GINKGOLIDE B PROMOTES PROLIFERATION AND FUNCTIONAL ACTIVITIES OF BONE MARROW-DERIVED ENDOTHELIAL PROGENITOR CELLS: INVOLVEMENT OF AKT/ENOS AND MAPK/P38 SIGNALING PATHWAYS

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Abstract

Bone marrow-derived, circulating endothelial progenitor cells (EPCs) contribute to neovascularization in various diseases, and represent a very interesting alternative cell source for enhancing vasculogenesis in regenerative medicine. In this study, we investigated the effects of Ginkgolide B (GB) on proliferation and differentiation of EPCs, and the involved signaling pathway *in vitro*. EPC proliferation, migration, adhesion and angiogenesis activities were assessed with the WST-8 assay, Transwell chamber assay, cell counting and angiogenesis kit, respectively. Apoptosis was detected with annexin V and propidium iodide staining. The protein expression of angiogenesis-related makers was detected by Western blot, and related gene expression was determined by real-time polymerase chain reaction (RT-PCR). The results showed that GB promoted the proliferation and endothelial gene expression, and markedly enhanced vascular endothelial growth factor-induced migration response and the capability to incorporate into the vascular networks in EPCs. GB protected EPCs from H_2O_2-induced cell death. GB induced the phosphorylation of eNOS, Akt and p38, which in turn promoted cell proliferation and function. In conclusion, the present study demonstrates that GB, at a near medical applied dose, increases the number and functional activities of EPCs with involvement of Akt/endothelial nitric oxide synthase and mitogen-activated protein kinase (MAPK)/ p38 signal pathways. These findings raise the intriguing possibility that GB may play an important role in the protection and revascularization of blood vessels.

Keywords: Endothelial progenitor cells, Ginkgolide B, proliferation, angiogenesis, Akt, endothelial nitric oxide synthase (eNOS), p38.

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Introduction

It is well known that circulating endothelial progenitor cells (EPCs) play important roles in maintaining vascular integrity and in injury repair. EPCs are a type of cell population that have the capacity to circulate, proliferate and differentiate into mature endothelial cells, but have not yet acquired characteristic mature endothelial cell markers and have not yet formed a lumen (Asahara *et al*., 1997; Peichev *et al*., 2000).

There was compelling evidence for bone marrow (BM)-derived EPCs to play a pivotal role in the postnatal vasculogenesis (Dimmeler *et al*., 2001; Sata, 2006). Circulating progenitor cells derived from the bone marrow were found to exhibit features of endothelial cells, ability to home to sites of ischemia, and contribute to form new blood vessels (Isner and Asahara, 1999; Carmeliet, 2000). Mobilization of EPCs augmented the neovascularization of ischemic tissue and represented a useful therapeutic strategy for clinical therapy (Asahara *et al*., 1997; Satoshi and Takayuki, 2005). Growth factors (such as VEGF, PDGF, Ang-1, and stromal-derived factor-1) induced the mobilization and expansion of EPCs and hematopoietic stem cell populations in the bone marrow (Rabbany *et al*., 2003), which are through direct activation of signaling receptors on various subsets of precursor cells and paracrine interactions with the bone marrow stromal cells.

The extracts from the medicinal plant, *Ginkgo biloba*, act as antioxidant and free radical scavenger, and have been shown to exhibit protective effects on tissue abnormalities that include myocardial ischemia-reperfusion injury (Shen *et al*., 1998), ischemic brain damage (Zhang *et al*., 2000), and neuronal apoptosis (Ahlemeyer *et al*., 1999). These effects are supposed to be beneficial in cardiovascular, cerebrovascular, and neurological disorders (Yoshikawa *et al*., 1999). The extracts of *Ginkgo biloba* mainly contain flavonoid glycosides and terpenoids (ginkgolide, bilobalide) . Ginkgolides have been demonstrated to function as antagonists of platelet-activating factor (Lamant *et al*., 1987), exhibit a wide range of pharmacological actions, such as inhibiting the aggregation of platelet (Akiba *et al*., 1998), arterial thrombosis (Bourgain *et al*., 1987), acute inflammation (Teather *et al*., 2002), allergic reaction (Coyle *et al*., 1989) and so on. Ginkgolides in the terpeniod extracts can be divided into isotypes A, B, C, M, and J among which Ginkgolide B (GB) ($C_{20}H_{24}O_{10}$) has the highest biological activity (Li *et al*., 2009).

Mobilization of EPCs or mononuclear cells containing EPCs from bone marrow contribute to postnatal neovascularization *via* vasculogenesis and to increase functional blood supply (Kureishi *et al*., 2000). In addition, clinical studies reported that with the increase of age, accumulation of risk factors led to a decreased number of circulating EPCs, impaired cell activities and accelerated senescence (Scheubel *et al*., 2003; Chang *et al*., 2010; Takahisa *et al*., 2010). To overcome this problem, attention has increasingly been paid to enhance mobilization and differentiation of EPCs. GB was found to protect endothelial cells (Li *et al*., 2009). Therefore, we hypothesized that GB may promote proliferation and functional activities of bone marrow-derived endothelial progenitor cells. In this study, we investigated the effects of GB, as potential angiogenic agent, on the proliferation and differentiation of EPCs, as well as its putative mechanisms.

Materials and Methods

Isolation and cultivation of EPCs

Bone marrow was collected from the drill holes of the pedicle during internal spine fixation of patients with lumbar degenerative diseases (42 patients, age range 38-73 years, mean age 61 years, degenerative lumbar spondylolisthesis, disc herniation with lumbar spondylolisthesis, lumbar spinal stenosis with instability). Informed consent for bone marrow collection was obtained from the patients, and all procedures were performed in accordance with the guidance and approval of a research ethics committee in the First Affiliated Hospital of Sun Yat-sen University (NO. 2008-55).

