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RESEARCH ARTICLE

Stimulative effects of *Polygonum amplexicaule* var. *sinense* on osteoblastic MC3T3-E1 cells

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Abstract

Context: *Polygonum amplexicaule* D. Don var. *sinense* Forb. (Polygonaceae) (PAF) is a well known traditional herb used to treat some diseases, such as fractures, rheumatoid arthritis, muscle injury, and pain. However, its pharmacological mechanism of promoting the healing of fractures is still unknown.

Objective: The present study was designed to investigate the effects of PAF ethanol extracts on the proliferation and differentiation of osteoblastic MC3T3-E1 cell *in vitro*, thereby to illuminate the pharmacological mechanism to promote the healing of fractures.

Materials and methods: The effects of PAF ethanol extracts on MC3T3-E1 cell proliferation and differentiation were detected by using CCK-8, cell cycle, alkaline phosphatase (ALP), and prostaglandin E_2 (PGE_2) assays *in vitro*.

Results: The results showed that PAF ethanol extracts significantly stimulated cell proliferation at 0.1–100 μg/mL and the proportion of cells in S-phase increased from 16.33 to 27.29% in osteoblastic MC3T3-E1 cells. Moreover, PAF ethanol extracts increased ALP expression in MC3T3-E1 cells at the concentration from 0.1 to 100 μg/mL and inhibited PGE_2 production induced by TNF-α in osteoblasts at the concentrations ranging from 10 to 100 μg/mL in MC3T3-E1 osteoblasts.

Discussion and conclusion: These results indicated that PAF directly stimulates cell proliferation and differentiation of osteoblasts; therefore, this study preliminarily explored the pharmacological mechanism of PAF to promote the healing of bone rheumatism and various fractures.

Keywords: PAF, proliferation, differentiation, osteoblasts, pharmacology

Introduction

In recent years, traditional Chinese medicines (TCMs) have become important sources for exploration of potential therapeutic agents because of their tremendous diversity. Various strategies have been employed to investigate the phytochemical and pharmacological effects of TCMs based on their therapeutic potentials (Brixen et al., 2004). *Polygonum amplexicaule* D. Don var. *sinense* Forb. (Polygonaceae) (PAF) is a famous herbal drug used effectively to treat some diseases such as fractures, rheumatism, osteoporosis, muscle injuries, and pain. PAF has been demonstrated to be an effective herb for the treatment of rheumatism and fractures in Chinese folk medicine. PAF has also been reported to effectively treat atherosclerosis in China. The antibiotic and antiviral effects of PAF were also examined, and positive results were obtained in China. With respect to the chemical composition of *P. amplexicaule*, Ren et al. (2009) isolated nine compounds from the root tubers of the herb, and Yang et al. (2007) isolated 21 compounds from its essential oil. Approximately 30 compounds were found during our research (data not shown). However, its pharmacological mechanism of promoting the healing of fracture is still unknown.

The MC3T3-E1 osteoblastic cell line is a widely adopted model for the research of osteogenesis *in vitro* (Quarlers et al., 1999). By use of the MC3T3-E1 cell model, several medical herbs such as Icariin (Zhao et al., 2010), *Actaea*

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racemosa (Ranunculaceae) (Chan et al., 2008), safflower seeds (Asteraceae) (Kim et al., 2008), *Ulmus davidiana* Planch (Ulmaceae) (Kang et al., 2006), *Drynaria fortunei* (Drynariaceae) (Jeong et al., 2004), Drynariae Rhizoma (Drynariaceae) (Jeong et al., 2005), soybean isoflavones (Choi et al., 2001; Suh et al., 2003), and *Homalomena occulta* (Homalomenae) (Hu et al., 2008) have been proved to be functional to promote osteogenesis. The purpose of this study is to explore the pharmacological mechanism of PAF to promote the healing of bone fracture by analyzing the proliferation and differentiation of osteoblastic MC3T3-E1 cell.

Materials and methods

Materials

MC3T3-E1 cell line was supplied by Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Tissue culture media and reagents, fetal bovine serum (FBS) were from Hyclone Inc. (Logan City, UT). Diethylstilbestrol (DB) was from Shanghai Sine Kangjie Pharmaceutical Co. Ltd. (Shanghai, China). All other reagents were of analytical grade and purchased from China National Pharmaceutical Industry Corporation. Ltd. (Shanghai, China).

