Food Chemistry 116 (2009) 990–994

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Beneficial effects of fucoidan on osteoblastic MG-63 cell differentiation

Young-Sook Cho [a,1], Won-Kyo Jung [b,1], Jung-Ae Kim [a], IL-Whan Choi [c], Se-Kwon Kim [a,d,*]

[a] Department of Chemistry, Pukyong National University, 559-1 Dae-Yeon 3, Dong NamGu, Busan 608-737, Republic of Korea
[b] Department of Marine Life Science, and Marine Life Research Center, Chosun University, Gwangju 501-759, Republic of Korea
[c] Department of Microbiology, and Bio-Marker Research Center for Personalized Therapy, College of Medicine, Inje University, Busan 614-735, Republic of Korea
[d] Marine Bioprocess Research Center, Pukyong National University, Busan 608-737, Republic of Korea

ARTICLE INFO

Article history:
Received 20 September 2008
Received in revised form 10 March 2009
Accepted 12 March 2009

Keywords:
Fucoidan
Osteoblastic differentiation
Alkaline phosphatase (ALP)
Osteocalcin (OC)
Bone morphogenetic protein-2 (BMP-2)

ABSTRACT

Fucoidan extracted from the marine brown alga *Undaria pinnatifida*, significantly induced osteoblastic cell differentiation. Our results indicated that a non-toxic sulphated polysaccharide, fucoidan, can increase activity of alkaline phosphatase (ALP) and level of osteocalcin (OC) as phenotypic markers for early-stage osteoblastic differentiation and terminally osteoblastic differentiation, respectively. Furthermore, the results showed positive effects of fucoidan on bone morphogenic protein-2 (BMP-2) as an important factor for bone formation, remodeling and mineralization. The present study may provide new insights in the osteoblastic differentiation of fucoidan and possibility for its application in bone health supplement.

1. Introduction

Osteoporosis is a reduction in skeletal mass due to an imbalance between bone resorption and bone formation, whereas bone homeostasis requires balanced interactions between osteoblasts and osteoclasts (Ducy, Schinke, & Karsenty, 2000; Teitelbaum, 2000). Current drugs and functional foods for bone health include bisphosphonates, calcitonin, estrogen, vitamin D analogues, ipriflavone and isoflavone. These are all bone resorption inhibitors, which maintain bone mass by inhibiting the function of osteoclasts (Rodan & Martin, 2000). It is desirable, therefore, to have satisfactory bone-building (anabolic) agent that would stimulate new bone formation and correct the imbalance of trabecular microarchitecture which is characteristic of established osteoporosis (Berg, Neumeyer, & Kirkpatrick, 2003; Ducy et al., 2000). Since new bone formation is primarily a function of the osteoblasts, agents which regulate bone formation act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation of the osteoblasts (Ducy et al., 2000). The recent studies have shown beneficial effects of sulphated monosaccharide (SGlc) and polysaccharide (anionic PEC) on osteoblastic cell differentiation and bone regeneration, respectively (Kim, Eresha, Niranjan, & Kim, 2007; Nagahata, Nakaoka, Teramoto, Abe, & Tsuchiya, 2005).