The procedures for isolation, cultivation and identification of human EPCs cultures followed previously published methods (Asahara *et al*., 1997; Kalka *et al*., 2000). Briefly, mononuclear cells were collected from the bone marrow by density gradient centrifugation using Ficoll-Paque™ PREMIUM (1.077; GE Healthcare; www2.gehealthcare.com) according to the manufacturer's instructions. The isolated cells were cultivated in dishes coated with fibronectin and induced by EGM-2 MV Single-Quots (Cambrex, Charles City, IA, USA) at 37 °C with 5% CO_2 in humidified air at density of $3-5 \times 10^6$/cm². After 3 d in culture, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), new medium was applied and the cultivation was maintained through 7 d. Immuno-fluorescence staining and flow cytometry were used to indentify EPCs. Quantitative fluorescence-activated cell sorting (FACS) was performed on a FACS Vantage SE flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The Weibel-Palade body in cells was visualized by transmission electron microscopy (TEM). The ability of tube formation by EPCs was determined by *in vitro* angiogenesis assay.

Chemicals

GB (purity ≥ 99% by HPLC) was obtained from the National Institutes for Food and Drug Control, Beijing, China (http://www.nifdc.org.cn/directory/web/WS02/CL0049/2191.html). GB was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 100 g/L.

EPCs proliferation assay

The effect of GB on EPCs proliferation was determined by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl) -2H-tetrazolium, monosodium salt (WST-8) assay kit (CCK-8, Dojindo, Rockville, MD, USA) (Nojima *et al*., 2009). After cultivation for 7 d, adherent cells were detached with 0.25% trypsin and cultured with non-FBS EBM-2 in 96-well culture plates at 8×10^3 cells per well for 24 h. Then, the medium was replaced by fresh non-FBS EBM-2 with different concentrations of GB (0, 1.25, 5, 20, 80 and 160 μg/mL) for different periods. At 24 or 48 h, CCK-8 was used according to the manufacturer's instructions. WST-8 was added into each well for 4 h before the measurement. The absorbance at 450 nm was measured using a microplate reader.

EPCs migration assay

EPC migration was evaluated by using a Transwell chamber assay as described before (Lee *et al*., 2009). In brief, human EPCs were maintained for 24 h in serum-free medium, pretreated with 0, 5, 20 or 80 μg/mL GB for 48 h. Detached with trypsin, 1×10^5 EPCs were placed in the upper chamber of a 24 well Transwell (3422, Corning, Corning, NY, USA). 600 μL EGM-2 media containing 50 ng/mL VEGF were placed into the lower compartment of the chamber. After 6 h incubation at 37 °C, EPCs on the top of the membrane was wiped off with a cotton swab. The Transwell membrane was rinsed with PBS (37 °C), and then incubated in 4% paraformaldehyde at room temperature for 15 min. For quantification, cells were stained with 0.1% crystal violet solution. Cells migrating into the lower chamber were counted manually in ten random microscopic fields.

EPC adhesion assay

The assay was done as described before (Lee *et al*., 2009). Human EPCs were starved, and treated with GB as above. Human EPCs were washed with PBS (containing 0.1% BSA) and gently detached with 0.25% trypsin. Human EPCs at 1×10^4/well in EGM-2, 2% FBS were replated onto fibronectin-coated 96-well culture plate, and incubated for 30 min at 37 °C. After incubation, non-adherent cells were removed by washing three times with PBS. The adherent cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, dyed with 100 μL 0.1% crystal violet for 30 min at the room temperature. Adherent cells were counted by independent blinded investigators.

In vitro angiogenesis assay

In vitro angiogenesis assay was performed with an In Vitro Angiogenesis Assay Kit (Chemicon, CA, USA) according to the manufacturer's instructions (Urbich *et al*., 2005). Briefly, ECMatrix™ solution was thawed on ice overnight, mixed with 10 × ECMatrix™ diluents and placed in a 96-well tissue culture plate at 37 °C for 1 h to allow the matrix solution to solidify. Human EPCs were starved, and treated with GB as described above. 10,000 cells/well were replated on the top of the solidified matrix

solution. Tubule formation was inspected under an inverted light microscope at 200× magnification. Tubule formation was defined as a structure exhibiting a length four times its width. Five independent fields were assessed for each well, and the average number of tubules/200× field was determined.

Effect of GB on the viability of H_2O_2-injured EPCs

Human EPCs were starved, and treated with GB as described above. After that, the culture medium was replaced with EBM-2 containing H_2O_2 (150 µmol/L) and the cells were cultured for another 6 h. Cell viability was measured by CCK-8 as described above. 2 x 10^6 cells/well were plated in 6-well plates for the Annexin V FITC/PI assay (Pharmingen, Becton Dickinson) to estimate apoptosis and necrosis. The percentage of apoptotic cells (annexin V positive and propidium iodide (PI) negative), necrotic cells (annexin V and PI positive) and cell debris (annexin V negative and PI positive), was analyzed.

Enzyme-linked immunosorbent assay (ELISA)

EPCs were cultivated and treated with GB as described in the viability/proliferation assay. After incubation with GB for 48 h, the supernatants were harvested and used for ELISA assay, according to the manufacturer's instructions (Excell, Shanghai, China).

Inhibition effect on proliferation of cell signaling pathway inhibitors

EPCs were cultivated and treated with GB as described in the viability/proliferation assay. 1 µM, 10 µM or 100 µM wortmannin (Sigma) was used alone or with 80 µg/mL GB for 24 h. PD98059, LY294002 (both from Cell Signaling Technology, Danvers, MA, USA) with the concentration of 1 µM, 10 µM or 100 µM was added alone or with 80 µg/mL GB for 48 h.

Western blot analysis

EPCs were collected and lysed with ice-cold lysing buffer (Novagen, Merck, Darmstadt, Germany). The protein concentration was determined by the Bradford assay. 20 µg of total proteins were separated on a 10% SDS polyacrylamide gel. Bands on the gels were blotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The proteins were detected with specific phospho-Akt, phospho-eNOS, phospho-p38 and Akt, eNOS, p38 antibodies (1:1000, all from Cell Signaling Technology). Protein bands were visualized with Chemiluminescence Reagent Plus (Thermo, Waltham, MA, USA) and exposed to X-ray film (Fujifilm, Tokyo, Japan). The bands were quantified with the Bandscan software 5.0.