Plant material

The fresh root tubers of PAF were collected by Mr. Chongrong Wang in October 2008 and authenticated by Dr. Dingrong Wan, Professor in Pharmacognosy at the College of Pharmacy, South-Central University for Nationalities (SCUN). The voucher specimen (SCUN0810) was deposited in the Herbarium of the College of Pharmacy, SCUN, China.

Extract and sample preparation

The dried (low temperature drying) root tubers of PAF (10 kg) were ground to coarse powder with a mortar and pestle. The coarse powder was extracted with 95% (v/v 20,000 mL) ethanol for three times at room temperature and concentrated under pressure condition to obtain total ethanol extract 1.185 kg (yield 19%). Ethanol extract sample and DB (as positive control) were weighed and diluted with Dulbecco's modified Eagle's medium (DMEM). The concentrations of ethanol extracts and DB were between 0.01 and 100 μg/mL.

Cell culture

MC3T3-E1 cells were cultured in DMEM supplemented with 10% FBS, 100 μg/mL penicillin, and 100 U/mL streptomycin in a mixture of 95% air and 5% CO_2 in a humidified incubator at 37°C.

Cell proliferative CCK-8 assay

The cells (2×10^4 cells/well) were seeded in 96-well plates and cultured for 24 h to obtain adherent monolayer cells. After washing twice with phosphate-buffered saline (PBS), the cells were treated with various concentrations of PAF ethanol extracts for 1–7 days, and cell proliferation was evaluated by Cell Count Kit-8 assay (CCK-8; Beyotime Inst Biotech., China) according to the manufacturer's instructions (Kim et al., 2010). In brief, 10 μL CCK-8 was added to each well (100 μL medium). After incubation for 2 h at 37°C, the absorbance at 450 nm was measured by an enzyme-linked immunosorbent assay (ELISA) plate reader. The experiments were carried out in five replicates and the results were expressed as mean ± standard deviation (SD).

Cell cycle analysis

The cells (2×10^5 cells/well) were seeded in six-well plates and cultured for 24 h to obtain adherent monolayer cells. The adherent cells were cultured for another 24 h in serum-free medium for synchronization then washed twice with 1× PBS; the cells were cultured with various concentrations of PAF ethanol extracts. The cells were harvested and fixed with 70% cold ethanol at 4°C overnight, then washed with PBS and centrifuged at 2000 rpm for 3 min. Next, the cells were incubated with 100 μL RNase (1 mg/mL) for 30 min at 37°C, then 50 μL (1 mg/ mL) propidium iodide (PI; Jingmei, Shenzhen, China) was added and the cells were incubated at 4°C for 30 min in the dark. The cell cycle was detected by flow cytometry (Kanazawa et al., 2008; Pozharski, 2010) (BD Biosciences, San Jose, CA). The data were analyzed by Modfit software (Verity Software House, Topsham, ME).

Alkaline phosphatase activity assay

MC3T3-E1 cells were cultured to grow to 80–100% confluent. The medium was replaced with phenol red-free alpha minimum essential medium (α-MEM) containing 5% charcoal–dextran-treated FBS (CD-FBS; Gibco, Grand Island, NY). Cells were cultured with various concentrations of ethanol extracts from PAF, supplemented with 10 mM β-glycerophosphate (β-GP) (G9422; Sigma Chemical Co., St. Louis, MO), which was added to initiate *in vitro* cells differentiation (Choi, 2005; Amos et al., 2008). After 3 days, the medium was removed and the monolayer cells were gently washed twice with PBS. The cells were harvested to 1.5-mL tubes and centrifuged for 20 min at 3000 rpm. Supernatant was discarded, and PBS (pH 7.2–7.4) was added to prepare a solution with 1×10^7 cells/mL. Freeze–thaw cycles were repeated three times to break the membrane of the cells to release intracellular components. Then the solution was centrifuged for 15 min at 12,000 rpm. The supernatant was harvested for the measurement of alkaline phosphatase (ALP) concentration by use of an ELISA kit (R&D System Inc., Minneapolis, MN). The experiments were carried out in five replicates and data were expressed as means ± SD.

Prostaglandin E_2 assay

The cells were cultured according to the method mentioned above except that the β-GP was replaced with 10–10 M tumor necrosis factor alpha (TNF-α). The

content of prostaglandin E_2 (PGE_2) in the supernatant was measured with an ELISA kit (R&D System Inc.) according to the manufacturer's instructions (Kajii et al., 1999). Immediately after the color development, the absorbance was read at 450 nm. The experiment was carried out in five replicates and data were expressed as means ± SD.

