Ginkgolide B enhances the differentiation of preosteoblastic MC3T3-E1 cells through VEGF: Involvement of the p38 MAPK signaling pathway

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Abstract. Ginkgolide B (GB) is one of the ginkgolides isolated from the leaves of the *Ginkgo biloba* tree. Our previous study indicated that GB promotes the proliferation, migration and adhesion of endothelial progenitor cells, and the induction of angiogenesis through vascular endothelial factor (VEGF). In the present study, the effects of GB on the differentiation of MC3T3-E1 cells and the signaling pathway involved were investigated *in vitro*. The MC3T3-E1 cell viability activities were assessed using an MTS assay. Measurements of alkaline phosphatase activity and Alizarin Red staining were used to identify osteoblastic differentiation of the MC3T3-E1 cells. The mRNA and secretion levels of VEGF were detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis and enzyme-linked immunosorbent assays, respectively. The protein expression levels of phosphorylation-associated markers were detected using western blot analysis and associated gene expression was determined using RT-qPCR analysis. It was found that GB significantly promoted alkaline phosphatase activity and osteoblastic mineralization in the MC3T3-E1 cells. In addition, the mRNA expression and secretion levels of VEGF in the MC3T3-E1 cells were significantly increased in MC3T3-E1 cells treated with GB. SB203580, a specific inhibitor of p38 mitogen-activated protein (MAP) kinase, markedly suppressed the GB-induced p38 kinase phosphorylation and GB-induced synthesis of VEGF. PD98059, an inhibitor of the upstream kinase, which activates p44/p42 MAP kinase, had minimal effect on the GB-induced phosphorylation of p44/p42 MAP kinase or the GB-induced synthesis of VEGF. Taken together, these results indicated that GB promoted osteoblastic differentiation of the MC3T3-E1 cells through VEGF, and that the p38, but not the p44/p42 MAP kinase signaling pathway, was involved in the GB-induced synthesis of VEGF.

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Introduction

Ginkgo biloba has been used as a medicine in China for >5,000 years. *Ginkgo biloba* extracts, isolated from the leaves of the *Ginkgo biloba* tree, contain flavonoid and terpenoid substances (1). It has antioxidant effects, acting as a scavenger of free radicals, has a relaxing effect on vascular walls, an antagonistic action on platelet-activating factor, beneficial effects on blood flow and microcirculation, and it stimulates neurotransmitters (2). A number of studies have demonstrated that ginkgolides exhibit protective effects on tissue abnormalities, which include myocardial ischemia reperfusion injury (3), ischemic brain damage (4) neuronal apoptosis (5), hypoxia-induced memory deficits and neuronal DNA damage (6). These effects are considered to be of benefit in the treatment of diseases that are associated with the production of free radicals, including ischemic heart disease, cerebral infarction, chronic inflammation and aging (7,8). The ginkgolides demonstrated to be present in the terpenoid extracts can be divided into isotypes A, B, C, M and J, with ginkgolide B (GB; $C_{20}H_{24}O_{10}$) exhibiting the highest biological activity (9).

The GB ginkgolide is a major constituent of the terpenoid fraction. A number of studies have shown that GB possesses neuroprotective effects on various brain injuries, including permanent focal ischemia, transient focal ischemia and ischemia-reperfusion injury (10), by exerting antioxidant effects, reducing brain edema or inhibiting cell apoptosis (11,12).

To the best of our knowledge, the majority of previous studies have focused on the neuroprotective effects of GB.

Our previous study (13) indicated that GB promotes bone marrow-derived endothelial progenitor cell proliferation, migration and adhesion, and the induction of angiogenesis through vascular endothelial growth factor (VEGF). As the effect of GB on osteoblast-like MC3T3-E1 cells has not been examined previously. The present study aimed to investigate the effect and underlying mechanism of GB on osteogenic differentiation in the MC3T3E-1 cell line. The results of the present study demonstrated that GB promoted osteoblastic differentiation of the MC3T3-E1 cells through the upregulation of VEGF, and that the p38 mitogen-activated protein (MAP) kinase signaling pathway is involved in GB-induced expression of VEGF. These findings suggest GB may be important in the treatment of osteoporosis.

Materials and methods

Cell culture. Murine osteoblastic MC3T3-E1 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in a 5% CO_2 atmosphere at 37˚C in α-MEM (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences). On reaching 80% confluence, the cells were passaged with 0.25% trypsin (BioSharp, Hefei, China) and transferred to new culture flasks at a ratio of 1:3. To induce osteogenic differentiation, the culture medium was replaced with differentiation medium (α-MEM containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid).

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). The GB was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at a concentration of 100 g/l.