Real-time RT-PCR assay

Total RNA of human EPCs was extracted by the TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA) as described by the manufacturer. The quantity and quality of the extracted RNA was confirmed by absorption measurement at 260 and 280 nm using the spectrophotometer (Beckman Coulter, Brea, CA, USA). cDNA synthesis was performed with 1 µg of total RNA treated with DNase (0.5 U/µg RNA) with the PrimeScript®

RT reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The expression of endothelial NOS (eNOS), kinase insert domain receptor (KDR), vascular endothelial growth factor (VEGF) and von Willebrand Factor (vWF) transcripts was detected with a real-time PCR kit (SYBR® Premix Ex Taq, Takara Bio) with specific primers (Takara Bio), using ABI Prism 7700 SDS apparatus (Applied Biosystems/Life Technologies). The mRNA fold changes were calculated on quadruple experiments by using the comparative critical threshold cycle (CT) $2^{\Delta\Delta CT}$ value.

Statistical analysis

All experiments were performed at least 3 times with cells from different donors, representative experiments are shown in the figures. The data shown are expressed as mean ± SEM. Student's *t*-test was performed for statistical comparisons between two groups. The level of significance was set at $p < 0.05$.

Results

The growth process of EPCs

When mononuclear cells isolated from human bone marrow were cultured in endothelial cell growth medium on fibronectin-coated dishes, both adherent and non-adherent cells demonstrated round morphology with different sizes as assessed by light microscopy during the first 3 days, suggesting the presence of various subpopulations within the human mononuclear cell fraction. Approximately 20% of the mononuclear cells transferred to culture plates grew as adherent cells during the first week of culture with a tendency to form clusters or colonies (Fig. 1A). When cultivated for 7 days, the adherent cells exhibited strong ability to take up Dil-acLDL and FITC-UEA-I, and the double positive rate was (95.1 ±4.0)% (Figure 1B), which were identified as differentiating EPCs. The Weibel-Palade body was observed in endochylema by TEM (Fig. 1D). During the second week of culture, the cells changed toward a spindle-shaped endothelium-like morphology. Then, attached cells formed a cobblestone-like structure at day 14 (Fig. 1A). 7 d-cultured EPCs (early EPCs) had more active angiogenic potential than 15 d-cultured EPCs (late EPCs) (Fig. 1C). In the culture process, the expression pattern of surface markers changed toward a more mature endothelial cell phenotype during the cultivation period of 2 weeks, which showed a significant increase when 14 d cultures were compared to 7 d cultures with regard to the expression of vWF (from 5.4% to 38.9%), KDR (from 66.7% to 81.8%) and a marked decrease in expression of CD133 (from 18.5% to 3.8%), and CD34 (from 45.4% to 36.1%) (Fig. 1E).

