Effects of mangiferin on cell proliferation and alkaline phosphatase activity of human fetal osteoblast cell line

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Abstract

Mangiferin is a naturally occurring polyphenolic compound of C-glycosylxanthone, isolated from fern *Davallia solida*. It has been reported that mangiferin has antioxidant, anticancer, antitumor, anti-inflammatory, anti-obesity, neuroprotective, cardio protective, anti-allergic and inhibits osteoclast formation and bone resorption. This study aimed to investigate the effects of mangiferin on cell proliferation and alkaline phosphatase activity of human fetal osteoblast cell line (hFOB 1.19). Cell proliferation which determined by Presto Blue^{TM} Cell Viability method showed that mangiferin significantly increased cell viability and proliferation at concentrations from 0.125 to 4 µg/ml with no cytotoxic effect. The stimulation was observed within the concentration range 0.125-1 µg/ml at 24 h and 0.5-1 µg/ml at 30 h. The alkaline phosphatase detection was measured both alkaline phosphatase staining using BCIP/NBT substrate solution and also alkaline phosphatase activity. After treatment for 4 days, the alkaline phosphatase activity was significantly increased at 126%, 147% and 159% from 0.25, 0.5, 1 µg/ml, respectively, compared to untreated control (100% control). In conclusion, mangiferin has the ability to increase the proliferation and promote alkaline phosphatase activity of a human fetal osteoblast cell line. This study provides useful information which might be lead to the development of mangiferin for the prevention and treatment of osteoporosis.

Introduction

Osteoporosis is a most common disease usually found among elderly population, especially post-menopausal women. Osteoporosis is decreased in numbers and dysfunction of osteoblast, whereas the osteoclast numbers and function is increased[1], leading to the imbalance between bone formation and resorption. The disease is characterized by low bone mass and disease progression leads to high risk of bone fragility, increasing in bone fractures, skeletal pain and the deterioration of bone. Currently, the treatment of postmenopausal osteoporosis including hormone replacement therapy (HRT) by estrogen alone or combined with a progestin is to replace a woman's depleting hormone levels and thus alleviate menopausal symptoms and bone loss. However, there are some side effects and health risks after long term of treatment such as breast cancer, venous thrombosis, myocardial infarction and cerebrovascular diseases[2]. Another group of osteoporosis drugs target on inhibition of osteoclast function and bone resorption consisting of selective estrogen receptor modulators (SERMs), calcitonin[3] and bisphosphonates[4]. Nevertheless, the treatment contains the adverse side effects such as nausea, vomiting, chest pain, sore throat and difficulty in swallowing[5]. Recently, the phytochemicals extracted from the natural product is an interesting source for preventive and therapeutic of disease including osteoporosis.

Traditional Chinese medicine has been used for treatment of osteoporosis disorders and bone diseases particularly by increasing bone formation and therapeutic effect on stimulating osteoblast differentiation[6]. The previous studies of traditional Chinese medicine in fern extracts for treatment of bone diseases such as the folk remedy Gu sui bu renowned for its therapeutic effects on bone injuries[7]. Stimulative effect of Gu sui bu from fern *Drynaria baronii* has been increased the ALP activity and promoted calcification of bone[8]. A pure compound, amentoflavone from fern *Selaginella tamariscina,* enhances osteogenic differentiation and bone formation of human mesenchymal stem cells (hMSC)[9]. Therefore, we interested to examine the effect of a phytochemical from fern extract for prevention and treatment of osteoporosis.

Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxy-9H-xanthene-9-one), a natural polyphenol having C-glycosylxanthone structure[10], is widely present in higher plants, mango fruit (*Mangifera indica* Linn.) and papaya[11]. Mangiferin has many pharmacological activities including antioxidant[12], anticancer[13], anti-inflammatory[14], anti-obesity[10], neuroprotective[15], cardioprotective[16] and anti-allergic properties. In addition, it has been reported that mangiferin inhibits the osteoclast formation and bone resorption[17]. However, the effect of mangiferin on the osteogenic differentiation of human osteoblast cells has not been reported. In this study, mangiferin isolated from rhizome root of fern *Davallia solida* was to investigate its effects on cell proliferation and alkaline phosphatase activity of human fetal osteoblast cell line.

Methodology
Materials
Mangiferin with a high purity was received from Dr. Nopporn Thasana, Laboratory of Medicinal Chemistry, Chulabhorn Research Institute. Mangiferin was collected from the Royal Project Foundation, Kasetsart University. Purified mangiferin isolated from rhizome root of fern *Davallia solida*, extracted by methanol, further analyzed by Nuclear magnetic resonance (NMR) spectroscopy and High-performance liquid chromatography (HPLC). Mangiferin was dissolved in dimethylsulfoxide (DMSO) to make a stock solution and stored at -20°C and diluted to the desired concentrations for the experiment.

