Phytopharmacology

Inforesights Publishing

Pycnogenol® enhances proliferation and mineralization in osteoblast-like MC3T3-E1 cells

Noboru Hasegawa[1], Lin Mei[2], Miyako Mochizuki[3]

[1]Department of Health and Medical Sciences, Ishikawa Prefectural Nursing University, 1-1, Gakuendai, Kahoku-City, Ishikawa 929-1212, Japan.
[2]School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, PR China.
[3]Higashiosaka Junior College, 3-1-1 Nishitsutsumigakuen-cho, Higashiosaka 577-8567, Japan.

[*]Corresponding author: hsgwn@ishikawa-nu.ac.jp; Tel: +81-76-281-8300, FAX: +81-76-281-8319

Received: 2 March 2012, **Revised**: 2 April 2013, **Accepted**: 9 April 2013

Abstract

Pycnogenol® (PYC) is a natural plant extract from the bark of *Pinus pinaster* Atiton that has potent anti-oxidant activities. Recent studies have shown that orally administered PYC can increase the biomechanical strength of bone and bone mineral density in ovariectomized rats. However, the effect of PYC on osteoblast mineralization has not yet been examined. We studied the differentiation and mineralization in mouse osteoblast-like MC3T3-E1 cells. PYC enhanced the metabolic activity dose dependently using an MTT assay by 120% (37.5 μg/ml) to 180% (375 μg/ml). The calcium depositions of the cells by alizarin red S staining were increased for 28 days after the culture with 375 μg/ml PYC. PYC activated the expression of estrogen receptor β (ERβ) about 2.8-fold for 28 days after the culture with 375 μg/ml PYC. These results suggest that PYC stimulates osteoblastic differentiation and mineralization via the expression of ERβ.

Keywords: Pycnogenol®; MC3T3-E1 cells; Differentiation; Mineralization, Estrogen receptor

Introduction

Postmenopausal osteoporosis associated with bone loss is a major bone disease among elderly women. Osteoporosis is a skeletal disorder characterized by decreased bone mass as a consequence of enhanced bone resorption relative to bone formation. Recent studies have shown the importance of oxidative stress in bone metabolism and bone loss. Green tea polyphenol, antioxidant vitamin, carotenoid and antioxidant lycopene have been shown to relieve osteoporosis by reducing bone loss in postmenopausal women (Shen, et al., 2009; Sugiura, et al., 2010; Mackinnon, et al., 2010). Pycnogenol® (PYC), a family of flavonoids isolated from French maritime pine bark (*Pinus pinaster* Aiton., synonym *Pinus maritima* Mill.), is standardized to contain 70±5% procyanidins, which consist of condensed catechin and epi-

 © 2013 Inforesights Publishing UK

catechin with potent antioxidant and ROS scavenging properties (Heather, 2010). Recent studies have confirmed that orally administered PYC can decrease bone turnover rate in ovariectomized rats, resulting in positive effects on the biomechanical strength of bone and bone mineral density (Mei, et al., 2012). The present study was designed to evaluate the effects of PYC on bone formation using cultured osteoblast. MC3T3-E1 cells, an osteoblast-line cell line, reported by retain the capacity to differentiate into osteoblast (Sudo, et al., 1983). In the present study, the *in vitro* effect of PYC on the differentiation and mineralization in MC3T3-E1 cells was investigated to determine any possible bioactivities in bone metabolism.

Material and methods

Cell Culture and Chemicals

Osteoblastic MC3T3-E1 cells (purchased from RIKEN Cell Bank, Tsukuba, Japan) were cultured at 37 °C in 5% CO_2 in an α-modified minimal essential medium (α-MEM, Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (BioWest S.A.S, France), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA). Disodium β-glycerophosphate (10 mM, Sigma-Aldrich, St. Louis, MO, USA) and L-ascorbic acid (50 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) were added to the culture medium to differentiate into osteoblasts. PYC was provided by DKSH Japan K. K., Tokyo, Japan (Product name: PYCNOGENOL®, certificate number: CA09-063, content of procyanidins: 67%).