GB promotes proliferation capacity of EPCs

The effect of GB on the proliferative activity of EPCs was investigated by CCK-8. As shown in Fig. 2A, incubation of human EPCs with GB for 48 h dose-dependently increased the number of EPCs, which was maximal at 80 µg/mL GB, but at a dose of 160 µg/mL, no obvious increase compared to control was observed. An increase in EPC proliferative
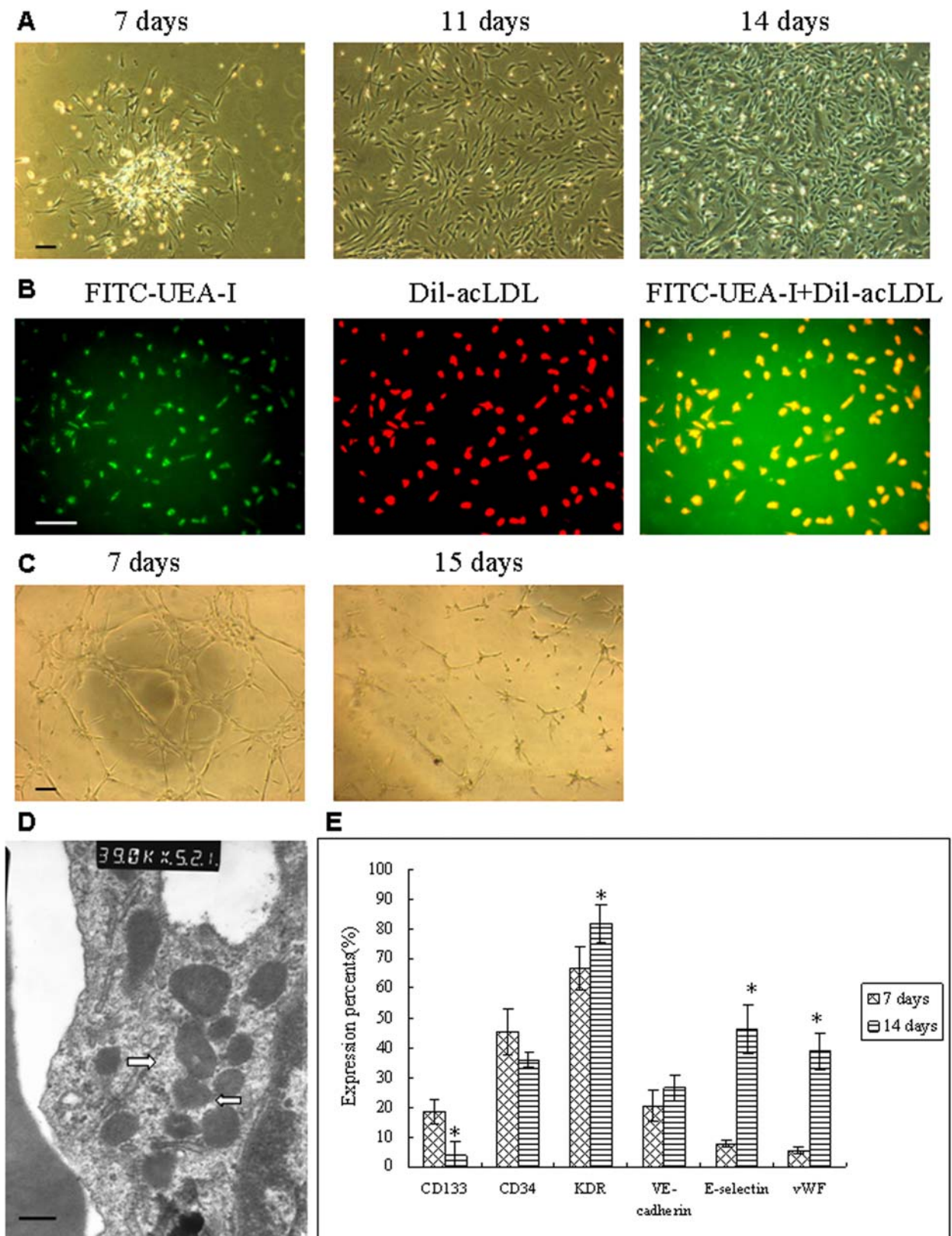
Fig. 1. (**A**) The morphology of EPCs in different growth periods. 7 days-cultured EPCs formed apparent colonies. 11 d-cultured EPCs were elongated and had a spindle shape. 14 d-cultured EPCs grown to confluence show a cobblestone-like monolayer. (**B**) EPC characterization by immunofluorescence. Binding to FITC-UEA-I, endocytosis of Dil-acLDL, and double positive cells appearing yellow in the overlay were identified as differentiating EPCs. (**C**) Differences between the two culture periods of EPCs of the ability to form tubes on the ECM *in vitro*. (**D**) A Weibel-Palade body (arrow) was observed in the endochylema by TEM. (**E**) The expression pattern of cell surface markers. The percentage of expression of CD133, CD34, KDR, VE-Cadherin, E-selectin and vWF was determined by flow cytometry at 7 d and 14 d culture. (*) $p < 0.05$ *versus* the results for 7 d. Bar in **A**, **B**, **C** = 100 μm, in **D** = 200 nm.
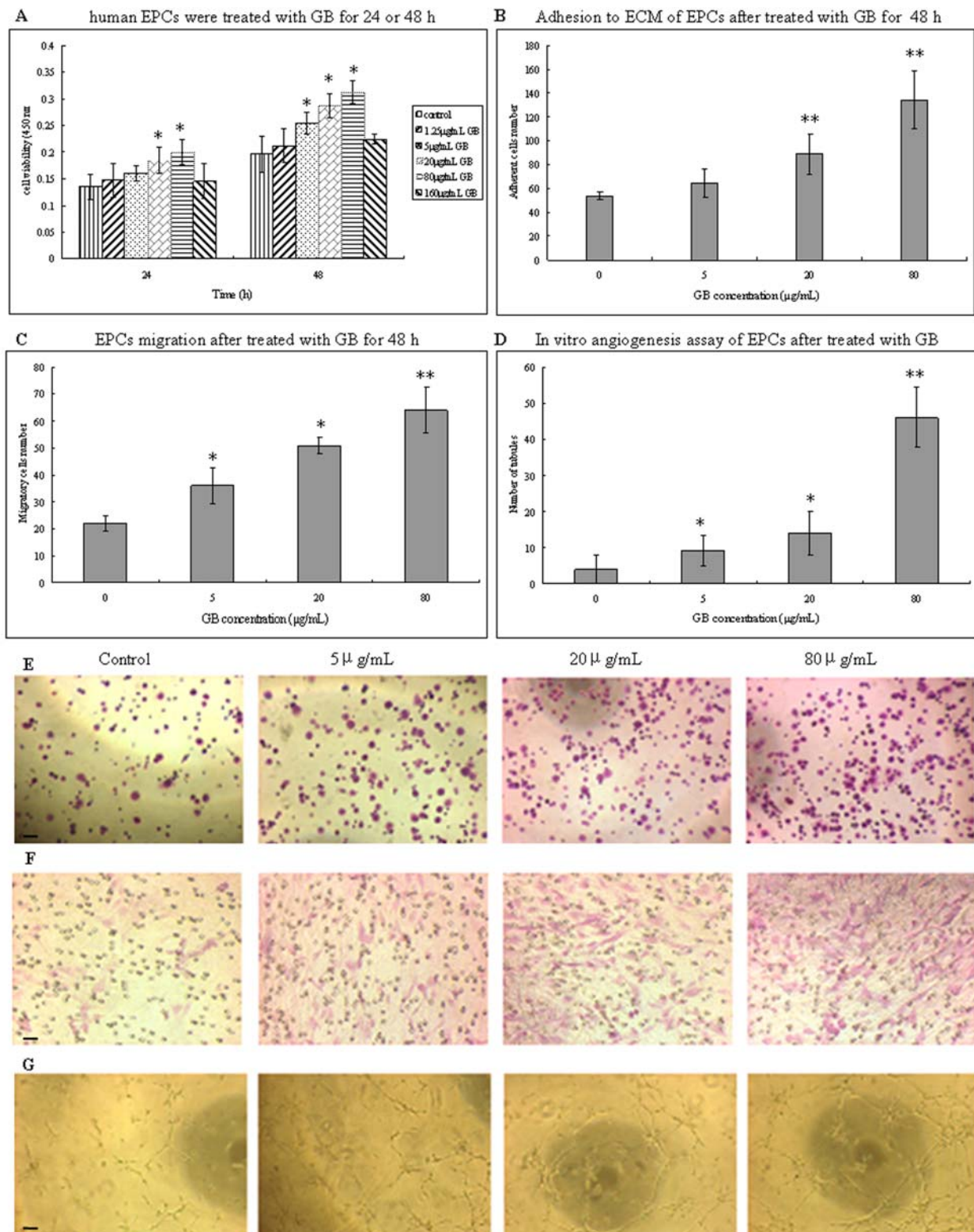
Fig. 2. (**A**) Human EPCs were starved in serum-free medium for 24 h, then treated with GB for another 24 h and 48 h. 1.25 and 160 μg/mL GB showed no effect on the proliferation of EPCs in 24 and 48 h. EPC proliferation was enhanced by 20 and 80 μg/mL GB for 24 h, and by 5, 20 and 80 μg/mL GB for 48 h, *N*=8. (**B**) After human EPCs were incubated with GB for 48 h, EPC adhesion to ECM was increased when the cells were incubated with 20 and 80 μg/mL GB, but not with 5 μg/mL