Natural Product Sciences
14(1) : 21-26 (2008)

Chiisanoside, A Lupane Triterpenoid from *Acanthopanax* Leaves, Stimulates Proliferation and Differentiation of Osteoblastic MC3T3-E1 Cells

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Abstract – The leaves of *Acanthopanax* species have traditionally been used as a tonic and a sedative as well as in the treatment of rheumatism and diabetes. Chiisanoside is the major active lupane triterpenoid of *Acanthopanax* leaves. To investigate the bioactivities of chiisanoside, which act on bone metabolism, the effects of chiisanoside on the function of osteoblastic MC3T3-E1 cells were studied. Chiisanoside (0.02~20 μM) significantly increased the growth of MC3T3-E1 cells and caused a significant elevation of alkaline phosphatase (ALP) activity, collagen content, and nodules mineralization in the cells ($P < 0.05$). The effect of chiisanoside (2 μM) in increasing ALP activity was completely prevented by the presence of tamoxifen, suggesting that the effect of chiisanoside might be partly estrogen receptor mediated. Moreover, cotreatment of p38 inhibitor SB203580 or JNK inhibitor SP600125 inhibited chiisanoside-mediated ALP upregulation, suggesting that the induction of differentiation by chiisanoside is associated with increased activation of p38 and JNK mitogen-activated protein kinases. Our data indicate that the enhancement of osteoblast function by chiisanoside may result in the prevention for osteoporosis.
Keywords – *Acanthopanax*, chiisanoside, osteoblastic MC3T3-E1 cells

Introduction

Osteoporosis is defined conceptually as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture. It can occur at any age and in any racial or ethnic group, though more common in post-menopausal women, and especially in Asia or Caucasia areas. The most serious consequence of osteoporosis is fracture, which is associated with increase in mortality, substantial morbidity and social costs (Cummings and Melton, 2002). Bone metabolic diseases develop when there is an imbalance between the formation and resorption of bone, which, in turn, depend on the interactions between osteoblasts and osteoclasts. The formation of bone involves a complex series of events, include the proliferation and differentiation of osteoprogenitor cells and result eventually in the formation of a mineralized extracellular matrix. To date, most effective osteoporosis therapies reduce bone loss but do not restore lost bone mass and strength. It is desirable, therefore, to have satisfactory bone-building (anabolic) agents that stimulate new bone formation and correct the imbalance of trabecular microarchitecture characteristic of established osteoporosis (Ducy *et al.*, 2000). Since new bone formation is primarily a function of the osteoblasts, agents which regulate bone formation act either by increasing the proliferation of cells of the osteoblastic lineage, or inducing differentiation of the osteoblasts (Ducy *et al.*, 2000; Lane and Kelman, 2003). Early intervention is now possible with the help of some effective medications, which may reduce the risk of first and recurrent fractures.

The plants belonging to *Acanthopanax* species (Araliaceae) are traditionally used in Korea as anti-rheumatoid arthritis, anti-inflammatory and anti-diabetic drugs and are recognized to have ginseng-like activities (Bae *et al.*, 2001; Lee *et al.*, 2002). Chiisanoside (Fig. 1), the main triterpenoid component of this plant, has been reported to have anti-hepatotoxic, anti-diabetic and antiviral effects, the latter of which was found to be associated with the inhibition of mitogen-induced lymphocyte proliferation (Hahn *et al.*, 1984; Kim *et al.*, 1999; Oh *et al.*, 2000). MC3T3-E1 cells, an osteoblast-like cell line, have been reported to retain the capacity to differentiate into osteoblasts (Kanno *et al.*, 2001) and to have both estrogen receptors (ER) α and β (Kleinerman *et*

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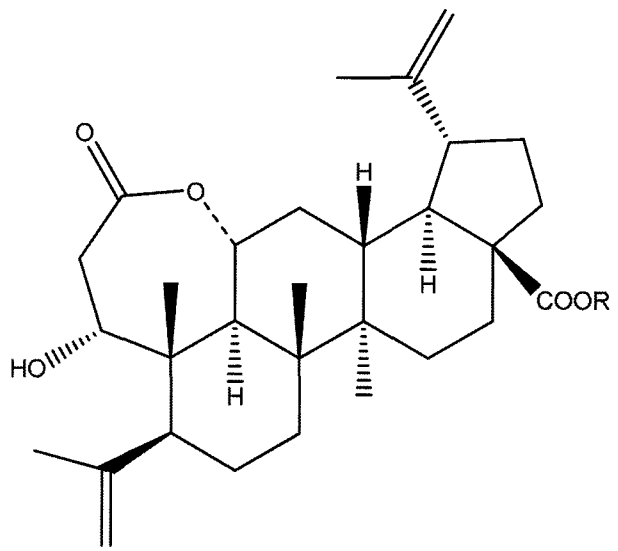