Proliferation assay

The metabolic activity of MC3T3-E1 cells was determined using an MTT assay kit (Cell Counting Kit-8, Dojindo, Kumamoto, Japan). After the cells were treated with PYC for 5 days, a 10μl MTT solution was added and incubated at 37 °C for 2 h. The absorbance at 450 nm was measured on a micro-plate reader (Multiskan JX, Thermo Fisher Scientific, Tokyo, Japan).

Mineralization assay

The calcium depositions of MC3T3-E1 cell cultures were stained by alizarin red S solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). PYC (375 μg/ml) was added throughout the culture. The cells were rinsed 3 times with PBS and fixed with 95% ethanol for 10 min, washed 5 times with distilled water, and stained with 2% alizarin red S for 5 min.

Western blotting

Intracellular protein extracts were analyzed on a 12% polyacrylamide mini-gel electrophoresis at room temperature. After the transfer onto PVDF membranes (Merck Millipore Corporation, Tokyo, Japan), the membranes were blocked, incubated with a 1:500 dilution of anti-mouse ERβ polyclonal antibody (Assay Biotechnology Company Inc., Sunnyvale, CA, USA), and incubated with a 1:300 dilution of anti-rabbit IgG (Biomedical Technologies, Inc., Stoughton, MA, USA) with SNAP i.d.™ (Merck Millipore Corp., Tokyo, Japan). The immunoreactive bands were detected using ECL detection reagent (WZ-ECL, Biological Industries

 © 2013 Inforesights Publishing UK

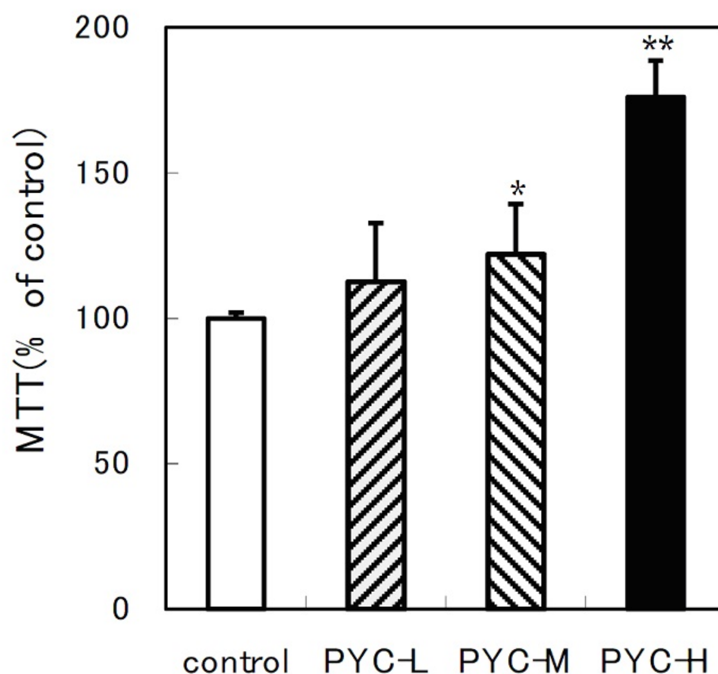

Figure 1. Effect of PYC on proliferation of MC3T3-E1 cells. PYC (PYC-L: 3.75 μg/ml; PYC-M: 37.5 μg/ml; PYC-H: 375 μg/ml) were added for 5 days. Proliferation level was evaluated by MTT in a differentiated control group. Results are presented as mean ± SEM of six experiments: * $p < 0.05$ as compared with differentiated controls, ** $p < 0.01$ as compared with differentiated controls (100%).

Israel Beit-Haemek, Ltd., Israel). Densitometry analysis was performed to quantify protein level (ImageJ 1.46). Protein content of whole cell lysates was determined by micro BCA assay (Thermo Scientific, USA).

Statistical analysis

Results are expressed as the mean ± SEM. A probability value of less than 0.05 was considered to indicate statistical significance.

Results

Proliferation of MC3T3-E1 cells

Figure 1 shows the level of proliferation of MC3T3-E1 cells. Cell metabolic activity was significantly increased dose-dependently in the PYC treatment group than in the control group.