GB, *N*=5. (**C**) Pretreatment with 5, 20 and 80 μg/mL GB for 48 h increased human EPC migration to the lower surface of the membrane of Transwell chambers, *N*=5. (**D**) GB preincubation for 48 h enhanced capillary-like tube formation of EPCs in a dose-dependent manner from 5 to 80 μg/mL, *N*=5. Representative pictures of adhesion, migration, and *in vitro* angiogenesis of 0 to 80 μg/mL GB pretreated human EPCs are shown in (**E**), (**F**), and (**G**) respectively. (*) $p < 0.05$, (**) $p < 0.01$ *versus* the control group. Bar = 100 μm.
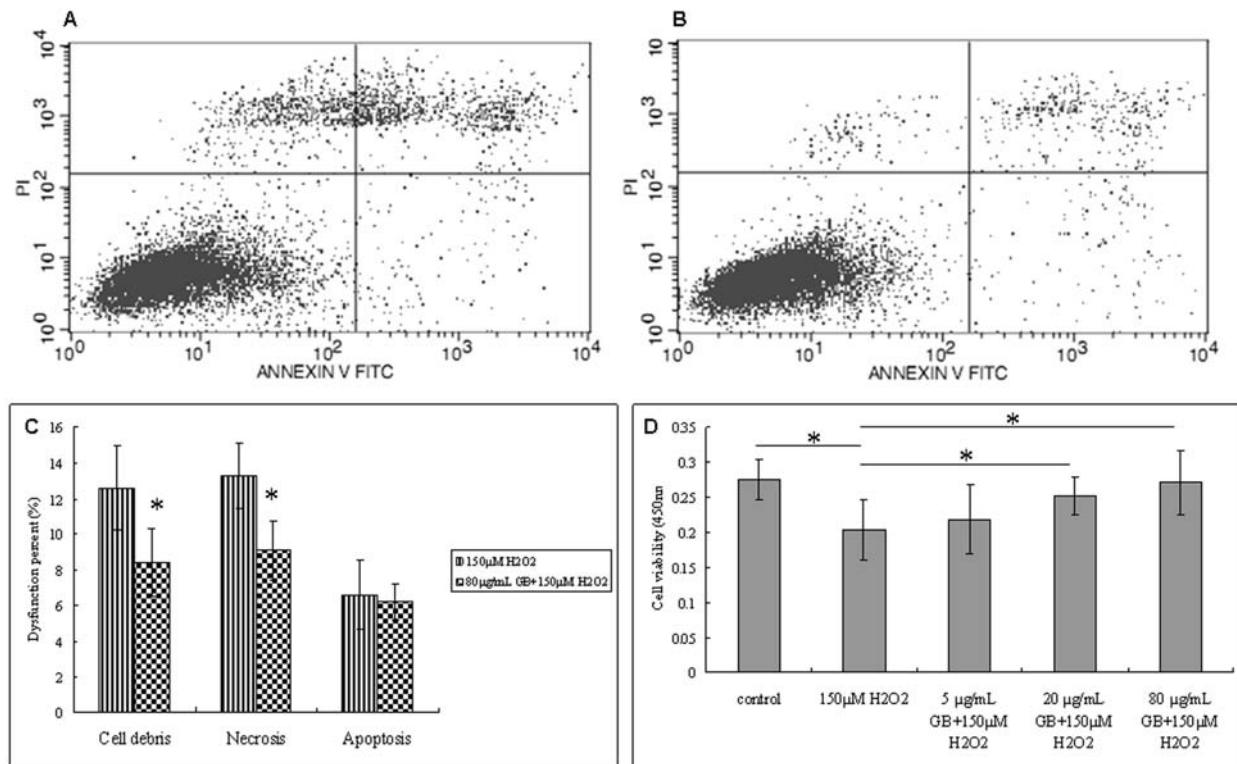
Fig. 3. Effects of GB on H_2O_2 induced cell dysfunction. Cells were pre-incubated with or without various concentrations of GB for 48 h, and then treated with 150 μM H_2O_2 for 6 h. Examples of dot plots, as determined by flow cytometry, following annexin V and PI double staining. The horizontal axis represents annexin V intensity, and the vertical axis shows PI staining. The lines divide each plot into four quadrants: lower left quadrant (living cells), lower right quadrant (apoptotic cells), upper left quadrant (necrotic cells), and upper right quadrant (debris). Representative results showed cell apoptosis, necrosis, and debris when cells were treated with 80 μg/mL GB (**B**) or without it (**A**), followed by 150 μM H_2O_2 for 6 h. (**C**) Inhibitory effect of GB on H_2O_2 induced cell apoptosis and necrosis was expressed as a percentage. The apoptosis, necrosis, and debris rates were 6.6%, 13.3% and 12.6% respectively without GB. After pretreatment with GB, the apoptosis, necrosis, and debris rate decreased to 6.2%, 9.1% and 8.4%, respectively. N=3. (**D**) Incubation with 150 μM H_2O_2 for 6 h significantly reduced the cell viability, GB alleviated the damage to EPCs by 150 μM H_2O_2 in a dose-dependent manner from 5 to 80 μg/mL. N=6. (*) $p < 0.05$ *versus* the control group.