Cell viability assay. The cytotoxic effects of GB on the MC3T3-E1 cells were evaluated using an MTS assay. The MC3T3-E1 cells were seeded in a 96-well plate at 10,000 cells per well and cultured for 24 h at 37˚C. Following rinsing with phosphate-buffered saline (PBS), the cells were treated with various concentrations of GB (0, 1.25, 5, 20, 80 and 160 μg/l) in fresh medium for 48 h. The viable cells were then treated with newly prepared medium containing 20 μl of 2 mg/ml MTS and 100 μl of α-MEM (10% FBS) in a CO_2 incubator for 3 h. The MTS was transformed by the living cells to a purple formazan dye, which was dissolved in 100 μl DMSO by shaking at 150 rpm for 10 min on an ELISA shaker (Gentaur, Brussels, Belgium). Finally, the relative colorimetric intensity of each well was evaluated using a Varioskan flash multimode reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) at a 490 nm wavelength.

Measurement of alkaline phosphatase (ALP) activity. The MC3T3-E1 cells were seeded in a 96-well plate at 10,000 cells per well, and placed in a 5% CO_2 incubator at 37˚C for 7 and 14 days, respectively. Following being rinsed with PBS, the MC3T3-E1 cells were treated with 20 μg/ml GB in fresh medium for 4 days. Following removal of the medium, the cells in each well were incubated with 100 μl of ALP buffer solution for 10 min. The cells of each well were treated with 100 μl of pNPP at 37˚C for 15 min to determine the ALP activity. Subsequently, 100 μl of 2 M NaOH was added to terminate the reaction. Finally, the conversion of p-nitrophenyl phosphate to p-nitrophenol was determined using a Varioskan flash multimode reader (Thermo Fisher Scientific Inc.) at a wavelength of 450 nm. The intensity of ALP activity relative to that of the control group in each well was determined.

Alizarin red staining. On days 14 and 21, the cultured cells were fixed for the detection of Alizarin Red staining (Sigma-Aldrich; Merck Millipore). Briefly, the cells were fixed with ice-cold 70% ethanol for 60 min, washed three times with PBS, and stained with Alizarin Red S (40 mM; pH 4.2) for 10 min, followed by rinsing with PBS three times. Images of the stained nodules were captured using a digital camera (Nikon C-SHG; Nikon Corporation, Tokyo, Japan). Subsequently, 50 μl 10% cetylpyridinium chloride was added to each well, cultured at room temperature for 20 min and the relative colorimetric intensity of each well was evaluated using a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Inc.) at a wavelength of 560 nm. Finally, according to the standard curve, the optical density value was converted to concentration.

Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The total RNA of the MC3T3-E1 cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following subsequent DNase digestion (Invitrogen; Thermo Fisher Scientific, Inc.), 500 ng of RNA was used to synthesize 10 μl of cDNA using an PrimeScript® RT Master Mix kit (Takara Biotechnology, Co., Ltd., Dalian, China). The qPCR reactions were performed in a CFX Connect™ real time system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using All-in-One™ qPCR mix (GeneCopoeia, Guangzhou, China). SYBR Green Master mix (GeneCopoeia) was used for all PCR experiments. Thermocycling conditions were as follows: 95˚C for 20 sec for pre-denaturation; and 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. The expression in each sample was evaluated in three technical replicates. The specificity of primer pairs and absence of primer dimers was validated by analysis of the dissociation curves and agarose gel electrophoresis. The comparative Cq method (14) was used for data analysis. The level of expression in each sample was normalized to the mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The mean expression from three experiments was calculated. The sequences of the primers used in the present study were as follows: VEGF, forward 5'GGACCCTGGCTTTACTGCTGTACC-3' and reverse 5'TCACCGCCTTGGCTTGTCACA-3'; Osx, forward 5'-GCAAGGCTTCGCATCTGAA-3 and reverse 5'-TAGCAGGTTGCTCTGCTC-3'; GAPDH, forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3'.

Western blot analysis. The proteins were isolated from MC3T3-E1 cells using RIPA lysis buffer (Google Biological Technology Co., Ltd., Wuhan, China) and concentration was determined by the BCA Protein assay kit (Beyotime Institute

of Biotechnology, Haimen, China) according to the manufacturer's protocols. The equal samples of proteins (30 μg) were separated on 10% SDS-PAGE gels and transferred onto a PVDF membrane followed by western blot analysis. Briefly, 5% milk in TBS containing 0.1% Tween-20 was used to block non-specific binding. The membrane was incubated with anti-VEGF rabbit polyclonal antibody (Merck Millipore; cat. no. AB1876), P-38 polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 9228), phosphorylated (phospho)-specific P-38 polyclonal antibody (Cell Signaling Technology, Inc.; cat. no. 4092), phospho-specific p44/p42 (Cell Signaling Technology, Inc.; cat. no. 4695), or p44/p42 MAP kinase antibodies (Cell Signaling Technology, Inc.; 9106) overnight at 4˚C at a dilution of 1:1,000, followed by extensive washing with PBS and incubation for 1 h at 37˚C with horseradish peroxidase-conjugated secondary antibody (1:20,000; Cell Signaling Technology, Inc.; cat. no.). Following antibody incubation and washes with PBS, an ECL kit (GE Healthcare Life Sciences, Chalfont, UK) was used for detection.

