$P<0.01$ vs puerarin (1.0 mmol/L).

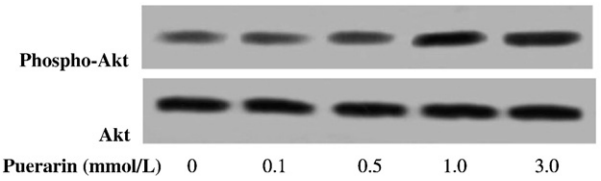

Fig. 5. Effect of puerarin on Akt phosphorylation on Ser^{473}. Mononuclear cells at day 4 were stimulated for 24 h with indicated concentrations of puerarin, and phosphorylation of Akt was determined with a phosphospecific Akt antibody. A representative blot from three independent experiments is shown.

phosphorylation of Akt, although it did not affect the total amount of Akt.

4. Discussion

In the present study, we have shown for the first time that puerarin prevented the onset of EPCs senescence, which was associated with a high proliferative capacity and profoundly increased clonal expansion potential. Recently, Assmus et al. have shown that EPCs' senescence was associated with the reduction of numbers and impairment of activity (Assmus et al., 2003). Imanishi et al. have documented that pro-atherosclerotic risk factor ox-LDL accelerated the onset of EPCs senescence, leading to cellular dysfunction (Imanishi et al., 2004). In addition, Ang II accelerates the onset of EPC senescence, which leads to the impairment of proliferative activity (Imanishi et al., 2005a). Subsequent animal experiment showed that the number of senescent EPCs was significantly greater in the angiotensin II (Ang II)-infusion rat, and Ang II treatment significantly decreased the functional activity in EPCs (Kobayashi et al., 2006). This study group has also documented that EPC senescence was accelerated in both experimental hypertensive rats and patients with essential hypertension (Imanishi et al., 2005c). Previous study has shown that the migratory capacity of EPCs was impaired in patients with hypertension (Vasa et al., 2001). More recently, high glucose, sirolimus and homocysteine have been shown to accelerate the onset of EPCs senescence, leading to the impairment of EPCs activity (Zhu et al., 2006; Imanishi et al., 2006; Kuki et al., 2006), while estrogen and Ginkgo biloba extract have been shown to reduce endothelial progenitor cell senescence through augmentation of telomerase activity, leading to the potentiation of proliferative activity and network formation (Imanishi et al., 2005b; Dong et al., 2007). Keeping these findings in mind, we speculated that EPCs senescence, might partly account for mechanisms by which puerarin increases EPCs numbers and activity.

The mechanisms, by which puerarin prevents the onset of cellular senescence and increases the proliferative capacity of EPCs, appear to involve telomerase activity. Telomerase, a ribonucleoprotein with reverse transcriptase activity, adds telomeric repeats at the linear end of eukaryotic chromosomes and thereby delays the development of senescence. Loss of telomerase activity has been suggested to constitute the molecular clock that triggers cellular senescence (Greider, 1990). Recently, Murasawa et al. have revealed that overexpression of *h*TERT by adenovirus-mediated gene delivery could result in a delay in senescence and recovery/enhancement of the regenerative properties of EPCs (Murasawa et al., 2002). However, ox-LDL, Ang II, high glucose, sirolimus and homocysteine have been shown to significantly diminish telomerase activity (Imanishi et al., 2004, 2005a, 2006; Kobayashi et al., 2006; Kuki et al., 2006; Zhu et al., 2006), which were considered to be associated with EPCs senescence induced by these factors, while estrogen and Ginkgo biloba extract have been shown to significantly increase telomerase activity (Imanishi et al., 2005a,b; Dong et al., 2007). In addition, the telomerase activities were also significantly lowered in both experimental hypertensive rats and patients with essential hypertension (Imanishi et al., 2005c). Our data demonstrated that puerarin dose dependently increased telomerase activity. Thus, it was suggested that puerarin prevented the onset of EPCs senescence most likely through activation of telomerase.

However, the molecular mechanisms by which puerarin increased telomerase activity remains to be determined. In this study, we have shown that puerarin significantly increased Akt phosphorylation. 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 (Kang et al., 1999; Breitschopf et al., 2001). Beside the direct phosphorylation of TERT, Akt might also act by increasing the activity of the endothelial NO synthase (eNOS), since NO has been demonstrated to activate telomerase and delays endothelial cell senescence (Vasa et al., 2000). However, the NO donor *S*-nitrosopenicillamine (SNAP) did not prevent the onset of EPCs senescence and inhibition of the NO synthase by N^G-monomethyl-L-arginine (LNMA) did not accelerate the onset of EPCs senescence, suggesting that the regulation of EPCs senescence is independent of NO (Assmus et al., 2003). Taken together, our data indicated that puerarin increased Akt phosphorylation in EPCs, which might lead to increase phosphorylation of TERT. The increase of phosphorylation of TERT might enhance telomerase activity and, thereby, prevented the onset of EPCs senescence. Further studies are required to elucidate our speculation.

In addition, we cannot rule out the possibility that a telomere-independent mechanism regulates replicative senescence, since cellular aging or senescence can be triggered by different pathways (Mathon and Lloyd, 2001). Assmus et al. have shown that statins inhibited senescence of EPCs independent of telomerase activity, but dependent on geranylgeranylpyrophosphate. Atorvastatin-mediated prevention of EPC senescence appears to be mediated by the regulation of various cell cycle proteins (Assmus et al., 2003). High glucose accelerates the onset of EPCs senescence most likely via the p38 MAPK pathway (Kuki et al., 2006). Sirolimus accelerated the onset of cellular senescence dependent on telomerase activity, as well as the cell cycle inhibitor p27^{kip1} (Imanishi et al., 2006). Thus, further studies are required to elucidate the mechanisms underlying the inhibitory effects of puerarin on senescence in EPCs.

5. Conclusions

In conclusion, the results of the present study demonstrated that puerarin delayed the onset of EPCs senescence, which may be related to activation of telomerase and Akt phosphorylation. The inhibition of EPCs senescence and induction of EPC proliferation by puerarin in vitro may improve the functional activity of EPCs in a way that is important for potential cell therapy.

6. Conflict of interest statement

None of the authors has a conflict of interest to declare in relation to this work.

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