Effects of puerarin on number and activity of endothelial progenitor cells from peripheral blood[1]

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ABSTRACT

AIM: To investigate whether puerarin can augment endothelial progenitor cells (EPCs) numbers, promote EPC proliferative, migratory, adhesive, and *in vitro* vasculogenesis capacity. **METHODS:** EPCs were characterized as adherent cells by double staining of DiLDL-uptake and lectin binding under a laser scanning confocal microscope. Expression of KDR, VEGFR-2, and AC133 was detected by flow cytometry. EPCs proliferation, migration and *in vitro* vasculogenesis were determined with MTT assay, modified Boyden chamber assay, and *in vitro* vasculogenesis kit, respectively. EPCs adhesive assay was performed by replating those on fibronectin-coated dishes, then adherent cells were counted. **RESULTS:** Incubation of isolated human MNCs with puerarin 0.1-3 mmol/L increased the number of EPCs, EPC proliferative, migratory, adhesive, and *in vitro* vasculogenesis capacity in a concentration- and time-dependent manner, which reached maximum at 3 mmol/L, 24 h (approximately 1-fold increase, *P*<0.01). **CONCLUSION:** Puerarin enhanced EPCs functional activity.

INTRODUCTION

Puerarin, a major effective ingredient extracted from the traditional Chinese medicine Ge-gen (Radix Puerriae; RP), has long been used to treat patients with coronary artery diseases (CAD). However, the mechanisms of puerarin are still not very clear. Recently, puerarin has been reported to have protective effects on endothelial dysfunction induced by many factors. Endothelial dysfunction ultimately loses a balance between the magnitude of injury and the capacity for repair[1]. A variety of evidence suggested that circulating endothelial progenitor cells (EPCs) constituted one aspect of this repair process[1,2]. EPCs have the capacity to circulate, proliferate, and differentiate into mature endothelial cells, but which have not yet acquired characteristic mature endothelial markers and have not yet formed a lumen[3,4]. Laboratory evidence suggested that these precursors participated in postnatal neovascularization and reendothelialization[1-3,5-7]. Endothelial dysfunction played an important role in the pathogenesis of CAD. In addition, it has recently been shown that patients at risk for CAD have decreased numbers of circulating EPCs with impaired activity[8].

We hypothesized that puerarin not only directly protected endothelial cell but also increased EPCs numbers and functions at the same time, thus accelerated endothelial repair process, which contributed to protective effects on endothelial cells and improved the clinical symptoms and prognosis of patients with CAD. To test this hypothesis, we observed the changes of numbers and activity of EPCs exposed to puerarin in this study.

MATERIALS AND METHODS

Isolation and cultivation of EPCs EPCs were cultured according to previously described techniques[1,9]. Briefly, total mononuclear cells (MNCs) were isolated from blood of healthy human volunteers by Ficoll density gradient centrifugation. Cells were plated on culture dishes coated with human fibronectin (Chemicon) and maintained in Medium 199 (Sigma) supplemented with 20 % fetal-calf serum, VEGF (10 µg/L, Chemicon), benzylpenicillin (100 kU/L), and streptomycin (100 mg/L). After 4-d culture, nonadherent cells were removed by washing with PBS, new media was applied, and the culture was maintained for 7 d. Informed consent was obtained from all volunteers and all of the procedures were done in accordance with national and international laws and policies.

Cellular staining Fluorescent chemical detection of EPCs was performed on attached MNCs after 7 d in culture. Direct fluorescent staining was used to detect dual binding of FITC-labeled Ulex europaeus agglutinin (UEA)-1 (Sigma) and 1,1-dioctadecyl-3,3,3, 3-tetramethylindocarbocyanine (DiI)-labeled acetylated low density lipoprotein (acLDL; Molecular Probe). Cells were first incubated with acLDL at 37 °C and later fixed with 2 % paraformaldehyde for 10 min. After being washed, the cells were reacted with UEA-1 (10 mg/L) for 1 h. After the staining, samples were viewed with an inverted fluorescent microscope (Leica) and further demonstrated by laser scanning confocal microscope (LSCM, Leica). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs[8,9]. Two or three independent investigators evaluated the number of EPCs per well by counting 15 randomly selected high-power fields (×200) with an inverted fluorescent microscope.