Enzyme-linked immunosorbent assay (ELISA). The MC3T3-E1 cells were cultivated and treated with GB, as described for the cell viability assay. Following incubation with GB for 24 and 48 h respectively, the supernatants were harvested and used for the ELISA assay (eBioscience, Inc., San Diego, CA, USA), according to the manufacturer's protocol.

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). All data were analyzed using one-way analysis of variance and Student's *t* test. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed independently at least three times.

Results

Effects of GB on the viability of MC3T3E1 cells. To determine the effects of various concentrations of GB on the cell viability of MC3T3E1 cells, which are have the potential to differentiate into osteoblast cells on culture in osteoblastic differentiation medium, an MTS assay was performed. The cells were treated with different concentrations of GB for 48 h and cell viability was assessed using the MTS assay. As shown in Fig. 1, GB marginally increased the viability of the MC3T3-E1 cells within a 1.25-160 μg/ml dose range, with 20 μg/ml GB showing the maximal effect.

Effects of GB on osteoblastic differentiation in MC3T3-E1 cells. ALP is a well-recognized marker for the differentiation of osteoblasts, and to determine the effects of GB on the activities of ALP in MC3T3 cells, ALP activity assays were performed. The MC3T3-E1 cells were cultured in α-MEM (control), GB (20 μg/ml), osteogenic medium and osteogenic medium supplemented with 20 μg/ml GB for 1 and 2 weeks, respectively. The ALP activity in the cells treated with osteogenic medium supplemented with GB was significantly higher, compared with those in the control cells and cells treated with osteogenic medium alone at 1 and 2 weeks, respectively (Fig. 2A). These results indicated that GB promoted ALP activity in the MC3T3-E1 cells at the early stage.

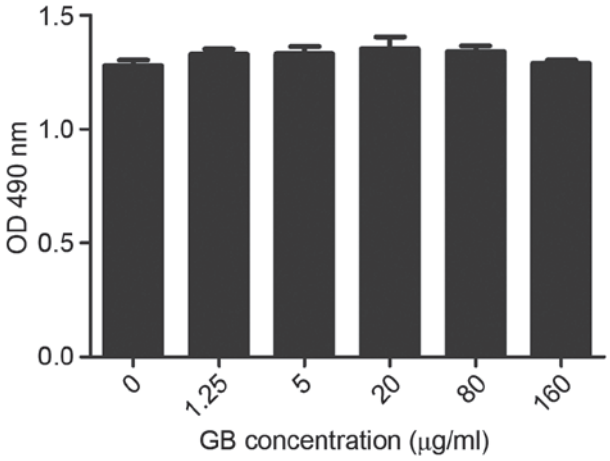

Figure 1. Effect of GB on the viability of MC3T3-E1 cells. The cells were seeded in 96-well plates (1x10^4 cells/well) for 24 h, and then treated with different concentrations of GB for another 48 h. Cellular proliferation was determined using an MTS assay. Each value represents the mean + standard deviation of three repeated experiments. GB, ginkgolide B; OD, optical density.

To determine the effect of GB on calcium deposition in the MC3T3-E1 cells, mineralization was examined using Alizarin Red staining, based on the results of the staining, quantification of the mineralization was performed. The MC3T3-E1 cells were examined following culture for 2 and 3 weeks in osteogenic medium with or without 20 μg/ml GB. GB significantly increased osteoblastic mineralization in the cells following treatment for 2 and 3 weeks, as visualized by the Alizarin Red S staining (Fig. 2B and C). There was an increase in the concentration of mineralized nodules in the group treated with osteogenic medium supplemented with GB, compared with the control group following culture for 2 and 3 weeks, respectively (Fig. 2B and C).

Effect of GB on the secretion of VEGF in MC3T3-E1 cells. To examine the effect of GB on the expression of VEGF in MC3T3-E1 cells, the present study used RT-qPCR analysis and ELISA assays to evaluate the mRNA expression and secretion levels of VEGF in the MC3T3-E1 cells. Over an incubation period of 24 h with GB, the mRNA expression of VEGF was significantly increased in the MC3T3-E1 cells treated with GB at 20 μg/ml, compared with the untreated MC3T3-E1 cells (P<0.05; Fig. 3A). However, the mRNA expression of VEGF decreased following incubation for 48 h with 20 μg/ml GB (Fig. 3A). Consistently, the concentration of VEGF in the supernatant was significantly increased in the MC3T3-E1 cells treated with 20 μg/ml GB, compared with the untreated MC3T3-E1 cells at 24 and 48 h, respectively (P<0.05; Fig. 3B).