Statistical analysis

All experimental results were performed with at least three replicates. Results were expressed as mean ± SD. Differences between groups were examined for statistical significance by Student's *t*-test, and $P < 0.05$ was considered statistically significant.

Results

The effect of PAF ethanol extracts on the proliferation of MC3T3-E1 cells

We detected the effects of the ethanol extracts of PAF on the proliferation of MC3T3-E1 cells. After the treatment with 1 μg/mL PAF extract for the indicated days, the numbers of MC3T3-E1 cells were measured with a CCK-8 assay. The results showed that the extract of PAF significantly promoted cell proliferation within 7 days compared with the medium groups (Figure 1A). The dose–response curves indicated that the extract (0.1–100 μg/mL) significantly promoted the proliferation of MC3T3-E1 cells with the optional concentration at 1 μg/mL ($P < 0.01$, Figure 1B). These results suggested that the herb PAF was able to promote the proliferation of osteoblastic MC3T3-E1 cells.

The effect of PAF ethanol extracts on cell cycle progression

Cell proliferation is regulated by cell cycle progression. MC3T3-E1 cells were treated with various concentrations of ethanol extracts, and cell cycle analysis was performed by PI staining and flow cytometry. The results showed that ethanol extracts dose-dependently up-regulated the cell proportion in S-phase significantly (Figure 2A and 2B). When cells were treated with 0.1–10 μg/mL of ethanol extracts, the proportion of cells in S-phase increased from 16.33 to 27.29% (Figure 2A and 2B). These results indicated that PAF ethanol extracts promoted cell proliferation by regulating cell cycle progression.

The effect of ethanol extracts on ALP expression in MC3T3-E1 cells

The healing of fracture is related to the differentiation of osteoblasts. In this study, the effect of ethanol extracts on the expression of ALP, a marker for osteoblast differentiation, was detected in MC3T3-E1 cell. The results showed that the treatment of MC3T3-E1 cells with 0.1–100 μg/mL of extract significantly up-regulated the expression of ALP (Figure 3), which was similar to that of the positive control DB. Therefore, the herb PAF has a markedly positive effect on the differentiation of MC3T3-E1 cells.

The effect of ethanol extracts on PGE_2 production in MC3T3-E1 cells

PGE_2 is an endogenous factor to stimulate osteolysis through the EP4 receptor (Tsutsumi et al., 2009). We examined the effect of ethanol extracts on PGE_2 production in MC3T3-E1 cells. As shown in Figure 4, the treatment of MC3T3-E1 cells with TNF-α increased the expression of PGE_2, but ethanol extracts (10–100 μg/mL) significantly inhibited the expression of PGE_2 induced by TNF-α.

Discussion

To investigate the pharmacological mechanism of PAF to promote the healing of fractures and rheumatism, we selected the well-recognized osteoblastic MC3T3-E1 cell line as *in vitro* model. As the healing of fractures and rheumatism is related to the proliferation and the differentiation of osteoblastic cell, we examined cell growth in osteoblastic MC3T3-E1 cells by CCK-8 assay *in vitro*. The results showed that the low dosage (1–10 μg/ mL) of