Flow cytometry analysis Fluorescence-activated cell sorting (FACS) detection of EPCs was performed on attached MNCs after 7 d in culture. Mononuclear cells were detached with 0.25 % trypsin followed by repeated gentle flushing through a pipette tip. Cells (2×10^5) were incubated for 30 min at 4 °C with phycoerythrin-conjugated monoclonal antibodies against kinase insert domain-containing receptor (KDR, R&D), CD34, AC133 (Miltenyi Biotec). Isotype-identical antibodies served as controls. After treatment, the cells were fixed in 1 % paraformaldehyde. Quantitative FACS was performed on a FACStar flow cytometer (COULTER).

Protocols Cells were serum depleted for 24 h before experiments. To demonstrate a concentration-dependent effect of puerarin (In this study, puerarin injection was used, which was purchased from Zhongce Pharmaceutical Corporation, Shandong Province, China) on EPCs. Cells were incubated with 0.1, 0.5, 1, and 3 mmol/L puerarin for 24 h, respectively. To determine reaction time course, cells were treated with puerarin 3 mmol/L for 6, 12, 24, and 48 h.

Migration assay EPC migration was evaluated by using a modified Boyden chamber assay[8]. In brief, isolated EPCs were detached using 0.25 % trypsin, harvested by centrifugation, resuspended in 500 µL M199, and counted, then 2×10^4 EPCs were placed in the upper chamber of a modified Boyden chamber. VEGF in serum-free M199 media was placed in the lower compartment of the chamber. After 24-h incubation at 37 °C, the lower side of the filter was washed with PBS and fixed with 2 % paraformaldehyde. For quantification, cells were stained with Giemsa solution. Cells migrating into the lower chamber were counted manually in 3 random microscopic fields. All groups were studied in triplicate.

Cell adhesion assay After a 24-h incubation with puerarin, human EPCs were washed with PBS and gently detached with 0.25 % trypsin. After centrifugation and resuspension in M199, 5 % FBS, identical cells were replated onto fibronectin-coated culture dishes and incubated for 30 min at 37 °C. Adherent cells were counted by independent blinded investigators[2].

EPC proliferation assay The effect of puerarin on EPCs proliferation was determined by MTT assay. After being cultured for 7 d, EPCs were digested with 0.25 % trypsin and then cultured in serum-free medium in 96-well culture plate (200 µL per well), to which was added puerarin (to make a series of final concentrations: 0.1, 0.5, 1, and 3 mmol/L). Each concentration included six wells, while the serum-free medium served as a control. After being cultured for 24 h, EPCs were supplemented with 10 µL MTT (5 g/ L) and

incubated for another 6 h. Then the supernatant was discarded by aspiration and the EPC preparation was shaked with 200 µL Me$_2$SO for 10 min, before the *OD* value was measured at 490 nm.

***In vitro* vasculogenesis assay** *In vitro* vasculogenesis assay was performed with *in vitro* Angiogenesis Assay Kit (Chemicon). The protocol was according to the manufacturer's instructions. Briefly, ECMatrix™ solution was thawed on ice overnight, then mixed with 10×ECMatrix™ Diluent and placed in a 96-well tissue culture plate at 37 ℃ for 1 h to allow the matrix solution to solidify. EPCs were harvested as described above and replated (10 000 cells per well) on top of the solidified matrix solution. Cells were grown with puerarin or vehicle control, and incubated at 37 ℃ for 12 h. Tubule formation was inspected under an inverted light microscope at 200×magnification. Tubule formation was defined as a structure exhibiting a length 4 times its width[10,11]. Five independent fields were assessed for each well, and the average number of tubules/200×field was determined.

Statistical analysis All data were presented as mean±SD. Differences between group means were assessed by an unpaired *t*-test for single comparisons and by ANOVA for multiple comparisons. *P*<0.05 was considered significant.