Effects of SB203580 and PD98059 on the GB-induced synthesis of VEGF in MC3T3-E1 cells. To determine whether p38 MAP kinase or p44/p42 MAP kinase are involved in the GB-induced VEGF synthesis, the present study examined the effects of

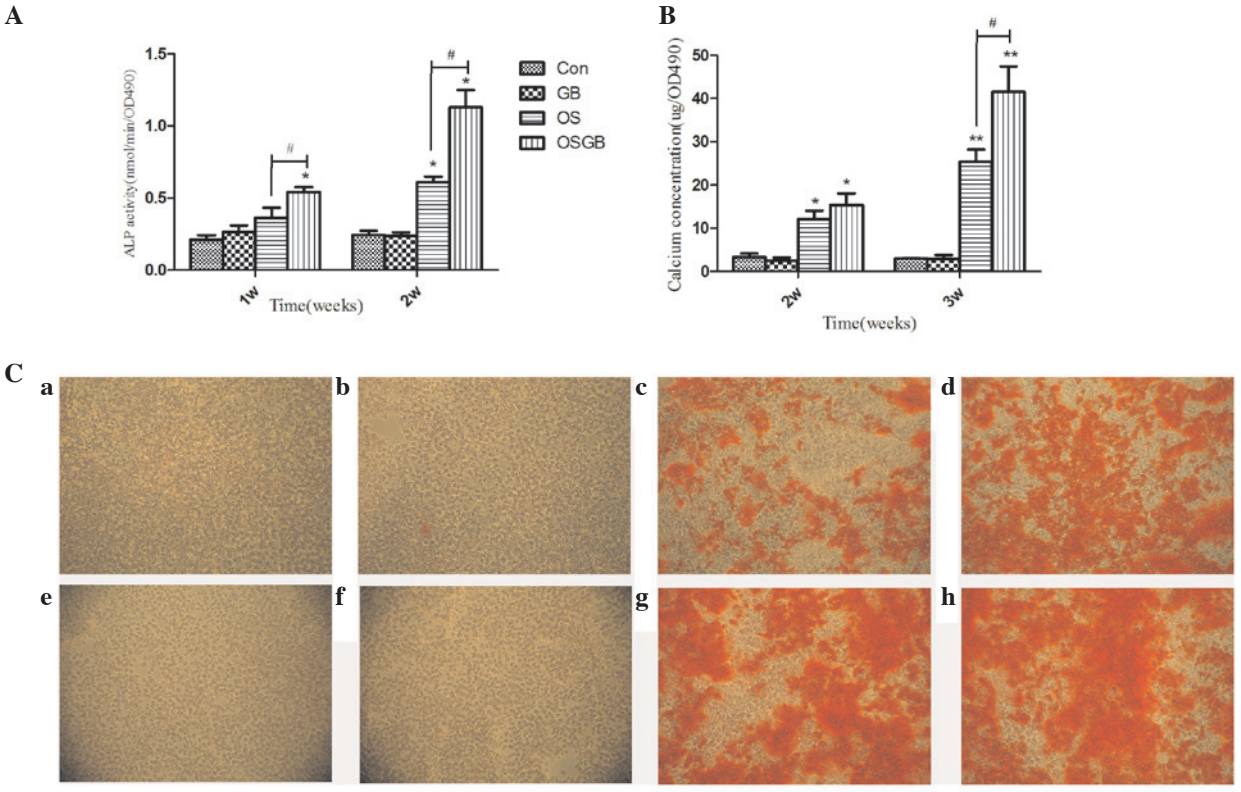

Figure 2. Effect of GB on the osteoblastic differentiation of MC3T3-E1 cells. (A) Measurements of ALP activity in MC3T3-E1 cells. The cells were cultured in α-MEM, GB, osteogenic medium and osteogenic medium supplemented with 20 μg/ml GB, respectively. Following treatment of 1 and 2 weeks, the cells were subjected to an ALP activity assay. The data are presented as the mean + standard deviation (*P<0.05, vs. Con; $^{\#}$P<0.05 vs. OS. n=3). (B) Quantitative analysis of mineralization, based on the results of the Alizarin Red staining. The data are presented as the mean + standard deviation (*P<0.05 and **P<0.01, vs. Con; $^{\#}$P<0.05, vs. OS; n=3). (C) Images in the top line show Alizarin Red S staining, with calcium deposition stained bright orange/red in the osteocytes at 2 weeks in the (a) Con, (b) GB, (c) OS and (d) OSGB groups. Images below show Alizarin Red S staining with calcium deposition stained bright orange/red in the osteocytes at 3 weeks in the (e) Con, (f) GB, (g) OS and (h) OSGB groups. Magnification, x100. GB, ginkgolide B; OS, osteogenic medium; OSGB, OS supplemented with GB; Con, control; ALP, alkaline phosphatase; OD, optical density.