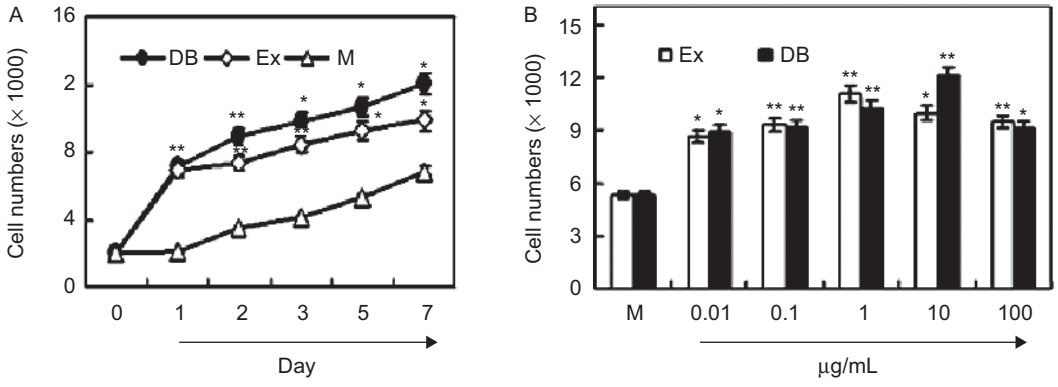

Figure 1. PAF ethanol extracts increase the proliferation of MC3T3-E1 cells. (A) PAF ethanol extracts induced time-dependent cell growth. MC3T3-E1 cells in 96-well plates were cultured with 1 μg/mL PAF extract for 7 days. Cell numbers were measured with a CCK-8 assay. (B) PAF ethanol extracts induced dose-dependent cell growth. Cells in 96-well plates were cultured with indicated ethanol extract for 3 days. Cell numbers were measured by a CCK-8 assay. Each point represents the mean ± SD of five determinations. *$P < 0.05$, **$P < 0.01$ compared with controls by Student's *t*-test. "EX" means ethanol extracts; "DB" means diethylstilbestrol (as positive control).

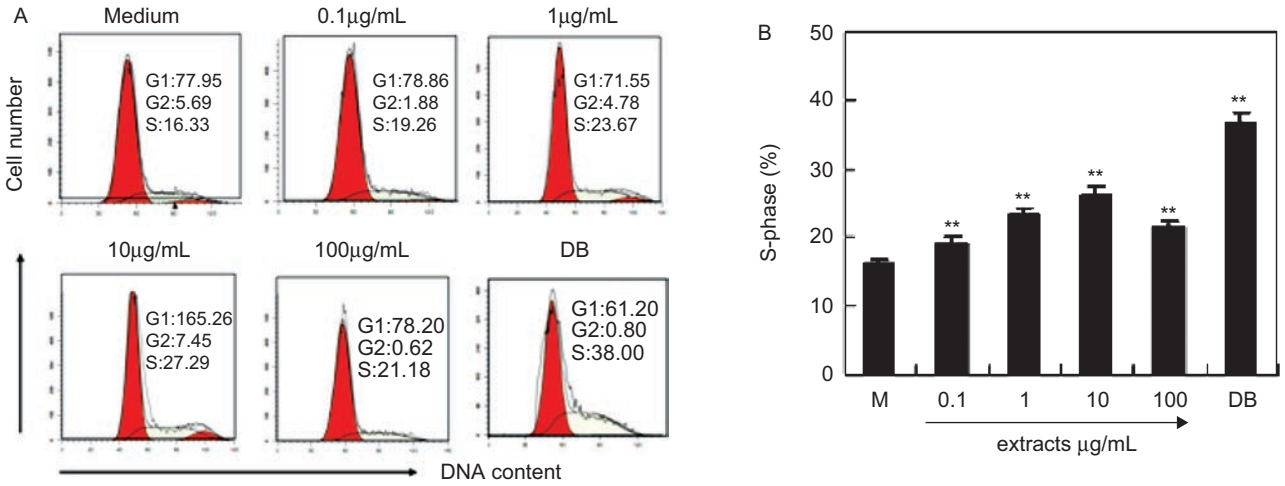

Figure 2. PAF ethanol extracts promotes cell cycle progression in MC3T3-E1 cells. Cells were seeded in six-well plates and cultured with indicated extract and 1 μg/mL DB for 3 days. Cell cycle was analyzed by PI staining. (A) The profile of cell cycle analysis. (B) The percentage of cells in S-phase. Results were expressed as means ± SD of five determinations. $^{**}P < 0.01$ compared with controls by Student's *t*-test. "DB" means diethylstilbestrol (as positive control).

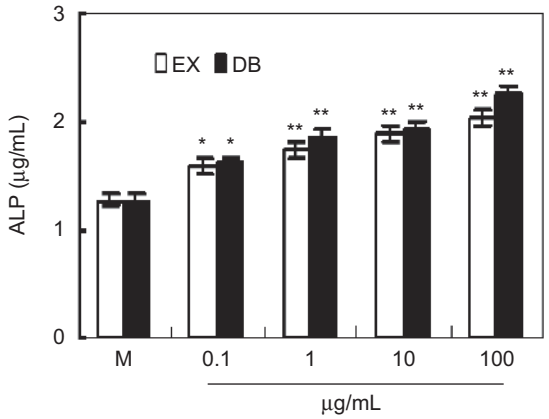

Figure 3. The effect of ethanol extracts on alkaline phosphatase (ALP) expression in MC3T3-E1 cells. Cells were cultured with indicated ethanol extracts or DB for 3 days in the presence of 10 mM β-glycerophosphate (β-GP). ALP concentration was detected by ELISA. Results were expressed as means ± SD of five determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with controls by Student's *t*-test. "EX" means ethanol extracts; "DB" means diethylstilbestrol (as positive control).