Brown seaweeds are known to produce different polysaccharides, namely alginates, laminarans and fucoidans (Painter, 1983; Percival, 1967). Among these polysaccharides, fucoidan has been mainly extracted from *Undaria pinnatifida* and *L. japonica* used as commercial seafoods in the East Asian countries like Korea, China, Japan, etc. Fucoidan, a sulphated polysaccharide, contains large proportions of L-fucose and sulphate, together with minor amounts of other sugars like xylose, galactose, mannose and glucuronic acid (Duarte, Cardoso, Noseda, & Cerezo, 2001; Percival, 1967). Recently, its biological activities such as antioxidative, anticoagulant, anti-thrombotic, anti-inflammatory, anti-tumoral and anti-viral have been reported (Chevolot, Mulloy, Ratiskol, Foucault, & Colliec-Jouault, 2001; Cumashi et al., 2007; Mourao, 2004; Maruyama, Tamauchi, Iizuka, & Nakano, 2006; Thompson & Dragar, 2004). However, to the best of our knowledge, there are no reports on beneficial effects of fucoidan on bone health or formation. Therefore, in this study, we examined the effects of fucoidan on osteoblastic MG-63 cell differentiation. Fucoidan extracted from *U. pinnatifida* may increase activity of alkaline phosphatase (ALP) and level of osteoclacin (OC), phenotypic markers for early stage and terminally differentiation, respectively. Furthermore, positive effects of fucoidan on bone morphogenic protein-2 (BMP-2) as an important factor for bone formation, remodeling and mineralization was investigated. The results may provide new insights in the osteoblastic differentiation of fucoidan and possibility for its application in bone health supplement.

* Corresponding author. Address: Department of Chemistry, Pukyong National University, 559-1 Dae-Yeon 3, Dong NamGu, Busan 608-737, Republic of Korea. Tel.: +82 51 620 6375; fax: +82 51 620 8147.
E-mail address: sknkim@pknu.ac.kr (S.-K. Kim).
[1] These authors contributed equally to this work.

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.03.051

2. Materials and methods

2.1. Chemicals and reagents

Fucoidan (total polysaccharide: 62.12% and sulphate: 34.20%) extracted from a brown seaweed *U. pinnatifida*, was purchased from HAEWON BIOTECH Inc. (Seoul, Korea). Fetal bovine serum (FBS), minimal essential medium (MEM), penicillin G, and streptomycin were purchased from GIBCO-BRL (Gaithersburg, MD, USA). BMP-2 ELISA kit was obtained from R&D Systems (Minneapolis, MN, USA). Osteocalcin ELISA kit was purchased from Takara (Tokyo, Japan), and p-nitrophenyl phosphate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). RT-PCR reagents were purchased from Promega (Madison, WI, USA).

2.2. Cell culture

The human osteosarcoma cell line, MG-63 (CRL-1427), was obtained from American Tissue Culture Collection (ATCC). MG-63 cells were cultured in minimal essential medium (MEM) (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/ml of penicillin G and 100 μg/ml of streptomycin).

2.3. Cell viability assay

MTT was used as an indicator of cell viability as determined by mitochondrial-dependent reduction to formazan. In brief, the cells were seeded and then treated with various reagents for the indicated time periods. After various treatments, the medium was removed and the cells were incubated with a solution of 0.5 mg/ml MTT. After incubation for 3 h at 37 °C and 5% CO_2, the supernatant was removed and the formation of formazan was observed by monitoring the signal at 540 nm using a microplate reader.

2.4. Alkaline phosphatase (ALP) activity

Cells were seeded into 96-well plates at a density of 5×10^3 cells/well and cultured for 24 h. The agent to be tested was added to the wells, and incubation continued for 2 days. The cells were then washed three times with physiological saline, and cellular protein concentration was determined by incubation in B (bicinchoninic acid) protein assay reagent containing 0.1% Triton X-100 for 1 h at 37 °C. The reaction was stopped by adding 1 M NaOH, and the absorbance measured at 560 nm. Alkaline phosphatase activity in the cells was assayed after appropriate treatment periods by washing the cells three times with physiological saline, then measuring cellular activity by incubation for 1 h at 37 °C in 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 10, containing 0.1% Triton X-100, 1.5 mM $MgCl_2$ and 15 mM p-nitrophenyl phosphate. The reaction was stopped by adding 1 M NaOH before measuring absorbance at 405 nm. A unit of phosphatase activity is defined as the amount of enzyme activity that will liberate 1 μm of p-nitrophenol per hour (Eichner, Brock, Heldin, & Souchelnytskyi, 2002).