RESULTS

Characterization of human EPCs Total MNCs isolated and cultured for 7 d resulted in a spindle-shaped, EC-like morphology (Fig 1). EPCs were characterized as adherent cells double positive for DiLDL-uptake and lectin binding by using LSCM (Fig 2). They were further documented by demonstrating the expression of KDR (78 %±7.8 %), CD34 (28.7 %±6.9 %), and AC133 (17.1 %±8.1 %) by flow cytometry (Fig 3).

Puerarin increased EPCs number *in vitro* Incubation of isolated human MNCs with puerarin increased the number of differentiated, adherent EPCs in a concentration-dependent manner, which became apparent at 0.5 mmol/L, with a peak at 3 mmol/L (about 1-fold increase, Fig 2 and 4A). In time-course experiments performed with puerarin 3 mmol/L, increase of EPCs number became apparent at 12 h and reached the maximum at 24 h (about 1-fold increase, Fig 4B).

Effect of puerarin on EPC proliferation Puerarin stimulated EPC proliferative activity in a concentration-dependent manner. The effect reached the maximum
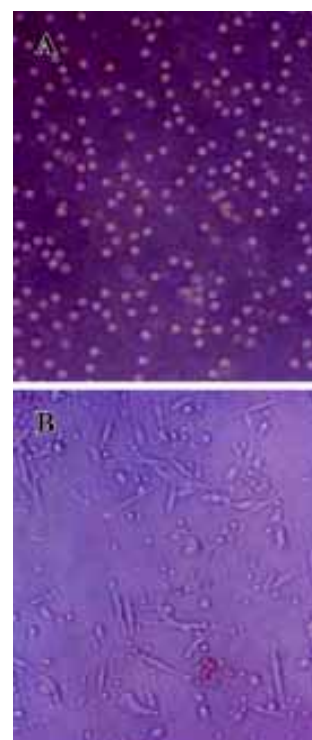

Fig 1. **(A) Mononuclear cells were plated on culture dishes coated with human fibronectin just after isolated from peripheral blood (×200). B: After a 7-d culture, nonadherent cells were removed and attached cells exhibited a spindle-shaped, endothelial cells like morphology (×200).**

at 3 mmol/L compared with control (0.75±0.07 *vs* 0.54± 0.06, *P*<0.01). Puerarin 3 mmol/L increased EPCs proliferative activity in a time-dependent manner, which became apparent at 12 h (*P*<0.05) and reached the maximum at 24 h (*P*<0.01, Fig 5).

Effect of puerarin on EPC migration Puerarin profoundly enhanced cell migration compared with control, maximal at 3 mmol/L (31.2±6.9 *vs* 13.8±3.8 per high-powered field, *P*<0.01). Puerarin 3 mmol/L also time-dependently enhanced EPC migratory activity, which became apparent at 12 h (*P*<0.05) and reached the maximum at 24 h (*P*<0.01, Fig 6).