Fig. 1. Structure of chiisanoside R = α-L-rhamnopyranosyl (1 → 4)-β-D-glucopyranosyl (1 → 6)-β-D-glucopyranosyl.

al., 1987; Tamir *et al.*, 2000). Those cells may provide very useful information about the effects of phytoestrogens on the differentiation of osteoblasts. In the present study, the *in vitro* effect of chiisanoside on the function of osteoblastic MC3T3-E1 cells was investigated in order to determine the possible bioactivities of chiisanoside on bone metabolism.

Experimental

Materials – Chiisanoside was isolated as white powder by silica gel and YMC reversed-phase C18 column chromatography from the leaves of *A. divaricatus* var. *albeofructus*. Chemical structure was identified by ^{1}H-NMR, ^{13}C-NMR and MS (mp 228 - 230 °C, m/z 953 [M-H]$^{-}$, 483 [M-(rha-glc-glc)-H]$^{-}$]. The purity was confirmed by HPLC was more than 99.5% (Kang *et al.*, 2003).

Cell culture and materials – Murine osteoblastic MC3T3-E1 cells were cultured at 37 °C in 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM; GibcoBRL, Grand Island, NY). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. When cells reached confluence, cells were subcultured using 0.02% EDTA-0.05% trypsin solution. Tamoxifen, SB203580, and SP600125 were purchased from Sigma Chemical (St. Louis, MO, USA).

Cell viability – Cells were suspended in medium supplemented with 10% FBS, and cell suspension containing 5×10^3 cells was added to the individual wells of 48-well microplates. The plates were incubated at 37 °C in a CO_2 incubator for 48 h. After discarding the culture medium and washing the cells with phosphate-buffered saline (PBS), serum-free medium containing 0.3% bovine serum albumin (BSA) and chiisanoside at appropriate concentrations was added to the cell culture and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. Surviving cells was counted by the MTT method. MTT 20 μL in 7.2 mM phosphate buffer solution, pH 6.5 (5 mg/mL), was added to each well, and the plates were incubated for an additional 2 h. After the removal of solutions in the well, dimethyl sulfoxide was added to dissolve formazan products, and the plates were shaken for 5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

Alkaline phosphatase activity – The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid, to initiate differentiation. The medium was changed every 2 - 3 days. After 8 days, the cells were cultured with medium containing 0.3% BSA and samples individually for 2 days. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. The cells were lysed with 0.2% Triton X-100, with the lysate centrifuged at 14,000 × *g* for 5 min. The clear supernatant was used to measure the ALP activity, which was determined using an ALP activity assay kit (Asan Co. Korea).

Collagen content – The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid. The medium was changed every 2 - 3 days. After 8 days, the cells were cultured with medium containing 0.3% BSA and chiisanoside for 2 days. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. Collagen content was quantified by Sirius Red-based colorimetric assay (Tullberg-Reinert and Jundt, 1999) Cultured osteoblasts were washed with PBS, followed by fixation with Bouin's fluid for 1 h. After fixation, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained by Sirius Red dye reagent for 1 h under mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove non-bound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm.

Calcium deposition assay – The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid. After 12 days, the cells were cultured with medium containing

0.3% BSA and chiisanoside individually for 2 days. On harvesting, the cells were fixed with 70% ethanol for 1 h, and then stained with 40 mM Alizarin Red S for 10 min with gentle shaking. To quantify the bound dye, the stain was solubilized with 10% cetylpyridinum chloride by shaking for 15 min. The absorbance of the solubilized stain was measured at 561 nm.

Statistics – The results are expressed as the mean ± SEM (n = 5). Statistical analysis was performed using a one-way ANOVA ($P < 0.05$) with the SAS statistical software.