2.5. Analysis of mineralization

The degree of mineralization was determined in the 24-well plates using Alizarin Red (Sigma Chemical, St. Louis, MO, USA) staining after 7 days treatment. Briefly, cells were fixed with 70% (v/v) ethanol for 1 h and were then stained with 40 mM Alizarin Red S in deionized water (pH = 4.2) for 15 min at room temperature. After removing Alizarin Red S solution by aspiration, cells were incubated in PBS for 15 min at room temperature on an orbital rotator. Then the cells were rinsed once with fresh PBS, and subsequently destained for 15 min with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH = 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured using a microplate reader.

2.6. Assaying the levels of osteocalcin and BMP-2

Osteocalcin and BMP-2 ELISA kits were used to detect osteocalcin and BMP-2 levels, respectively. Briefly, cells were treated with various concentrations of fucoidan. The culture medium was collected and measured for osteocalcin and BMP-2, respectively. These samples were placed in 96-well microtiter plates coated with monoclonal detective antibodies and incubated for 2 h at room temperature. After removing unbound material with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20), horseradish peroxidase conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a coloured solution, with colour intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm. Results were presented as the percentage of change of the activity compared to the untreated control (Kuo, Hsu, Chang, & Chang, 2005).

2.7. RT-PCR analysis

Expression levels of BMP-2 mRNA were examined using RT-PCR. We also performed RT-PCR for GAPDH independently as an internal control. RNA was isolated with TRIzol reagent. Aliquots (2 μg) of total RNA were reverse transcribed to cDNA using AMV reverse transcriptase. The oligonucleotides used for PCR were: 5′-CCACGTCTTCACATTTGGTG-3′(forward primer) and 5′-AGACTGCGCCTAGTAGTTGT-3′ (reverse primer) for human ALP mRNA and 5′-ATGTTCGCCTGAAACAGAGACCCA-3′ (forward primer) and 5′-CTTACAGCTGGACTTAAGGCGTTTC-3′ (reverse primer) for human BMP-2 mRNA, 5′-ACCACAGTCCATGCCATCAC-3′ (forward primer) and 5′-TCCACCACCCTGTTGCTTGTA-3′ (reverse primers) for human GAPDH, 5′-CCCAAAGGCTTCTTGTTG-3′ and 5′-CTGGTAGTTGTTGTGAGCAT-3′, and 5′-ATGAGAGCCCTCACACTCCTC-3′ (forward primers) and 5′GCCGTAGAAGCGCCGATAGGC (reverse primers) for osteocalcin (Drissi, Hott, Marie, & Lasmoles, 1997). The PCR products were detected by 1.5% agarose gel electrophoresis and photographed.

2.8. Intracellular calcium imaging

MG-63 Cells were loaded by incubation with 2 μM of Fura-2-AM (Molecular Probes Co., Eugene, OR, USA) in culture medium without serum for 30 min at RT in loading solution: 115 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM Hepes, and 10 mM glucose, pH 7.42. After washed continuously with nominally divalent cation-free buffer, Fura-2 fluorescent images were analyzed using a Zeiss LSM 510 laser scanning confocal device (Zeiss, Switzerland). The fluorescence emission at 510 nm with alternate excitation wavelengths of 345 and 380 nm was measured from individual cells.

2.9. Statistical analysis

Data were expressed as means ± SD ($n = 3$). Statistical comparisons of the results ere made using analysis of variance (ANOVA). Significant differences ($P < 0.05$) between the means of the control and test groups were analyzed by Dunnett's test.

3. Results and discussion

Fucoidan is one of the representative sulphated polysaccharides derived from marine brown algae. Sulphated polysaccharides such

as glucosamine sulphate, chitosan sulphate, heparin, and heparan sulphate are known to be macromolecules associated with the cell surface and the extracellular matrix (Nagahata et al., 2005; Xiao, Haase, Young, & Bartold, 2004), and have been shown to interact directly with a number of growth factors, including BMPs, via highly negatively charged polysaccharide chains. Indeed, heparin-affinity chromatography was used to purify the BMP activity from extracts prepared by employing demineralized bone matrix (Wang et al., 1990).