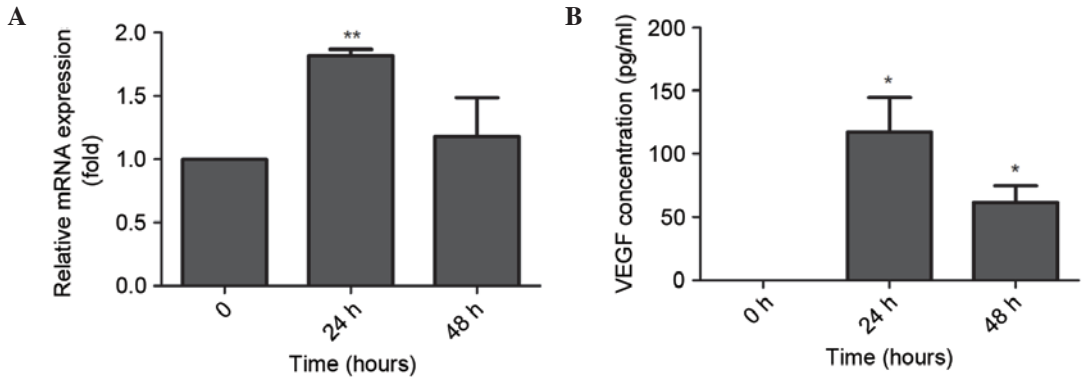

Figure 3. Effect of GB on the expression of VEGF in MC3T3E1 cells. (A) Cells were treated with 20 μg/ml GB for 24 and 48 h, respectively. The relative mRNA expression levels of VEGF were determined using reverse transcription-quantitative polymerase chain reaction analysis. Results are expressed as the mean + standard deviation (**P<0.01, vs. control; n=3). (B) Effect of GB on the secretion of VEGF in MC3T3E1 cells. The cells were treated with 20 μg/ml GB for 24 and 48 h, respectively. The cell supernatants were then collected and used for enzyme-linked immunosorbent assay analysis. Compared with the control group, the secretion of VEGF was increased by the presence of GB at 20 μg/ml. Results are expressed as the mean + standard deviation (*P<0.05, vs. Control; n=3). GB, ginkgolide B; VEGF, vascular endothelial factor.

SB203580, which specifically inhibits p38 MAP kinase, and PD98059, which inhibits the upstream kinase that activating p44/p42 MAP kinase, on the synthesis of VEGF induced by GB. SB203580, which by itself had minimal effect on the synthesis of VEGF, significantly reduced the GB-stimulated mRNA expression (Fig. 4A) and synthesis (Fig. 4B) of VEGF at a concentration of 30 μM. By contrast, PD98059 had no significant effect on the mRNA expression of VEGF (Fig. 4C) or GB-stimulated secretion of VEGF (Fig. 4D).

Effects of GB on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells. In order to