ethanol extracts stimulated cell growth. At the same time, the results of the cell cycle assay demonstrated that PAF ethanol extract effectively promoted cell cycle progression, the proportion of cells in S-phase of cells significantly increased (from 16.33 to 27.29%). These results indicated that PAF ethanol extract promoted cell proliferation by regulating cell cycle progression. Moreover, the effect of ethanol extract on cell growth in osteoblastic MC3T3-E1 cells was comparable with DB (as positive control).

ALP is the most widely recognized biochemical marker for osteoblastic activity (Yamaguchi & Gao, 1998). Although its precise mechanism of action is poorly understood, this enzyme is believed to play an important role in bone metabolism (Mizutani et al., 1998). Therefore, we examined the effects of extract from the root tubers of PAF on the ALP activity of osteoblastic MC3T3-E1 cells. The results showed that the treatment of MC3T3-E1 cells with 0.1–100 μg/mL of ethanol extract significantly up-regulated the expression of ALP, and the effect of ethanol extracts on ALP expression was similar to that of the positive control DB.

PGE_2 is an endogenous factor to stimulate osteolysis through the EP4 receptor (Tsutsumi et al., 2009). It is known that some bone-resorbing agents like TNF-α (Kwan et al., 2004; Kitaura et al., 2005; Son et al., 2006) act on osteoblasts and stimulate PGE_2 release from osteoblasts (Igarashi et al., 1997). The released PGE_2 acts on stroma cells and enhances factors that support the differentiation from stem cells to osteoclasts to augment osteolysis. Furthermore, it is known that the presence of PGE_2 caused a significant decrease in bone ALP activity and a corresponding increase in bone acid phosphatase activity. In the present study, EG inhibited PGE_2 production induced by TNF-α in osteoblasts, suggesting that EG may inhibit the osteolysis by down-regulating the release of PGE_2 induced by TNF-α. This result partly elucidated the pharmacological mechanism of PAF to promote the healing of bone fractures and rheumatism.

TCMs have been developed for over 5000 years and are known to have lower toxicity, showing an advantage over chemical synthesized medicine, although the precise pharmacological mechanism of these agents remained to be explored. PAF is known for its functions in promoting tissue regeneration and granulation. It has been used in the treatment of fractures, rheumatism, and bone injuries. This medicinal herb has been used for hundred of years, and its safety and efficacy are well-established through a long history of human use, but the pharmacological mechanism is still unknown. In this study, we found that PAF extract promoted the proliferation and differentiation of osteoclasts, which play important roles for osteogenesis. These results partly elucidated the pharmacological mechanism

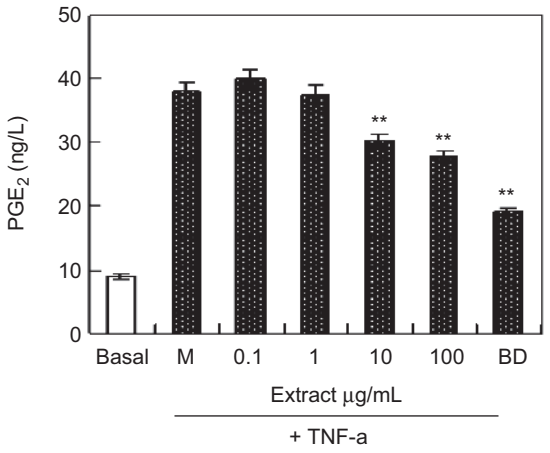

Figure 4. The effect of ethanol extracts on prostaglandin E_2 (PGE_2) production in MC3T3-E1 cells. Cells were cultured with indicated ethanol extracts and 1 μg/mL DB for 3 days in the presence of 10^{-10}M TNF-α. PGE_2 concentration was measured by ELISA. Results were expressed as means ± SD of five determinations. $^{**}P < 0.01$ compared with control by Student's *t*-test. "DB" means diethylstilbestrol (as positive control).

of PAF to promote the healing of bone fractures and rheumatism.

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Declaration of interest

This scientific treatise is an original research, and there is no plagiarism. Biodiversity was not harmed when samples of this medicinal herb were collected.

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