Effect of puerarin on EPC adhesiveness To study whether puerarin can alter adhesiveness of cultured human EPCs, EPCs were incubated with puerarin for 24 h. After being replated on fibronectin-coated dishes, EPCs pre-exposed to puerarin exhibited a significant increase in the number of adherent cells at 30 min. The increase in the number of adherent cells was in a concentration-dependent manner with a maximal effect achieved at 3 mmol/L (Fig 7A). Puerarin 3 mmol/L time-dependently increased EPC adhesive activity, which became apparent at 12 h (*P*<0.05) and reached the
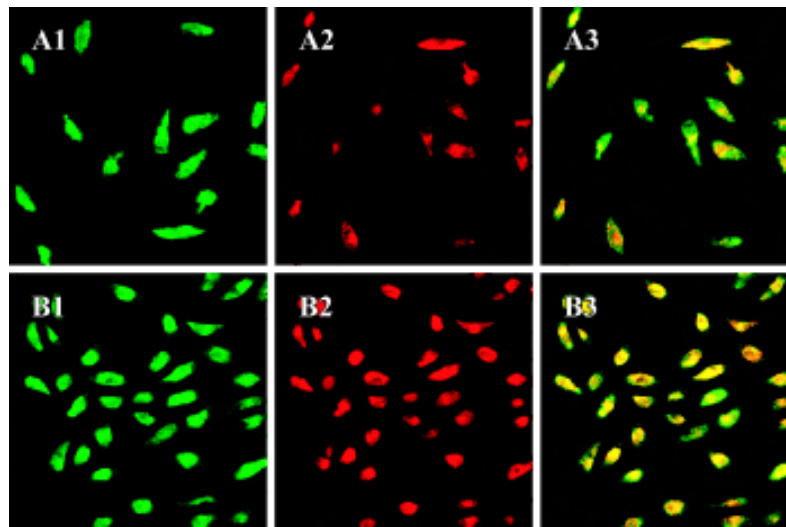
Fig 2. Double staining of mononuclear cells with ectin binding (green, exciting wave-length of 477 nm) and DiLDL uptake (red, exciting wave-length of 543 nm) under a laser scanning confocal microscope. Double positive cells appearing yellow in the overlay were identified as differentiating EPCs (×400). A1-A3: control group. B1-B3: puerarin group 3 mmol/L. Puerarin 3 mmol/L increased EPCs numbers compared with control group.

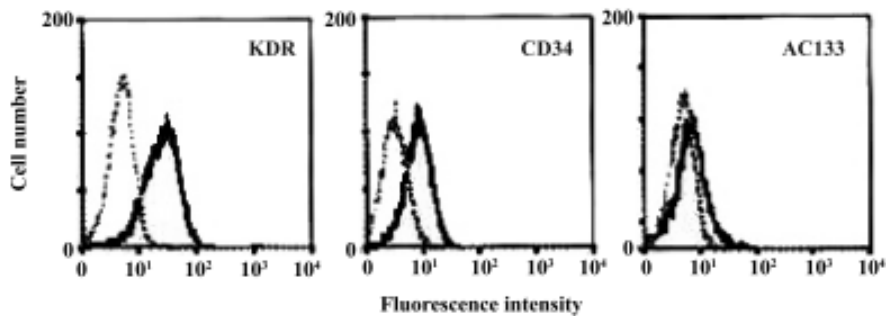**Fig 3. Flow cytometry analysis of adherent cells at d 7 of culture. *n*=6. Adherent cells were positive for KDR (78.0 %±7.8%), CD34 (28.7 %±6.9 %), and AC133 (17.1 %±8.0 %). Data were percentage of positive cells.**

maximum at 24 h (P<0.01, Fig 7B).

Effects of statins on EPC vasculogenesis Circulating EPCs home to sites of neovascularization and differentiate into endothelial cells *in situ*[3, 12] in a manner consistent with a process termed vasculogenesis. *In vitro* vasculogenesis assay showed that incubation of EPCs with puerarin for 24 h increased tubule number in a concentration-dependent manner, which reached peak at 3 mmol/L. Moreover, tubules in the puerarin-treated wells were qualitatively different and more complex than those in the control wells (Fig 8).

DISCUSSION

Puerarin is one of isoflavones extracted from Gegen which is the root of a wild leguminous creeper, *Pueraria lobata* (Willd) Ohwi. Experimental data have

shown that puerarin exhibits pleiotropic effects that can beneficially impact on occlusive vascular diseases, including: (1) dilating coronary arteries significantly and decreasing myocardial oxygen consumption; (2) decreasing platelet accumulation and platelet-rich thrombus formation; (3) improving microcirculation both in animals and in patients; (4) inhibiting the proliferation of vascular smooth-muscle cells; (5) inhibiting the activity of rennin-angiotensin-aldosterone system, and so on[13-15]. Moreover, puerarin has recently been shown to have protective effects on endothelial dysfunction induced by lipid peroxide, chemical hypoxia, and hydroxyl-free radicals[14-16].