Mineralization of MC3T3-E1 cells

Figure 2 shows the level of mineralization of MC3T3-E1 cells. The area stained red indicates the sites where calcium was deposited. Cells in the control group showed no mineralization nodules (A), while cells in the presence of β-glycerophosphate and L-ascorbic acid formed nodules (B). PYC activated and promoted the mineralization of MC3T3-E1 cells (C).

© 2013 Inforesights Publishing UK

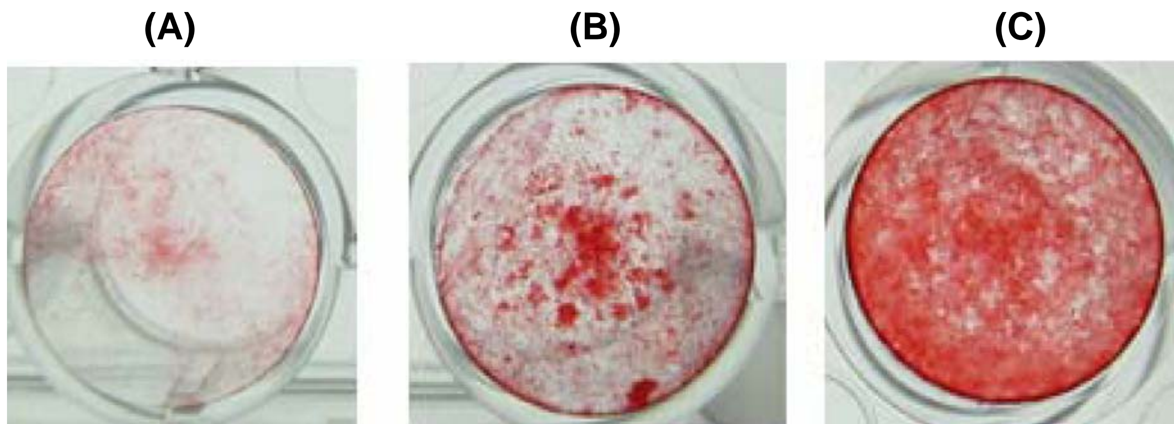

Figure 2. Effect of PYC on mineralization of MC3T3-E1 cells that were differentiated for 28 days. Level of mineralization was evaluated by alizarin red S staining in a control group (A), differentiated group (B), and PYC (375μg/ml) -treated differentiated group (C).

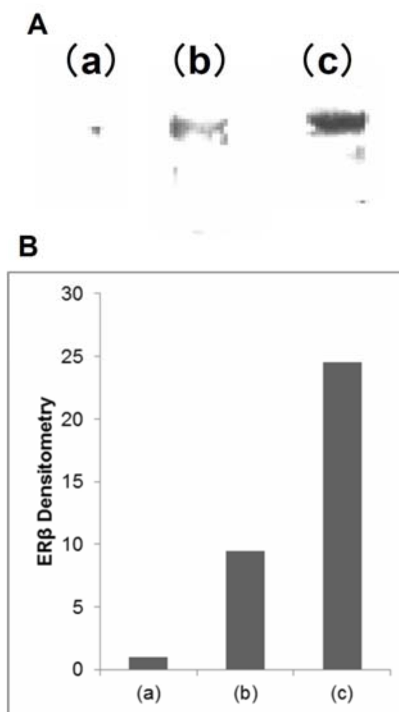

Figure 3. Effect of PYC on expression of ERβ in MC3T3-E1 cells that were differentiated for 28 days. Level of expression of ERβ was evaluated by Western blot analysis in a control group (a), differentiated group (b), and PYC (375μg/ml) -treated differentiated group (c).

ERβ expression of MC3T3-E1 cells

MC3T3-E1 cells were reported to use ERβ for the differentiation of osteoblasts. Cells in the control group showed no expression of ERβ, but cells in the presence of β-glycerophosphate and L-ascorbic acid expressed ERβ. PYC activated the expression of ERβ about 2.8-fold.

MC3T3-E1 cells were reported to use ERβ for the differentiation of osteoblasts. Figure 3A shows the level of ERβ expression in MC3T3-E1 cells. Cells in the control group showed no expression of ERβ (a), but cells in the presence of β-glycerophosphate and L-ascorbic acid

 © 2013 Inforesights Publishing UK

expressed ERβ (b). PYC activated the expression of ERβ (c) 2.6- fold higher than the differentiated osteoblasts (Fig. 3B).