In the present study, to identify beneficial effects of fucoidan on bone health and mineralization, we examined whether fucoidan extracted from *U. pinnatifida* can increase activity of alkaline phosphatase (ALP) and level of osteoclacin (OC), phenotypic markers for early stage and terminally differentiation, respectively. We also investigated positive effects of fucoidan on production of bone morphogenic protein-2 (BMP-2) and hydroxyapatite particle as an important factor for bone formation and mineralization.

We first determined the effect of various concentrations of fucoidan on the cell proliferation of MG-63 by MTT assay. Our results showed that fucoidan did not exhibit significant effects on cell proliferation at the concentrations used (10–250 μg/ml) after 24 h of treatment in cell line (Fig. 1). In addition, treatment of 100 μg/ml of fucoidan did not exhibit significant effects on cell viability for 72 h (Fig. 2).

The effect of fucoidan on the maturation of osteoblasts was studied by determining alkaline phosphatase activity in MG-63 cell. The results showed that fucoidan increased alkaline phosphatase activity and mRNA expression in a dose-dependent manner after 48 h of treatment in both cell lines (Fig. 3).

The effect of fucoidan on terminal differentiation of osteoblast-like cell was also studied by determining the osteocalcin concentrations in the culture medium. Osteocalcin is a specific cell marker protein for the terminal cell differentiation of osteoblast which is selected into cell culture medium by the osteoblast. MG-63 treated with fucoidan for 48 h experienced significant increases in osteocalcin synthesis. Furthermore fucoidan stimulated osteocalcin synthesis in a concentration-dependent manner (Fig. 4).

According to the Alizarine Red-S staining data (Fig. 5a), fucoidan (100 μg/ml) increased the amounts of hydroxyapatite in the cell. The amount of mineral intercellular level was higher in a concentration-dependent manner. To clearly understand the influence of fucoidan on mineralization, another important process in differentiation, calcium deposition was analyzed using confocal microscopy. Previously known as Furaptra, Fura-2 is a Ca^{2+} indicator used for intracellular measurements of these two divalent (Hofer, Fasolato, & Pozzan, 1998). Fucoidan (100 μg/ml) showed significantly stimulatory effect on mineralization by the increase of intracellular calcium (Fig. 5b).

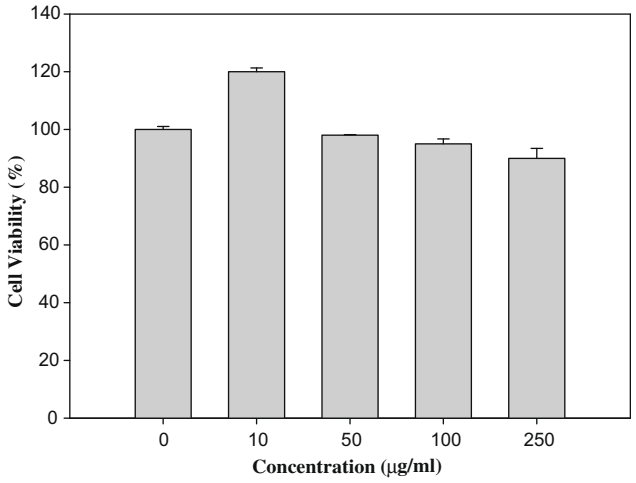

Fig. 1. Effects of fucoidan on viability of MG-63 cell in dose-dependant manner. Cells were treated with various concentration of fucoidan for 24 h.