Results and Discussion

Osteoporosis associated with estrogen deficiency is the most common cause of age-related bone loss. Hormone replacement therapy (HRT) can resolve most postmenopausal problems. However, compliance with HRT is poor because of its associated risks of breast and endometrial cancers with long-term use (Persson *et al.*, 1999). In the search for an alternative treatment, the potential health benefits of phytoestrogens have been suggested (Tham *et al.*, 1998). There is considerable evidence indicating that phytoestrogens, like certain selective estrogen receptor modulators, have estrogenic action on bone and the cardiovascular system, and have antiestrogenic action on the breast and uterus (Brzezinski and Debi, 1999). Phytoestrogens have been proposed to prevent bone resorption and promote bone density (Alekel *et al.*, 2000). In the present study, we investigated the effect of chiisanoside on the function of osteoblasts using pre-osteoblastic target cell line, MC3T3-E1, which has been a well-characterized as an *in vitro* model for osteoblast differentiation (Sudo *et al.*, 1983). MC3T3-E1 cell growth was elevated significantly by the presence of chiisanoside (0.02~20 μM) (Fig. 2). The effect of chiisanoside on osteoblast differentiation was first assessed by measuring the ALP activity, one of the major osteoblast differentiation markers. Chiisanoside significantly increased the ALP activity at concentrations of 0.2~20 μM. At the concentration of 2 μM, chiisanoside increased ALP activity up to 131% compared to that of control (Fig. 3). Since chiisanoside significantly increased ALP activity in osteoblastic MC3T3-E1 cells, we further investigated the effect of chiisanoside on collagen synthesis using Sirius Red-based colorimetric assay. Chiisanoside significantly increased collagen synthesis at concentrations of 0.02-20 μM (Fig. 4). Next, we examined the effects of chiisanoside on mineralization, another important process in differentiation, by measuring the calcium deposition by

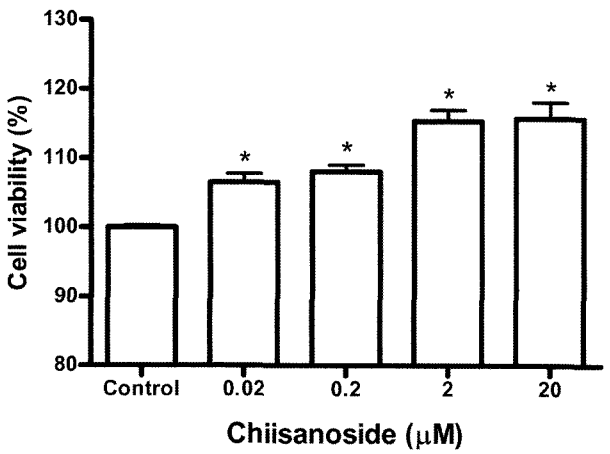

Fig. 2. Effects of chiisanoside on the viability of MC3T3-E1 cells. Data are expressed as a percentage of the control. The control 0.408 ± 0.005 OD. $^{*}P < 0.05$ vs. control.

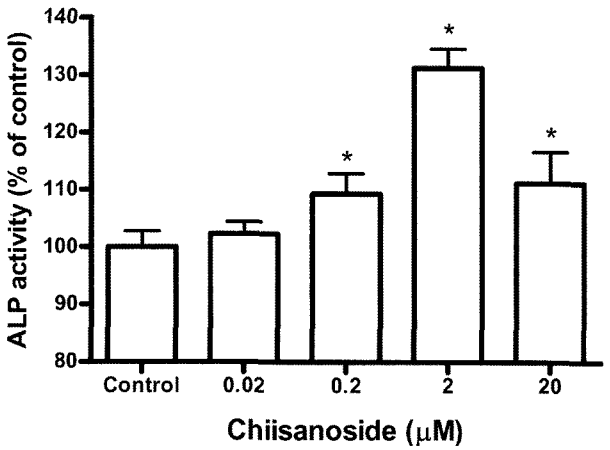

Fig. 3. Effects of chiisanoside on the alkaline phosphatase activity of MC3T3-E1 cells. Data are expressed as a percentage of the control. The control ALP activity was 0.510 ± 0.015 Unit/10^6 cells. $^{*}P < 0.05$ vs. control.