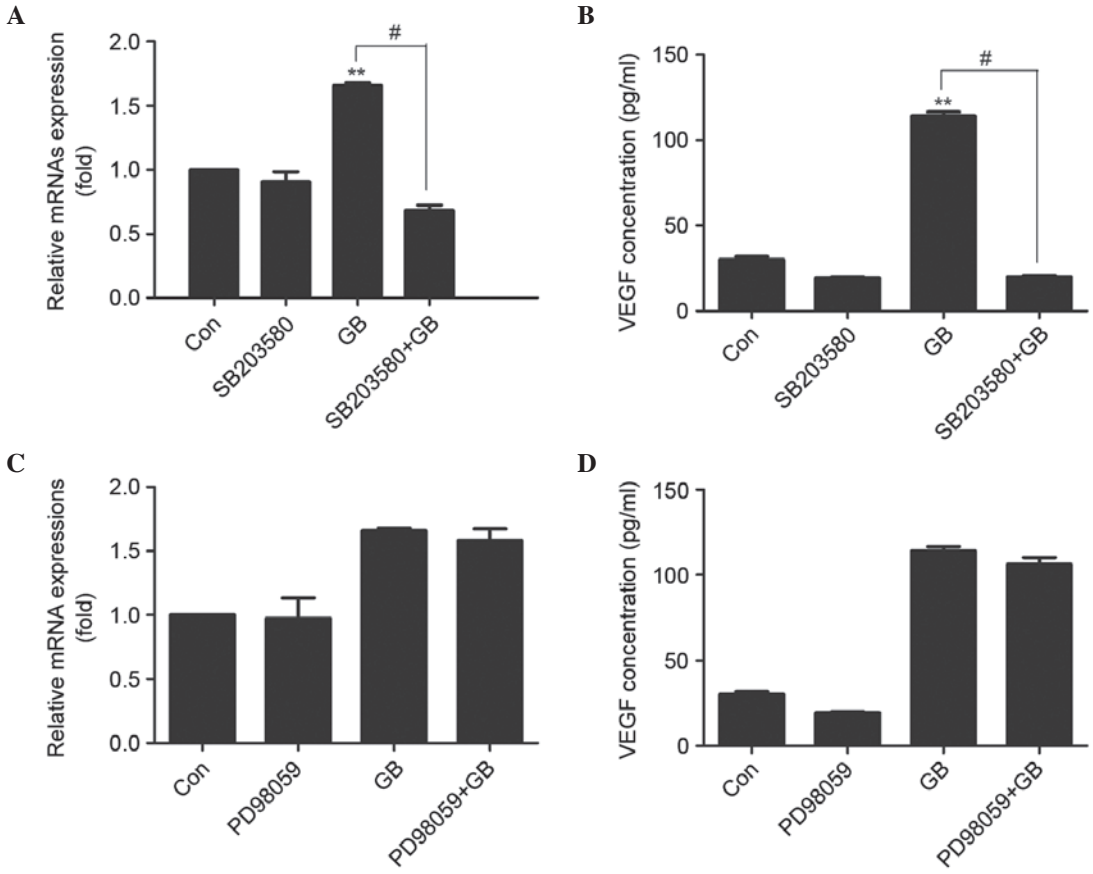

Figure 4. Effect of SB203580 and PD98059 on the expression of VEGF in MC3T3E1 cells. (A) Cells were pretreated with 30 μM SB203580 for 1 h, following which the cells were treated with 20 μg/ml GB for 24 h. The RNA was extracted and mRNA expression levels of VEGF were determined using RT-qPCR analysis. Results are expressed as the mean + standard deviation (**P<0.01, vs. Con; $^{\#}$P<0.01; n=3) (B) Cells were pretreated with 30 μM SB203580 for 1 h, followed by treatment with 20 μg/ml GB for 24 h. Cell supernatants were then collected and used for enzyme-linked immunosorbent assay analysis. The results are expressed as the mean + standard deviation (**P<0.01, vs. Con; $^{\#}$P<0.01; n=3). (C) Cells were pretreated with 50 μM PD98059 for 1 h, followed by treatment with 20 μg/ml GB for 24 h. The RNA was extracted and the mRNA expression levels of VEGF were determined using RT-qPCR analysis. The results are expressed as the mean + standard deviation. (D) Cells were pretreated with 50 μM PD98059 for 1 h, followed by treatment with 20 μg/ml GB for 24 h. Cell supernatants were then collected and used for enzyme-linked immunosorbent assay analysis. Results are expressed as the mean + standard deviation. GB, ginkgolide B; VEGF, vascular endothelial growth factor; Con, control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

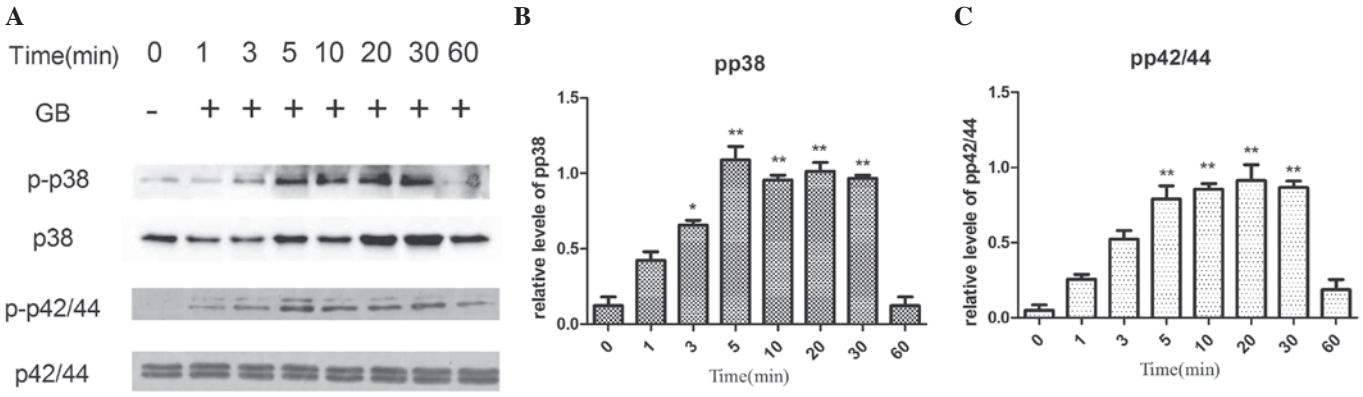

Figure 5. Effects of GB on the phosphorylation of p38 and p42/44 MAP kinase in MC3T3-E1 cells. The cultured cells were stimulated with 20 μg/ml GB for 1, 3, 5, 10, 20, 30 and 60 min, respectively. (A) The extracts of cells were subjected to SDS-PAGE and subsequent western blot analysis with antibodies against p-p38 or p42/44 MAP kinase and p38 or p42/44MAP kinase. (B and C) Quantification of the levels of p-p38 and p42/44 be densitometry. *P<0.05, **P<0.01 vs. 0 min GB, ginkgolide B; MAP, mitogen-activated protein; p-, phosphorylated.