However, endothelial dysfunction ultimately looses a balance between the magnitude of injury and the capacity of repair. Local migration and proliferation of endothelial cells adjacent to the site of injury had been
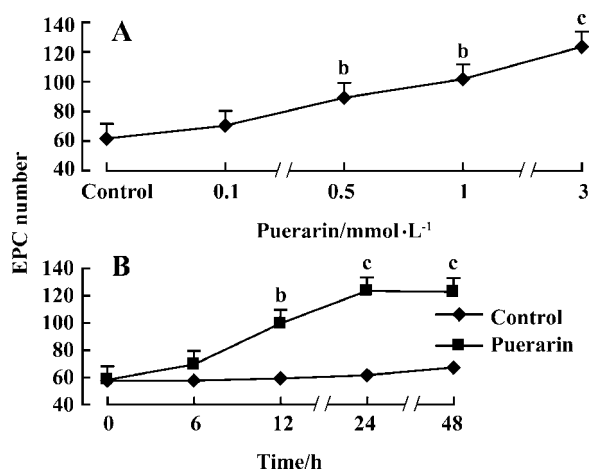
Fig 4. Puerarin increased differentiated human MNCs number in a concentration-dependent manner (A) and in a time-dependent manner (B). *n*=6. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* control.
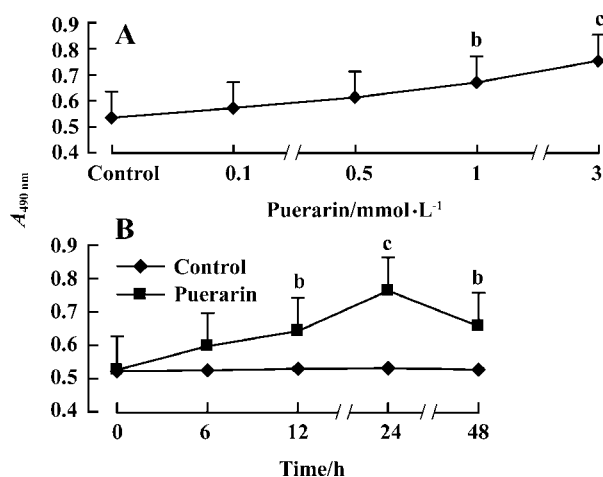

Fig 5. Puerarin stimulated human EPCs proliferation in a concentration-dependent manner (A) and puerarin 3 mmol/L stimulated human EPCs proliferation in a time-dependent manner (B). *n*=6. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* control.
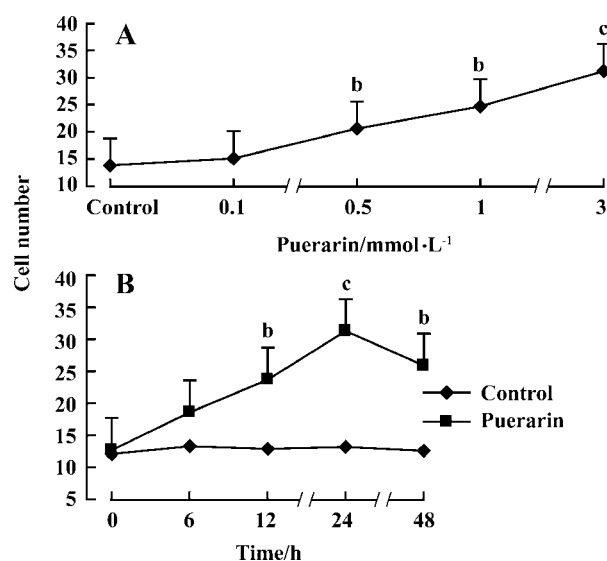