Cell culture
The human fetal osteoblast cell line 1.19 (hFOB1.19) was purchased from American Type Culture Collection (ATCC, CRL No.11372). The cells were cultured in 1:1 mixture of phenol red-free DMEM/Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (MERCK), 14.3 mM $NaHCO_3$, 100 U/ml penicillin, 100 µl g/ml streptomycin (Gibco, USA) and 0.3 mg/ml G418 Sulfate (Bio Basic, Canada). The cells were cultivated at 37°C with 5% CO_2. The medium was replace every 3 days, and the cells were subcultured using 0.05 % Trypsin-EDTA (CAPRICORN).

Cell viability and proliferation assay
Cell viability and proliferation was assessed by Presto Blue^{TM} cell viability method. The hFOB1.19 cells at a density of 5×10^3 cells/well were seeded in 96-well black plates and allowed to adhere at 37°C for 24 h. After washing with a phosphate buffered saline (PBS) buffer, the cells were treated with the medium at different concentrations of mangiferin (0.125, 0.25, 0.5, 1, 2, 4 and 8 µg/ml) for 24, 30 and 36 h. Cells were then added with 10 µl of PrestoBlue cell viability reagent (Invitrogen) and were incubated for 30 min at 37°C with 5% CO_2. Finally, the cells were detected by the fluorescence signal at excitation 530/25 and emission 590/35 using a microplate reader spectrophotometer (BioTek). All experiments were performed in triplicate, and the relative cell viability and proliferation (%) was expressed as a percentage relative to the untreated control cells (100%).

Alkaline phosphatase (ALP) staining

The cells (3×10^4 cells/well) were seeded into 24-well plates and incubated for 24 h. After that, the hFOB1.19 cells were treated with various concentrations of mangiferin (0.25, 0.5 and 1 µg/ml) and incubated for 4 and 7 days. Then, the cells were washed with PBS and fixed with 500 µl of 10% neutral buffered formalin for 15 min at room temperature. After washing step, the cells were stained with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) (Amresco) at room temperature for 1 h in dark. Finally, the cell layers were washed with PBS and photographed under an inverted light microscope (IX71, Olympus).

ALP activity

The hFOB1.19 cells (6×10^4 cells/well) were cultured into a 12 well plate for 24 h and subsequently treated with various concentrations of mangiferin (0.25-1 µg/ml). After treatment for 4 and 7 days, the cells were washed with PBS, scraped and centrifuged at 10,000 rpm for 5 min to collect the cell pellet. The cells were added by lysis buffer containing 50 mM Tris-HCl, pH 7.5 and proteinase inhibitor cocktail (Amresco) and lysed by sonication. After centrifugation, the clear supernatant was collected for ALP activity assay and the protein concentration was determined using Bradford protein assay (Bio-rad). The enzymatic reaction was performed with 5 mM *p*-nitrophenol phosphate substrate (pNPP) (Biobasic), 100 mM Glycine buffer (pH 10.4), 5 mM $MgCl_2$, 0.15 M alkaline buffer (Sigma-Aldrich) and amounts of cell lysate for 30 min at 37°C. The reaction was stopped with 0.1 N NaOH and measured the absorbance at 405 nm using a microplate reader (BioTek). Alkaline phosphatase activity was quantified by a production of *p*-nitrophenol (µM) product per mg protein per minute and calculated as the percentage compared to untreated control (100% control).