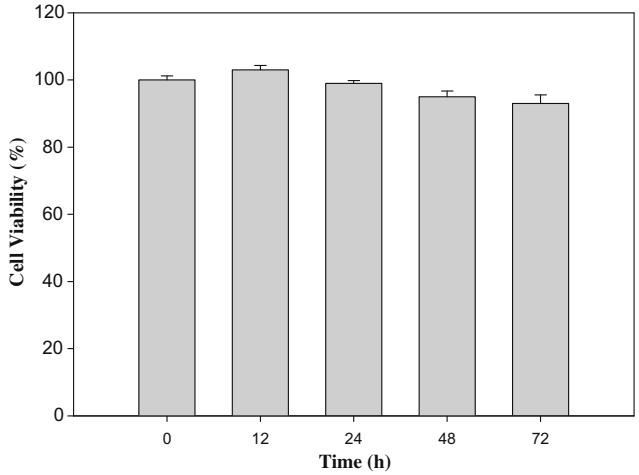

Fig. 2. Effects of fucoidan (100 μg/ml) on viability of MG-63 cell in time-dependant manner.

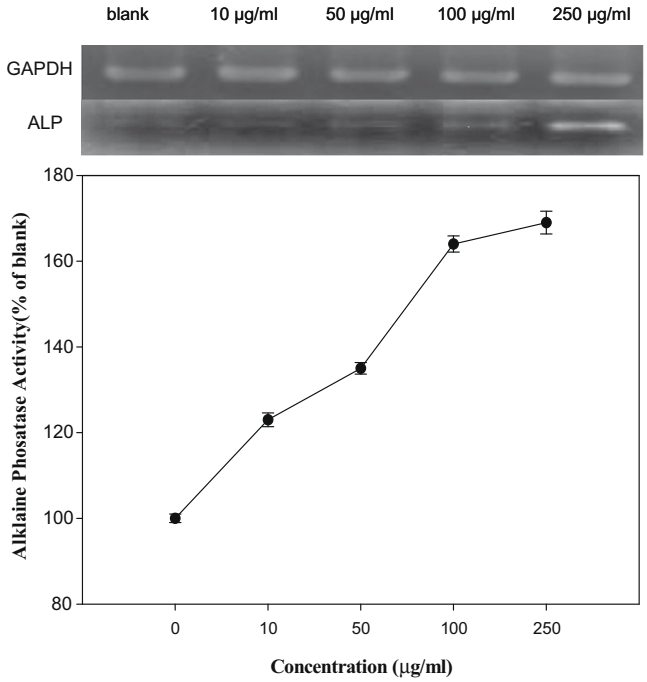

Fig. 3. Alkaline phosphatase assay. The MG-63 cells were treated with various concentrations of fucoidan for 48 h. Alkaline phosphatase activity was assessed by the conversion of p-nitrophenyl phosphate in 0.1 M $NaHCO_3$–Na_2CO_3 buffer, pH 10, containing 1.5 mM $MgCl_2$ and 0.1% Triton.

The expression of the BMP-2 gene using RT-PCR and of bone morphogenetic proteins synthesis in the presence or absence of fucoidan using BMP-2 ELISA kits was also examined. The results indicated that fucoidan could cause a significant increase in BMP-2 mRNA and protein synthesis in MG-63 cells. After 24 h of fucoidan treatment, production of BMP-2 was significantly increased in a dose-dependent manner (Fig. 6).

Bone morphogenetic proteins (BMPs) play an important role in the process of bone formation and remodeling (Sykaras & Opperman, 2003). It has been well documented that stimulation of osteoblast cell differentiation is characterized mainly by increased expression of alkaline phosphatase, and osteocalcin (Xiao et al.,