Fig 6. (A) Puerarin increased migration rate of human EPCs in a concentration-dependent manner. (B) Puerarin 3 mmol/L increased migration rate of human EPCs in a time-dependent manner. *n*=6. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* control.
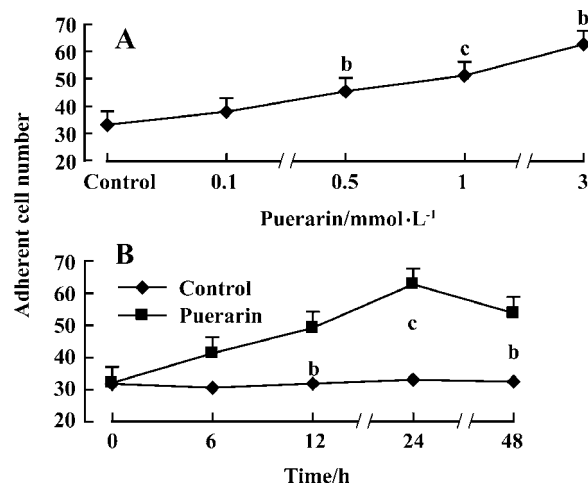

Fig 7. (A) Incubation of isolated human MNCs with puerarin for 24 h concentration-dependently increased the number of adherent cells after 30 min. (B) Incubation of isolated human MNCs with puerarin 3 mmol/L time-dependently increased the number of adherent cells. *n*=6. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* control.

regarded as the principal mechanism of endothelial repair until Asahara and colleagues, in 1997, described circulating EPCs[3]. More recently, 2 groups have documented in animals and human subjects that EPCs contribute up to 25 % of endothelial cells in newly formed vessels[17,18]. These cells are bone marrow-derived and have the capacity to home in sites of endothelial injury. Here they incorporate into the endothelium and thereby repair the defects[19]. Moreover, Vasa *et al* have recently reported that patients with CAD revealed reduced levels and functional impairment of EPCs, which was

correlated with risk factors for CAD[8]. Therefore, the augmentation of EPC numbers by pharmacological modulation may be a novel strategy to improve neovascularization after ischemia.

The results of the present study demonstrated that puerarin could augment EPCs number, and promote EPCs proliferative, migratory, adhesive, and *in vitro* vasculogenesis capacity. Given the well-established role
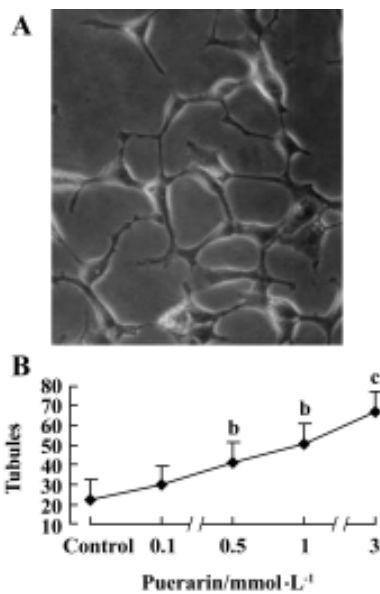
Fig 8. (A) Typical vascular tubes can be seen in some fields after incubation of endothelial progenitor cells (EPC) with puerarin. (B) Incubation of EPCs with puerarin increased tubule number in a concentration-dependent manner. *n*=6. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control.

of EPCs participating in neovascularization and reendothelialization, stimulation of EPCs by puerarin may contribute to the clinical benefit of puerarin therapy in patients with CAD. Thus, our results might suggest a novel mechanism of action of puerarin: namely, puerarin not only directly protects endothelial cell but also increases EPCs numbers and functions at the same time, thus accelerates endothelial repair process, which contributes to protective effects on endothelial cells and improves the clinical symptoms and prognosis of patients with CAD. Since puerarin have the merits of low toxicity and rare complications, it might be a promising medication to improve postnatal neovascularization and reendothelialization in patients with CAD.

The mechanisms by which puerarin increase EPC numbers and activity remain to be determined. There are several possible scenarios by which puerarin could increase the number of circulating EPCs. One explanation might be decreased apoptosis of premature progenitor cells. Indeed, CD34-positive EPCs were shown to be very sensitive to apoptosis induction. Moreover, puerarin is known to be able to protect apoptotic endothelial cells. Another explanation is that puerarin may interfere with the signaling pathways regulating EPC differentiation or mobilization.

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