activity at 80 μg/mL GB became apparent at 24 h and reached a maximum at 48 h.

Adhesion to ECM was increased by GB

To investigate the possibility that GB alters the adhesiveness of cultured human EPCs, EPCs were incubated with GB for 48 h. After replating on fibronectin-coated dishes, EPCs pre-exposed to GB exhibited a significant increase in the number of adhesive cells at 30 min. The number of adhesive cells began to increase after treated with 5 μg/mL GB and in a dose-dependent manner ($p < 0.01$; Figs. 2B, 2E).

GB enhances the migratory activity of EPCs

It is known that promotion of cell mobility and maintenance of cell survival signaling are essential for capillary tube formation. Thus, we further investigated whether GB can affect the migrative activity of EPCs. The cellular response of GB-treated EPCs to VEGF-induced migration was examined using the Transwell assay. GB (22 ±2.6 *vs*. 64 ±6.5 cells/field for control and 80 μg/mL GB, respectively; $p < 0.01$) also concentration-dependently enhanced EPCs

migratory activity, which became apparent at 5 μg/mL ($p < 0.05$) and reached a maximum at 80 μg/mL (Figs. 2C, 2F).

GB augments the capillary-like tube formation

In vitro angiogenesis assay was used to investigate the ability of EPCs to participate in vascularization, which is the most important activity of EPCs. The response of the EPCs to GB is shown in Fig. 2D, and Fig. 2G. Tubule number increased in a dose-dependent fashion in response to GB at 48 h of incubation, with peak tubule numbers observed in the presence of 80 μg/mL GB. Moreover, tubules in GB-treated wells were qualitatively different and more complex than those in control wells.

Protective effect of GB on the H_2O_2-injured EPCs

EPCs were exposed to increasing concentrations of H_2O_2 from 0 to 1,000 μmol/L for 6 h (data from the pilot study not shown). Our studies showed that 150 μmol/L H_2O_2 is the proper concentration. The treatment of cells with GB in the presence of 150 μmol/L H_2O_2 protected the cells against the cytotoxicity effect of H_2O_2 (Fig. 3D). Following H_2O_2 treatment, EPCs apoptosis and necrosis were examined.
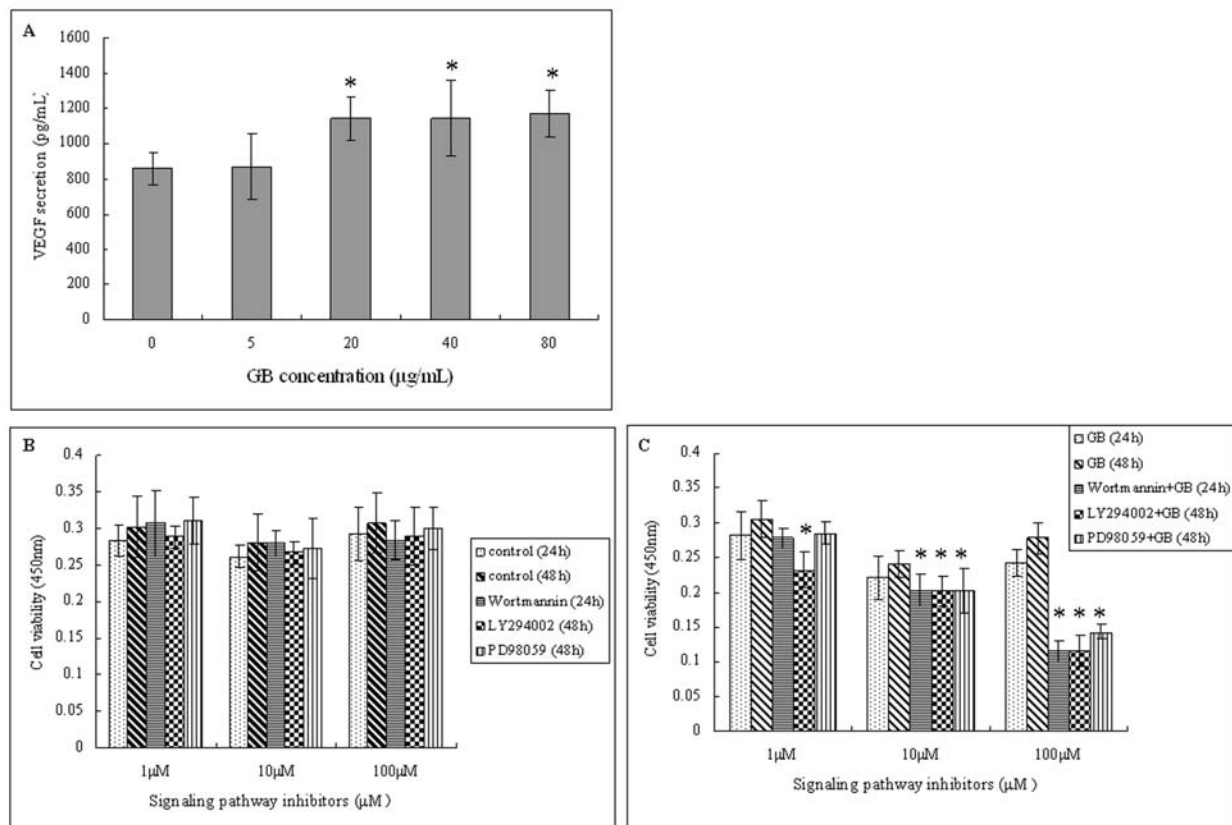
Fig. 4. (**A**) The VEGF secretion of EPCs treated with or without GB for 48 h. Cell supernatants were then collected and used for ELISA analysis. Compared with the control group, VEGF secretion was increased by the presence of GB at 20, 40 and 80 μg/mL. (**B**) To characterize the relative roles of Akt and MAPK/ERK pathway on GB-induced proliferation, PI3K and MEK1 inhibitors (wortmannin and LY294002 for PI3K, PD98059 for MEK1) were used. At concentrations of 1, 10 and 100 μM, the growth of EPCs was not affected by wortmannin for 24 h, by LY294002 or PD98059 for 48 h, (**C**) Wortmannin (10 and 100 μM) in 24 h, LY294002 (1, 10 and 100 μM) or PD98059 (10 and 100 μM) for 48 h significantly attenuated the cell proliferation induced by 80 μg/mL GB. (*) $p < 0.05$ *versus* the control group.

Flow cytometry analysis indicated the protective effect of GB against oxidative damage induced apoptosis by H_2O_2 (Figs. 3A, 3B). The dysfunctional cell rates decreased from 32.5% (apoptosis rate 6.6%, necrosis rate 13.3%, and debris rate 12.6%, without pretreatment with GB) to 23.7% (apoptosis rate 6.2%, necrosis rate 9.1%, and debris rate 8.4%, pretreated with GB) (Fig. 3C).

Effects of GB on VEGF secretion
Because of the important role of VEGF in the regulation of angiogenesis and cell function, we evaluated the secretion level of VEGF in EPCs. Over a period of incubation of 48 h with GB, the concentration of VEGF in the supernatant became significantly greater for EPCs treated with GB at 20, 40 or 80 μg/mL than for untreated (control) EPCs (859.6 ±69.2 pg/mL *vs*. 1174.5 ±110.6 pg/mL, for control and 80 μg/mL GB, respectively; $p < 0.05$; Fig. 4A).