determine whether GB activates p44/p42 MAP kinase and/or p38 MAP kinase, the present study investigated the effects of GB on these MAP kinases and, specifically, their levels of phosphorylation. Treatment with GB led to an increase in the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase in a time-dependent manner (Fig. 5). The significant

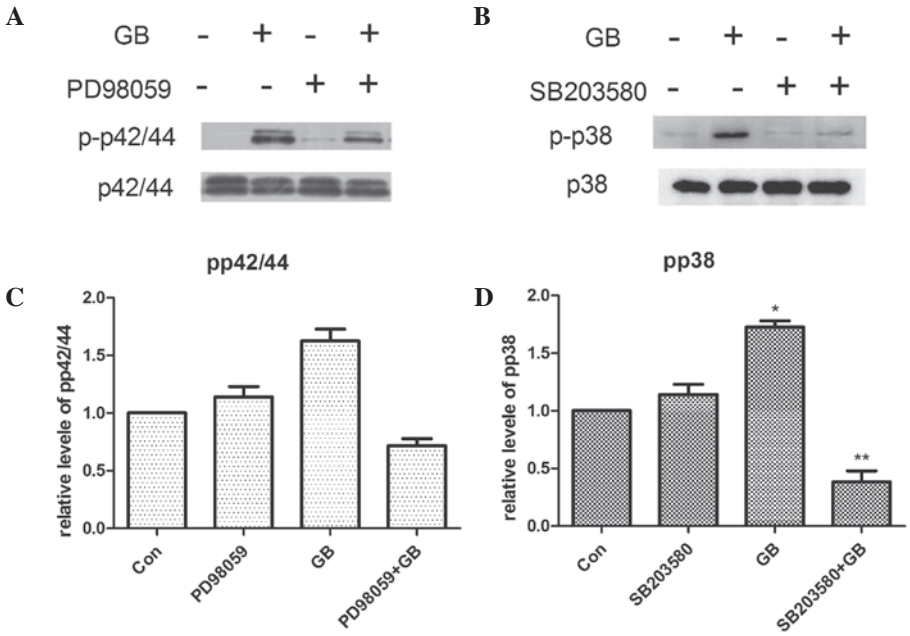

Figure 6. Effects of SB203580 and PD98059 on the GB-induced phosphorylation of p38 MAP kinase and p44/p42 MAP kinase. (A) Effect of PD98059 on GB-1-induced phosphorylation of p42/44 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 50 μM of PD98059 for 60 min, and then stimulated with 20 μg/ml GB for 5 min. Extracts of cells were subjected to SDS-PAGE and incubated with p-p42/44 MAP kinase antibodies or p42/44 MAP kinase antibodies. Similar results were obtained with two additional and different cell preparations. (B) Effect of SB203580 on the GB-1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM of SB203580 for 60 min, and then stimulated with 20 μg/ml GB for 5 min. Extracts of the cells were subjected to SDS-PAGE and incubated with p-p38 MAP kinase antibodies or p38MAP kinase antibodies. Similar results were obtained with two additional and different cell preparations. (C and D) Quantification of the levels of p-p42/44 and p-p38 by densitometry analysis. *P<0.05, **P<0.01 vs. the Con group. GB, ginkgolide B; MAP, mitogen-activated protein; p-, phosphorylated; Con, control.

stimulatory effects of GB on the phosphorylation of p44/p42 MAP kinase and p38 MAP kinase were observed 5 min post-stimulation (Fig. 5).

Effects of SB203580 and PD98059 on the GB-induced phosphorylation of p38 MAP kinase and p44/p42 MAP kinase in MC3T3-E1 cells. To investigate whether p38 or p44/p42 MAP kinase is involved in the phosphorylation induced by GB in the MC3T3-E1 cells, The MC3T3-E1 cells were pretreated with 30 μM SB203580 or 50 μM PD98059 prior to treatment with 20 μg/ml GB. As shown in Fig. 6, PD98059 had minimal effect on the GB-induced phosphorylation of p44/p42 MAP kinase (Fig. 6A). SB203580 alone had minimal effect on the phosphorylation of p38 MAP kinase; however, it significantly decreased the GB-induced phosphorylation of p38 MAP kinase (Fig. 6B).

Discussion

Traditional Chinese medicines have long been used among the Chinese population for the treatment of several diseases and conditions. Several *in vitro* and *in vivo* studies have demonstrated that Chinese herbs have positive effects on bone formation, which occur by promoting osteoblastic proliferation and inhibiting osteoclastic formation (15). Li *et al* (16) found that Naringin, a Chinese herbal medicine, promotes the proliferation and differentiation of bone marrow mesenchymal stem cells and effectively reverses ovariectomy-induced osteoporosis in rats. In a study by Huh *et al* (17), it was found that *Puerariae radix* is vital in osteoblastic bone formation and that this may be associated with the angiogenic property of the herb via the increased mRNA expression of VEGF.