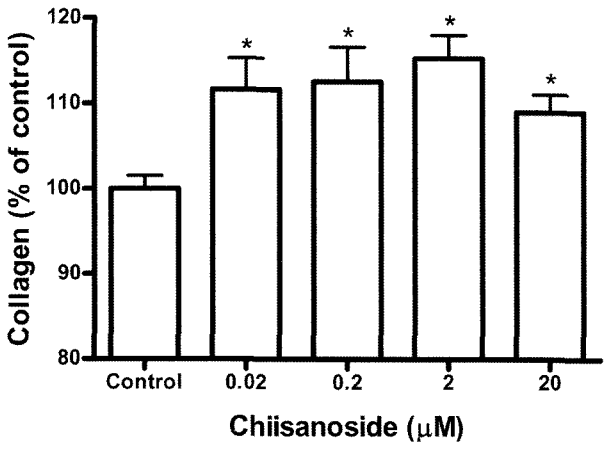

Fig. 4. Effects of chiisanoside on the collagen content of MC3T3-E1 cells. Data are expressed as a percentage of the control. The control 22.69 ± 0.349 μg. $^{*}P < 0.05$ vs. control.

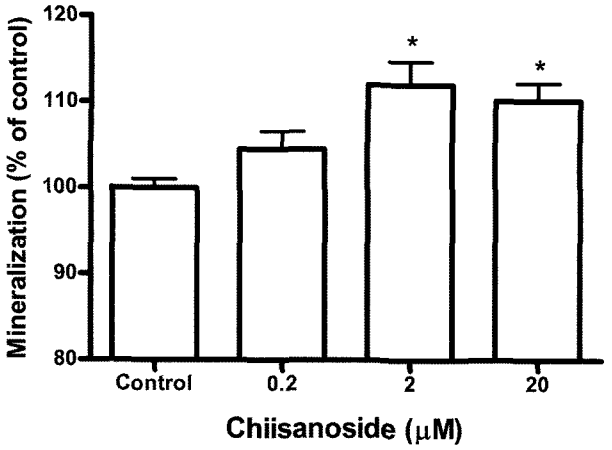

Fig. 5. Effects of chiisanoside on the mineralization of MC3T3-E1 cells. Data are expressed as a percentage of the control. The control mineralization value was 0.640 ± 0.006 OD. * $P < 0.05$ vs. control.

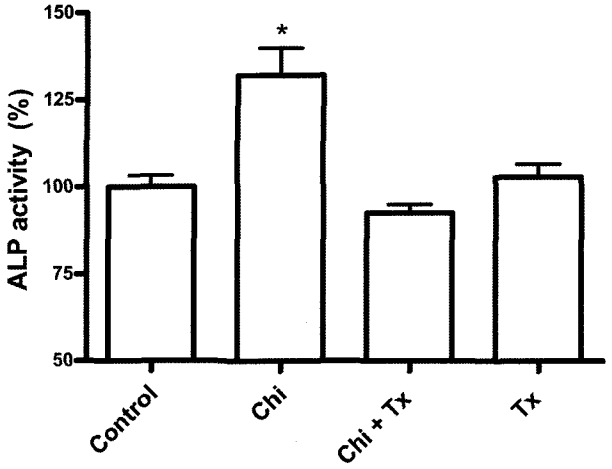

Fig. 6. Effect of tamoxifen on ALP activity in the presence of chiisanoside. One hour before the treatment with 2 μM chiisanoside (Chi), cultures were pre-treated with 1 μM tamoxifen (Tx). Data are expressed as a percentage of the control. * $P < 0.05$ vs. control.

Alizarin Red staining. In consistent with the effects on ALP activity and collagen synthesis, chiisanoside showed significant stimulatory effect on mineralization (Fig. 5). Our results demonstrate that chiisanoside significantly increases the proliferation and differentiation of osteoblastic MC3T3-E1 cells.

Phytoestrogens are known to exert stimulatory effects on osteoblast differentiaion *via* estrogen receptor (ER)-mediated pathway. Therefore, we examined the involvement of ER in the stimulatory effects of chiisanoside on osteoblast differentiation, by using tamoxifen, an ER antagonist. The addition of tamoxifen abolished the stimulatory effects of chiisanoside on osteoblast differentiation (Fig. 6). These results suggested that chiisanoside enhanced osteoblast differentiation, in part, *via* an ER-dependent pathway. Estrogens have been shown to exert a variety of beneficial effects in men and women. They are recognized to protect against postmenopausal symptoms, osteoporosis, heart attack and other cardiovascular problems, and possibly Alzheimer's disease. Recently, mounting evidence suggests that phytoestrogens may exert beneficial effects on the above-mentioned chronic diseases (Brandi, 1997). The data presented here demonstrate the estrogenic effect of chiisanoside. Chiisanoside may be available as a lead compound for phytoestrogen agents that can offer estrogenic activity as well as treatment of postmenopausal symptoms, osteoporosis and other cardiovascular diseases. However, further studies are required to determine the mechanism by which chiisanoside possesses its estrogenic effect.