The activation of AKT and ERK pathways mediates the effects of GB on EPCs
We examined the signaling molecules that were responsible for cell growth upon GB stimulation. To characterize the relative roles of PI3K/Akt and MAPK/ERK on GB-

induced proliferation, three different selective inhibitors (wortmannin and LY294002 for PI3K, PD98059 for MEK1) were used. At the concentrations of 1 μM, 10 μM and 100 μM , all three inhibitors did not affect the growth of control EPCs (Fig. 4B). But the cell number when treated with GB and inhibitors were significantly decreased compared to treatment with GB alone ($p < 0.05$) (Fig. 4C). These results suggested a link between GB-induced EPCs proliferation and both Akt and ERK-activated signaling pathways.

To further investigate the molecular mechanisms underlying the effects of GB on EPCs, the phosphorylation status of Akt, eNOS and p38, which are implicated in EPCs proliferation and function, was examined by Western blot analysis. Under the conditions used in the present experiments, GB stimulated Akt expression, an effect that was more evident at the higher concentrations, while the total Akt levels remained unchanged. Since VEGF requires Akt to augment EPCs numbers, this suggested an essential role for Akt in regulating EPCs differentiation (Figs. 5A, 5B). In addition, eNOS phosphorylation in EPCs was also significantly induced after GB treatment. To further define the roles of MAPK in the GB-induced effects on EPCs, we
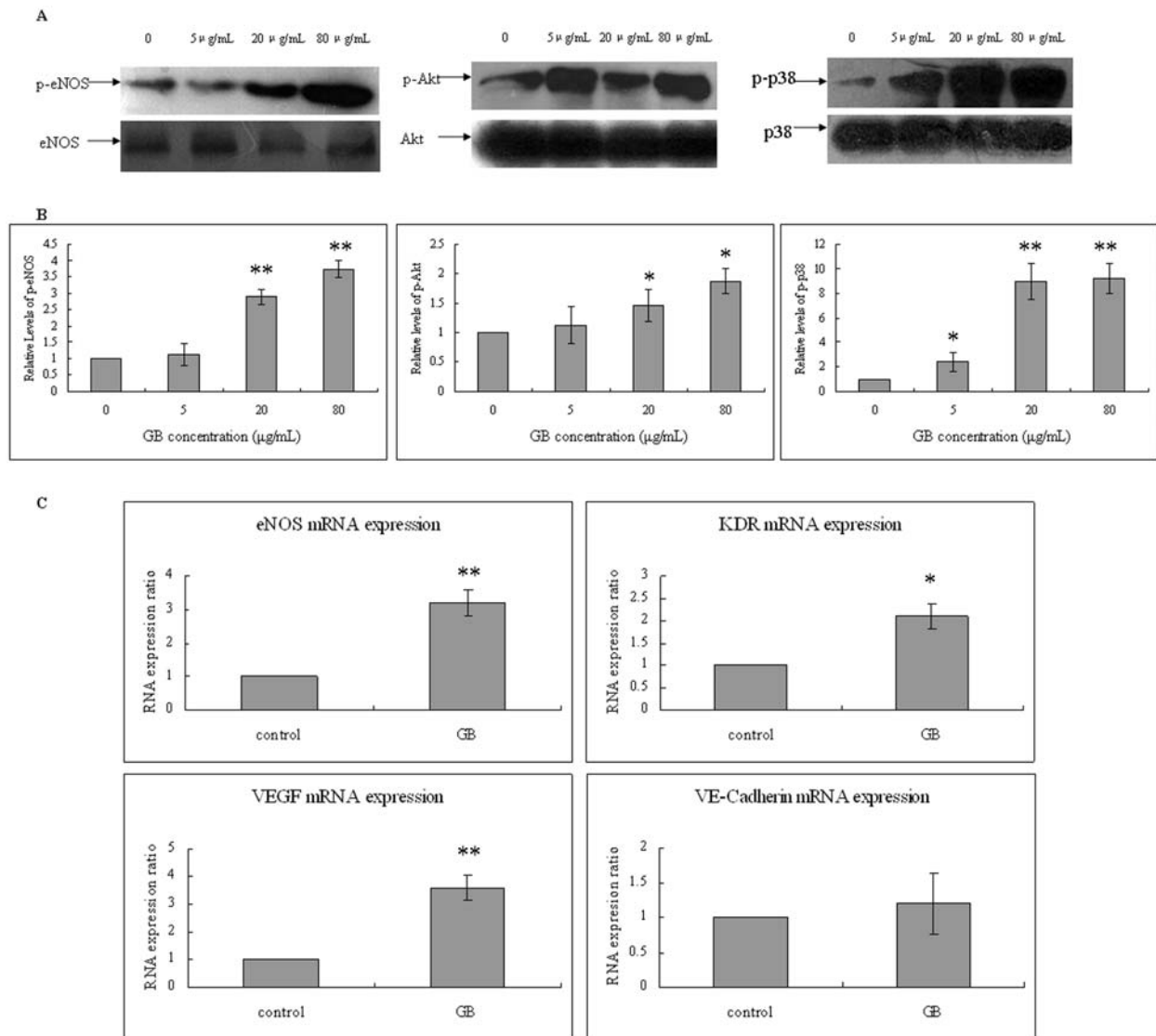
Fig. 5. GB induces the phosphorylation of Akt, eNOS and p38. (**A**) EPCs were treated with the indicated concentrations of GB for 48 h. The phosphorylation states of the indicated kinases were then examined by Western blotting. (**B**) Quantification of the levels of phospho-Akt, phospho-eNOS and phospho-p38 by densitometry analysis. (**C**) GB induces endothelial gene expression. EPCs were incubated without (control) or with 80 µg/mL GB for 48 h. Total RNA was extracted and the expression of indicated genes was determined by RT-PCR. (*) $p < 0.05$, (**) $p < 0.01$ *versus* the control group.

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analyzed the expression of phosphorylated p38 following GB treatment. As demonstrated in Fig. 5A, GB activated the phosphorylated p38 levels significantly, suggesting that p38 is involved in the stimulatory effect of GB on EPCs.