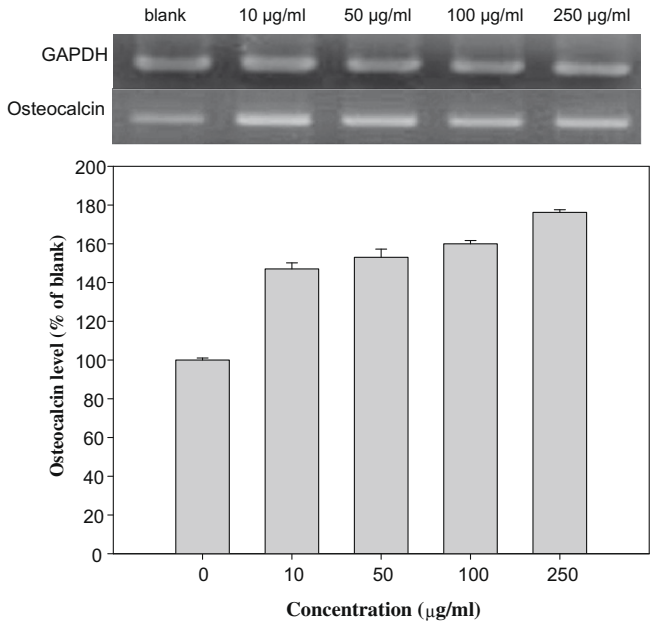

Fig. 4. Effects of fucoidan on the osteocalcin secretion of MG-63 cell. Cells were treated with various concentrations in the presence of FBS for 2 days.

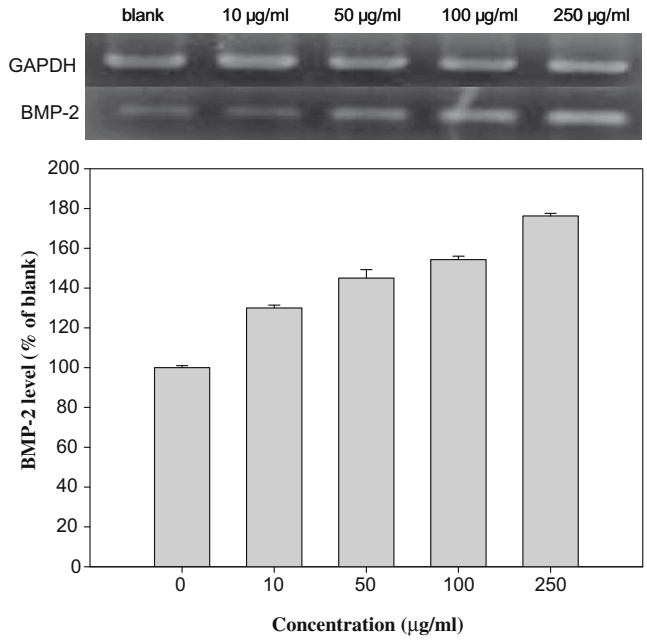

Fig. 6. Effects of fucoidan on the mRNA expression and production of BMP-2 in a does-dependent manner.

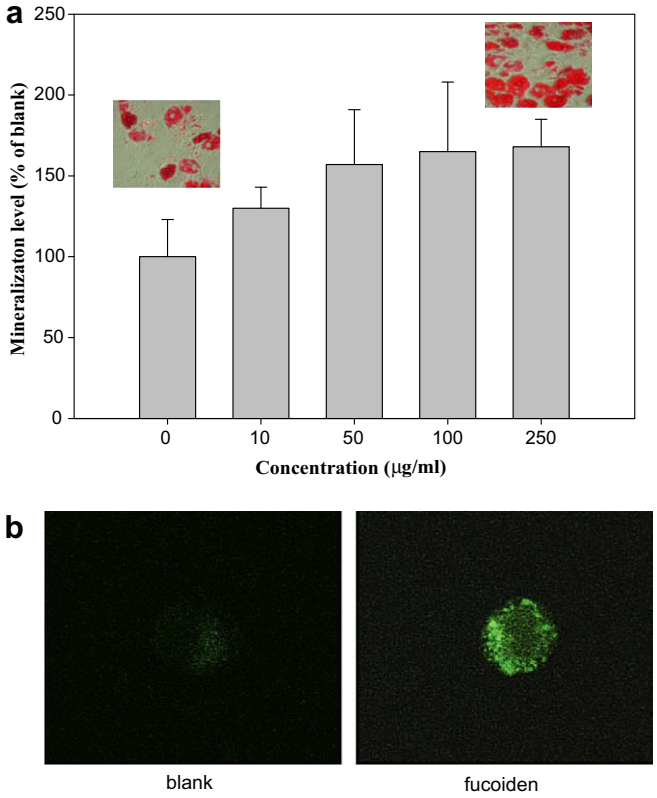

Fig. 5. Effects of fucoidan on the increase of hydoxyapaptite and calcium in MG-63 cell (a) Alizarine Red-S staining 7 days after cultures: Photograph of representative stained cell culture. The production of mineralization was increased by fucoidan in a dose-dependent manner. (b) Fucoidan (100 μg/ml) showed a significant effect on the increase of intracellular calcium.