Recent studies have shown that some intracellular signaling pathways, which mediate the biological effects, are induced by physical stimuli. The mitogen-activated protein kinase (MAPK) family has been found to mediate the signal transduction of external stimulation into intracellular signals, which regulate cell growth and differentiation (Bogoyevitch, 2000). The extracellular signal-regulated kinase (ERK1/2) pathway is primarily responsive to growth factors and mitogens, but it is also activated by hypoxia or osmotic stress and appears to be involved predominantly in cell growth, division, and differentiation (Seko *et al.*, 1996). Though weakly activated by growth factors, c-Jun NH2-terminal kinase (JNK) shows a strong response to cellular stresses such as UV irradiation, protein synthesis inhibitors, and reperfusion following ischemia (Le *et al.*, 2001). On the other hand, p38 MAPK is activated in cellular responses to various environmental stresses, such as hyperosmolarity (Widmann *et al.*, 1999). JNK and p38 are important signaling pathways for osteoblast differentiation induced by several osteotropic factors (Guicheux *et al.*, 2003). Therefore, evaluation of the role of MAP kinases in chiisanoside-induced osteoblastic differentiation was performed using selective inhibitors of p38 and JNK pathways. The p38 inhibitor SB203080 and JNK inhibitor SP600125 significantly reduced the stimulation of ALP by chiisanoside (Fig. 7). These observations strongly suggested that p38 and JNK are involved in mediating the stimulation of ALP induced by chiisanoside. Chattopadhyay *et al.* (2004) demonstrated that JNK was involved in osteoblast proliferation. Recent findings also revealed that activation of the MAPK p38 was required for osteoblast differentiation, and p38 activation induced the expression of differentiation markers, such as ALP and mineral

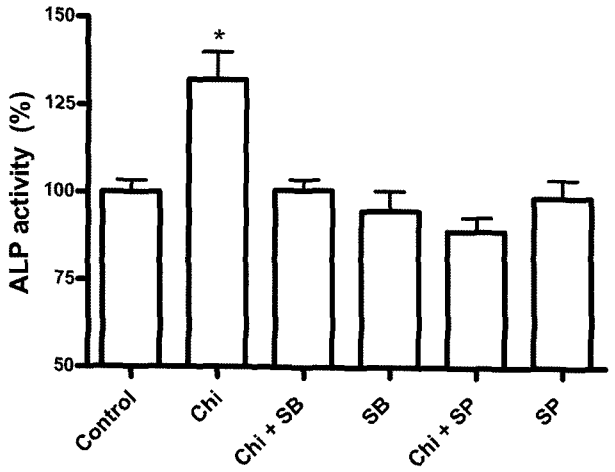

Fig. 7. Effect of p38 and JNK inhibitors on ALP activity in the presence of chiisanoside. One hour before the treatment with 2 μM chiisanoside (Chi), cultures were pre-treated with 1 μM SB203580 (SB) or 3 μM SP600125 (SP). Data are expressed as a percentage of the control. * $P < 0.05$ vs. control.

deposition (Sowa *et al.*, 2002; Guicheux *et al.*, 2003). p38 MAPK activation in cells has been reported to enhance osteoprotegerin (OPG) synthesis (Tazoe *et al.*, 2003). Bone morphogenetic protein-4 (BMP-4), which induces differentiation of osteoblasts, has been reported to increase OPG synthesis in a mouse bone-marrow-derived stromal cell line, ST2, via the p38 MAPK pathway, but not in the ERK1/2 pathway, because OPG synthesis in ST2 stimulated with BMP-4 was reduced by the p38 MAPK inhibitor SB203580 but not by an ERK1/2 inhibitor PD98059 (Tazoe *et al.*, 2003). Our observations indicate that the chiisanoside-induced differentiation response is mediated by the p38 and JNK kinases pathway. However, the relationship between chiisanoside and bone metabolism should be further investigated.

In conclusion, chiisanoside, the main triterpenoid component of *Acanthopanax* species, stimulated the growth and differentiation of osteoblastic MC3T3-E1 cells and its effect on cell differentiation was associated with estrogen receptor, p38, and JNK signaling pathway. This therefore suggests that chiisanoside may be beneficial in stimulating the osteoblastic activity resulting in bone formation.

Acknowledgements

This research was supported by a grant from Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government. We are grateful to KBSI for the provision of the spectroscopic instrument.

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(Accepted February 11, 2008)