Results and Discussion

*Effect of mangiferin on cell viability and proliferation*

Bone remodeling is a process where mature bone tissue is removed from the skeleton and new bone tissue is formed that undergoes continuous throughout life. The molecular mechanism of bone formation involves three major phases including cell proliferation, extracellular matrix maturation and extracellular matrix mineralization[18]. The effect of mangiferin on both cell viability and proliferation of hFOB1.19 was evaluated by Presto Blue[TM] cell viability method. The principle is a resazurin-based metabolic assay to quantitatively measure the proliferation of cells. When added to cells, the PrestoBlue® reagent is modified by the reducing environment of the viable cell and turns red in color, becoming highly fluorescent[19]. In this study, various concentrations of magiferin (0.125-8 µg/ml) were added to culture media for 24, 30 and 36 h with a final concentration of 0.1% DMSO in all concentrations. As shown in Figure 1, we found that mangiferin significantly increased the cell proliferation of human osteoblast cells at concentrations from 0.125 to 4 µg/ml compared to the untreated control. No cytotoxic effect was detected at all concentrations tested on hFOB1.19. However, the prominent stimulation for the proliferation was observed within the range 0.125 to 1 µg/ml at 24 h and 0.5 to 1 µg/ml at 30 h (Figure 1). Therefore, the results summarized that mangiferin was able to stimulate the cell proliferation between 24 h to 30 h in a dose-dependent manner as compared with untreated control cells (DMSO as a control).
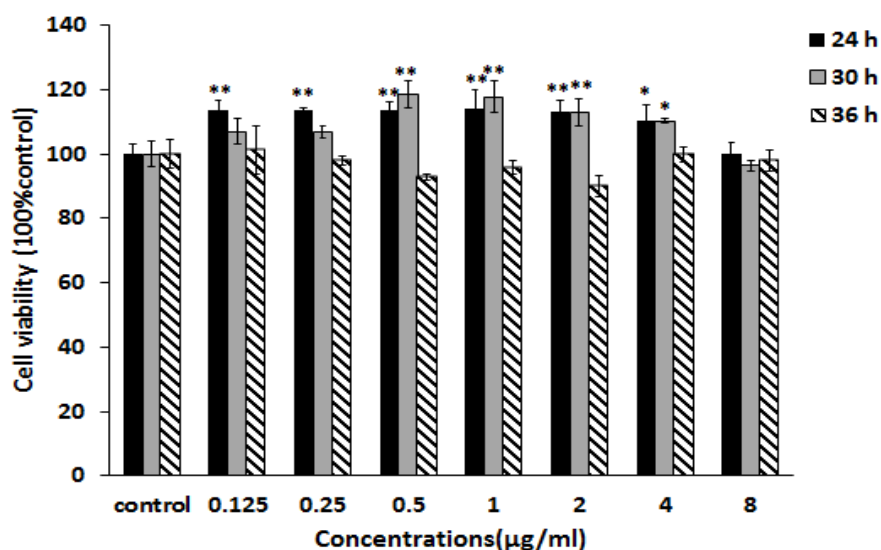
Figure 1. Effect of mangiferin on cell viability of hFOB1.19 cells. Cell viability and proliferation was determined using Presto Blue™ cell viability method. Cells were treated with different concentrations of mangiferin (0.125, 0.25, 0.5, 1, 2, 4 and 8 µg/ml) for 24, 30 and 36 h. Data shown represent the mean ± SD performed in triplicate. The mean difference was significant * $P < 0.05$, ** $P < 0.01$.

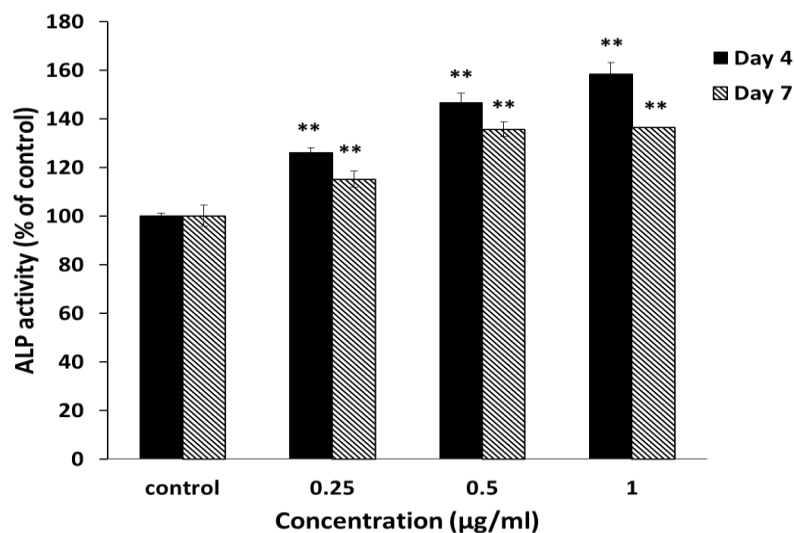*Effect of mangiferin on alkaline phosphatase staining of hFOB1.19*