GB upregulates the expression of endothelial genes
VEGF, eNOS, KDR and VE-adherin are well known as endothelial genes that are involved in the functions of EPCs. To examine whether GB treatment affected the expression of these genes in EPCs, we performed quantitative real-time (RT)-PCR. As shown in Fig. 5C, the expression level of eNOS increased in GB-treated human EPCs over threefold when compared with a vehicle-treated control ($p < 0.01$). The expression of KDR was also enhanced by GB treatment, although to a lesser extent (p

< 0.05; Fig. 5C). Additional experiment was performed to determine the expression level of VEGF, which is a gene implicated in the proliferation and differentiation of EPCs in an autocrine manner. There was a significant increase in the expression of VEGF ($p < 0.01$; Fig. 5C). But GB treatment did not seem to significantly affect the expression of VE-cadherin in EPCs.

Discussion

For the first time, the present study demonstrated that GB, at a near medical applied dose, enhanced the *ex vivo* expansion of EPCs with increased functional activities. GB increased the number of EPCs in a dose and time dependent

manner which became apparent at 20 μg/mL at 24 h, peaking at 80 μg/mL at 48 h. Homing and incorporation of EPCs into the sites of revascularization probably are determined not only by the number of circulating EPCs but also by EPC maturation and/or differentiation. In this regard, our present data showed that GB also promoted EPCs migration and adhesion in an approximate dose and time dependent manner. Moreover, GB effectively induced EPCs tubule formation *in vitro* accompanied by upregulation of VEGF. VEGF is known to be the master stimulus of angiogenesis through binding to two receptor tyrosine kinases, Flt-1 and KDR, which belong to the PDGF receptor superfamily (Ferrara, 1999). Based on the aforementioned analysis, in turn, GB could regulate EPCs mobilization and differentiation, and contribute to the pro-angiogenic effects of those cells.

Dimmeler *et al*. have shown that VEGF, as well as statins, induced EPC differentiation *via* the PI3K/Akt pathway, as demonstrated by the inhibitory effect of pharmacological PI3K blockers or the over expression of a dominant negative Akt construct. Statins increased the numbers of EPCs *via* a PI3K dependent pathway, which is putatively related to the release of nitric oxide, causing a reduction in the rate of apoptosis of EPCs (Dimmeler *et al*., 2001; Imanishi *et al*., 2008). Activation of Akt promoted endothelial cell survival by inhibiting apoptosis (Dimmeler *et al*., 1998), stimulated endothelial NO synthesis (Fulton *et al*., 1999), and mediated VEGF-induced endothelial cell migration (Kawasaki *et al*., 2008). Recent studies have also provided increasing evidence that MAPK signaling is critical during the endothelial differentiation of vascular progenitor cells (Xu *et al*., 2008). In order to detect the role of PI3K/Akt and MAPK in the GB-induced angiogenic response of EPCs, we performed additional experiments in which MAPK and Akt activation was blocked by the pharmacological agents wortmannin, LY294002, and PD98059 respectively. Our data showed that at concentrations that did not affect the growth of control EPCs, all three inhibitors significantly attenuated the cell proliferation induced by GB, suggesting that activation of the Raf/MEK and PI3K/Akt pathways is crucial for promoting cell growth and function. To explore whether these kinases play a role in the GB induced angiogenic response of EPCs, and to elucidate the molecular mechanisms allowing commitment of EPCs to enhanced cell functional activity in the presence of GB, the phosphorylated status of Akt, eNOS and p38 following GB treatment was assessed. Our data indicated that GB induced the transient activation of Akt, eNOS and p38. Collectively, these data may support that GB promotes the proliferation and differentiation of these cells and enhances their function *via* the activation of Akt and MAPK/p38.

Although the proportional contribution of angiogenesis and vasculogenesis to neovascularization of adult tissue remains to be determined, it has been shown, either by mobilization of EPCs or by injection of *in vitro*-cultured hematopoietic stem cells, that EPCs improved neovascularization of the hind limb and cardiac function (Kalka *et al*., 2000; Murohara, 2003). EPCs in peripheral blood and bone marrow have been implicated in revascularization, vascular repair, and smoothly blood supply (Asahara *et al*., 1997; Dimmeler *et al*., 2001; Kalka *et al*., 2000; Walter *et al*., 2002), represented a very interesting alternative cell source for enhancing the vasculogenesis in regenerative medicine (Avci-Adali *et al*., 2011; Finkenzeller *et al*., 2007). Therefore, further understanding of the mechanisms that regulate EPCs mobilization and differentiation may provide new insights for the use of GB.

Many antioxidant and anti-inflammatory mechanisms are involved in the protection of injured vascular endothelial cells by the use of pharmaceutical agents. In the present study, exposure of H_2O_2 to EPCs had induced oxidative damage and loss of cell viability. This finding is in accordance with previous results that reported the oxidant-mediated cellular injury by H_2O_2 on human umbilical vein endothelial cells and other cell types like human fibroblast cells and endometrial cells (Spencer *et al*., 2001; Estany *et al*., 2007; Cianchetti *et al*., 2008). As far as the results of the current study are concerned, the harmful effect of H_2O_2 and the protective effect of GB are mainly related to their oxidant and antioxidant activity. Since the WST-8 assay is a measure of total cell viability and the results should reflect changes in both cell proliferation as well as cellular damage, we characterized the specific effects of GB on levels of apoptosis and necrosis. The data showed that GB preserved the cell viability as well as protected against H_2O_2-induced cell damage in EPCs.

In summary, the results of the present study demonstrate that GB promotes EPCs proliferation, migration, adhesion and induction of angiogenesis *in vitro* through upregulation of VEGF. There are several possible scenarios by which GB could increase the number and functional activities of BM-EPCs. One explanation might be the decrease of apoptosis of premature progenitor cells. Secondly, GB may interfere with the signal pathways regulating EPCs differentiation. These findings raise the intriguing possibility that GB may play an important role in the protection and revascularization of blood vessels. Further studies are needed to determine whether GB could increase EPC-mediated new vessel formation *in vivo*, which is beneficial to inducing neoangiogenesis for regenerative medicine.

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Editor's Note: Since all questions/comments by the reviewers were answered by text changes, there is no "Discussion with Reviewers" section.