Discussion

Osteoblast proliferation is important in maintaining bone mineral density. Oxidative stress has been found to exert an inhibitory effect on osteoblast differentiation (Bai, et al., 2004). There are many reports concerning the activated effect of natural products, including polyphenol in the mineralization of MC3T3-E1 cells (Hagiwara, et al., 2011; Bu, et al., 2009). We reported that PYC increased both proliferation and mineralization in MC3T3-E1 cells. PYC was found to be able to counteract the effects of ROS on osteoblast differentiation.

Estrogen deficiency is the major cause of osteoporosis in postmenopausal women. Estrogen is important in maintaining the balance between osteoblasts and osteoclasts in bone homeostasis (Manolagas, et al., 2002). Estrogen binding to receptors (ERα and ERβ) induces estrogen receptor dimerization and translocation to the nucleus (Gruber, et al., 2004). Osteoblastic production was regulated by ERβ via the p38 mitogen-activated protein kinase signaling pathway (Wang, et al., 2012). We showed that PYC could cause proliferation and mineralization via the activation of ERβ. These results suggest that PYC stimulates osteoblastic differentiation via ROS scavenging properties and the expression of ERβ. These findings suggest that PYC is useful in preventing osteoporosis in postmenopausal women.

Conflict of interest

The authors declare no conflict of interest.

References

Bai XC, Lu D, Bai J, Zheng H, Ke ZY, Li XM, Luo SQ. (2004). Oxidative stress inhibits osteoblastic differentiation of bone cells by ERK and NF-kappaB. *Biochemical and Biophysical Research Communications* 314, 197-207.

Bu SY, Hunt TS, Smith BJ. (2009). Dried plum polyphenols attenuate the detrimental effects of TNF-alpha on osteoblast function coincident with up-regulation of Runx2, Osterix and IGF-I. *Journal of Nutritional Biochemistry* 20, 35-44.

Gruber CJ, Gruber DM, Gruber IM, Wieseer F, Huber JC. (2004). Anatomy of the estrogen response element. *Trends in Endocrinology & Metabolism* 15, 73-78.

Hagiwara K, Goto T, Araki M, Miyazaki H, Hagiwara H. (2011). Olive polyphenol hydroxytyrosol prevents bone loss. *European Journal of Pharmacology* 662, 78-84.

Heather O. (2010). Scientific and clinical monograph for PYCNOGENOL®. American Botanical Council: Austin, TX.

Mackinnon ES, Rao AV, Josse RG, Rao LG. (2011). Supplementation with the antioxidant lycopene significantly decreases oxidative stress parameters and the bone resorption marker N-telopeptide of type I collagen in postmenopausal women. *Osteoporosis International* 22, 1091-1101.

Manolagas SC, Kousteni S, Jilka RL. (2002). Sex steroids and bone. *Recent Progress in Hormone Research* 57, 385-409.

Mei L, Mochizuki M, Hasegawa N. (2012). Protective effect of pycnogenol® on ovariectomy-induced bone loss in rats. *Phototherapy Research* 26, 153-155.

 © 2013 Inforesights Publishing UK

Schen CL, Chyu MC, Yeh JK, Felton CK, Xu KT, Pence BC, Wang JS. (2009). Green tea polyphenol and Tai Chi for bone health: designing a placebo-controlled randomized trial. *BMC Musculoskeletal Disorders* 10, 110.

Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. (1983). In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *Journal of Cell Biology* 96, 191-198.

Sugiura M, Nakamura M, Ogawa K, Ikoma Y, Ando F, Shimokata H, Yano M. (2011). Dietary patterns of antioxidant vitamin and carotenoid intake associated with bone mineral density: findings from post-menopausal Japanese female subjects. *Osteoporosis International* 22, 143-152.

Wang YD, Tao MF, Wang L, Cheng WW, Wan XP. (2012). Selective regulation of osteoblastic OPG and RANKL by dehydroepiandrosterone through activation of the estrogen receptor β-mediated MAPK signaling pathway. *Hormone and Metabolic Research* 44, 494-500.

 © 2013 Inforesights Publishing UK