  Osteoblasts are bone-forming cells that express diverse osteoblastic markers during the differentiation process including the function of ALP protein. Alkaline phosphatase (ALP) is an enzyme that is involved in the bone mineralization. It is an early marker of osteoblast differentiation, its increased expression in matrix maturation stage and is associated with the progressive differentiation of osteoblasts. To evaluate the ability of mangiferin on the osteoblastic differentiation, human osteoblast cells (hFOB1.19) was treated with mangiferin (0.25-1 µg/ml). The ALP staining was performed for qualitative analysis the level of ALP activity using BCIP/NBT as a substrate after treatment for 4 and 7 days. As shown in Figure 2, the results showed that the higher intensity of blue-purple precipitate appeared after treatment at 0.25-1 µg/ml mangiferin compared to the untreated control (Figure 2A and B). These results indicated that mangiferin had the stimulating ability to increase the ALP level within the human osteoblastic cells.
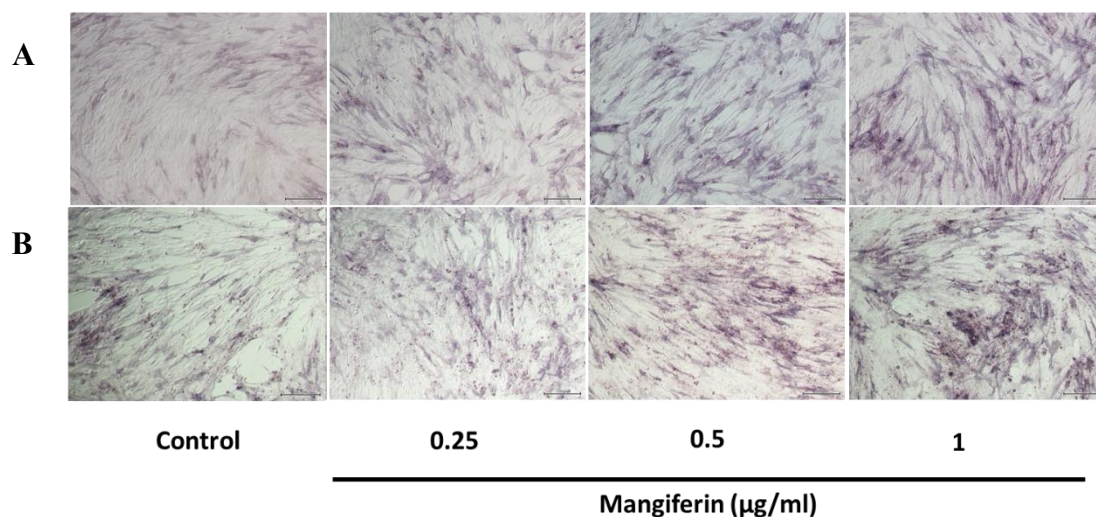
Figure 2. Alkaline phosphatase staining after treatment with mangiferin on hFOB1.19 cells. Cells were treated with various concentrations of mangiferin (0.25, 0.5 and 1 µg/ml), incubated for 4 and 7 days and stained with BCIP/NBT substrate solution. (A) ALP staining at day 4; (B) ALP staining at day 7. Magnification = 10x, Bar = 200 µm.

Effect of mangiferin on alkaline phosphatase activity of hFOB1.19

ALP is an osteogenic differentiation marker secreted by osteoblast during the process of cell differentiation and osteogenesis. The cellular alkaline phosphatase (ALP) activity is the most widely recognized an early marker of osteoblast differentiation[20] and an important indicator of bone formation[21]. ALP hydrolyzes a variety of phosphate compounds, release inorganic phosphate (Pi) into the extracellular matrix[22]. This study, the enzymatic assay is based on hydrolysis of the colorless, synthetic substrate *p*-nitrophenyl phosphate to produce a yellow-colored product, *p*-nitrophenol and inorganic phosphate. ALP removes the phosphate group to generate p-nitrophenol, which is deprotonated under alkaline conditions to produce *p*-nitrophenolate that has strong absorption at 405 nm. The quantitative analysis of ALP activity in cell lysate was further determined by measuring amount of product after enzymatic activity. As shown in Figure 3, ALP activity was significantly increased in all doses of mangiferin treatment at day 4 and 7 in a dose-dependent manner compared to the untreated control. The level of ALP activity on day 4 showed the highest activity at 126%, 147% and 159% from 0.25, 0.5, 1 µg/ml, respectively, compared to untreated control (100%control) (Figure 3). These results suggested that mangiferin from fern *Davallia solida* had the enhancing ability to differentiate human osteoblastic cells.

Figure 3. Alkaline phosphatase activity of hFOB1.19 cells after mangiferin treatment.Cells were treated with various concentrations of mangiferin (0.25, 0.5 and 1 μg/ml), incubated for 4 and 7 days and determined the ALP activity using *p*-nitrophenyl phosphate (pNPP) as substrate. Data shown represent the mean ± SD performed in triplicate. The mean difference was significant * $P < 0.05$, ** $P < 0.01$ compared with untreated control.

Conclusion

In this study we demonstrated that mangiferin from fern *Davallia solida* effectively stimulated cell proliferation and promoted the alkaline phosphatase activity in osteoblast differentiation process of a human fetal osteoblast cell line. However, the mechanisms underlying the stimulating effect of mangiferin on hFOB1.19 cells need further investigations. Mangiferin may be served as a promising drug candidate for developing new preventive and therapeutic agents for osteoporosis patients.

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