GB, a herbal extract from the leaves of the *Ginkgo biloba* tree, has been shown to inhibit platelet-activating factor (18). Its suggested biological effects include the scavenging of free radicals, reducing oxidative stress, neural damage and platelet aggregation, and possessing anti-inflammatory, antitumor and anti-aging activities (19). Our previous study demonstrated that GB increases the number and functional activities of EPCs, with involvement of Akt/endothelial nitric oxide synthase and MAPK/p38 signaling pathways. However, to the best of our knowledge, whether GB induces osteogenic activity in mouse osteoblast-like MC3T3-E1 cells has not been reported. In the present study, it was found that GB promoted osteoblastic differentiation of MC3T3-E1 cells through the upregulation of VEGF, and the p38 MAP kinase signaling pathways was involved in the GB-induced synthesis of VEGF.

A number of previous reports have demonstrated a close association between angiogenesis, fracture healing and bone growth. It is known that, during bone formation, angiogenesis and osteogenesis are coupled spatially and temporally (20). The critical step in endochondral ossification is the introduction of highly vascularized bone to replace the avascular cartilage. The mechanism underlying angiogenesis involves in the action of VEGF, which is a potent angiogenic peptide and has mitogenic and chemotactic effects on endothelial cells (21,22).

There are an increasing number of reports indicating that VEGF has a positive effect in regulating osteoblast activity. D'Alimonte *et al* (23) demonstrated that VEGF enhances

the proliferation and osteogenic differentiation of human dental pulp stem cells *in vitro*. Hah *et al* (24) demonstrated that VEGF stimulates the differentiation of cultured human periosteal-derived cells into the osteoblastic and that VEGF may act as an autocrine growth factor for the osteoblastic differentiation of cultured human periosteal-derived cells. Tan *et al* (25) reported that VEGF promotes bone remodeling by direct effects on osteoblasts via regulating the gene expression of ALP, osteocalcin and osteoprotegerin through the VEGF receptor 2 signaling pathway. In the processes of bone formation and fracture healing, a study by Deckers *et al* (26) demonstrated that the VEGF produced by osteoblast-like cells enhances osteoblastic differentiation by stimulating endothelial cells to secrete growth factors and cytokines, which induce mesenchymal cells into osteogenic differentiation. In the present study, it was found that GB significantly increased ALP activity and osteoblastic mineralization in MC3T3-E1 cells. In addition, GB significantly increased the mRNA expression and secretion levels of VEGF in the MC3T3-E1 cells. These findings suggested that GB promoted the osteogenic differentiation of MC3T3-E1 cells through the upregulation of VEGF.

A previous study demonstrated that p38 MAP kinase, but not p42/p44 MAP kinase, is involved in prostaglandin E1-induced VEGF synthesis in MC3T3-E1 cells (27). Kozawa *et al* (28) showed that endothelin-1 stimulates VEGF synthesis via endothelin A receptor in osteoblasts, and that p38 MAP kinase is involved in endothelin-1-induced VEGF synthesis. Tokuda *et al* (29) reported that p44/p42 MAP kinase and p38 MAP kinase are involved in transforming growth factor-β-stimulated VEGF synthesis in osteoblasts. In the present study, in order to detect the role of p44/p42 and p38 MAP kinase in the GB-induced the expression of VEGF in MC3T3-E1 cells, the effects of GB on the phosphorylation of these MAP kinases were examined. It was found that GB induced the phosphorylation of p44/p42 and p38 MAP kinase in a time-dependent manner. Furthermore, SB203580, a specific inhibitor of p38 MAP kinase, markedly suppressed the GB-induced p38 kinase phosphorylation and GB-stimulated VEGF synthesis. PD98059, an inhibitor of the upstream kinase, which activates p44/p42 MAP kinase, had minimal effect on the GB-induced phosphorylation of p44/p42 MAP kinase or GB-induced VEGF synthesis. These data indicated that the p38, but not the p44/p42, MAP kinase signaling pathway is involved in the GB-induced expression of VEGF.

A series of studies have examined the role of MAP kinases in modulating osteogenic differentiation. Greenblatt *et al* demonstrated that p38 promoted skeleton formation and bone homeostasis through runt-related transcription factor 2, which is a key transcription factor associated with osteoblast differentiation (30,31). A study by Hu *et al* confirmed that p38 has a positive effect in the early and late osteogenesis of osteoblasts, bone marrow osteoprogenitor cells and the MC3T3-E1 osteoblast line (32,33).

In conclusion, the results obtained in the present study demonstrated that GB enhanced the osteoblastic differentiation of MC3T3-E1 cells through VEGF, and that the p38 MAP kinase signaling pathway was involved in the GB-induced expression of VEGF. These findings suggest that GB is a potential candidate target for treating or preventing osteoporosis.

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