2004). Especially, recombinant human bone morphogenetic protein (rhBMP)-2 plays an important role in bone formation and has been shown to enhance fracture healing. In fracture healing, there is increased BMP receptor expression in osteogenic cells near the fracture, in fibroblast-like spindle cells, and infibroblasts involved in endochondral ossification (Onishi et al., 1998). Interestingly, the promotion of bone-healing benefits by BMP-2 is believed to be due to their ability to stimulate the proliferation and differentiation of mesenchymal and osteoprogenitor cells, and both are angiogenic. Bone morphogenetic proteins (BMPs) are members of the TFG-β superfamily, which regulate embryonic development and also induce ectopic bone formation in developed issues (Hogan, 1996; Urist, 1965). In cells of the osteoblast lineage, BMPs 2, 4, and 7 induce expression of alkaline phosphatase, type I collagen, and other non-collagenous bone proteins found in osteoid; a phenotype consistent with differentiated osteoblasts (Cheifetz, Li, McCulloch, Sampath, & Sodek, 1996; Cheng, Shao, Charlton-Kachigian, Loewy, & Towler, 2003; Li, Cheifetz, McCulloch, Sampath, & Sodek, 1996).

4. Conclusions

Our investigation indicates that the presence of fucoidan causes significantly induced osteoblastic cell differentiation. Fucoidan could increase the levels of alkaline phosphatase, osteocalcin, and BMP-2 associated with the bone mineralization in the osteoblastic cell. From the results, the present study provides the proofs on the positive effects of fucoidan for the osteoblastic cell differentiation. To evaluate possibility for its application in bone health supplement, its bioavailability will be examined by further in vivo study.

Acknowledgement

This research was supported by a grant from Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Land, Transport and Maritime, Republic of Korea.

References

Berg, C., Neumeyer, K., & Kirkpatrick, P. (2003). Teriparatide. *Natural Review Drug Discovery, 2*, 257–258.

Cheifetz, S., Li, I. W., McCulloch, C. A., Sampath, K., & Sodek, J. (1996). Influence of osteogenic protein-1 (OP-1;BMP-7) and transforming growth factor-beta 1 on bone formation in vitro. *Connect Tissue Research, 35*, 71–78.

Cheng, S. L., Shao, J. S., Charlton-Kachigian, N., Loewy, A. P., & Towler, D. A. (2003). MSX2 promotes osteogenesis and suppresses adipogenic differentiation of multipotent mesenchymal progenitors. *Journal of Biological Chemistry, 278*, 45969–45977.

Chevolot, L., Mulloy, B., Ratiskol, J., Foucault, A., & Colliec-Jouault, S. (2001). A disaccharide repeat unit is the major structure in fucoidans from two species of brown algae. *Carbohydrate Research, 330*, 529–535.

Cumashi, A., Ushakova, N. A., Preobrazhenskaya, M. E., D'Incecco, A., Piccoli, A., Totani, L., et al. (2007). A comparative study of the antiinflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology, 17*, 541–552.

Drissi, H., Hott, M., Marie, P. J., & Lasmoles, F. (1997). Expression of the CT/CGRP gene and its regulation by dibutyryl cyclic adenosine monophosphate in human osteoblastic cells. *Journal of Bone and Mineral Research, 12*, 1805–1814.

Duarte, M. E., Cardoso, M. A., Noseda, M. D., & Cerezo, A. S. (2001). Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*. *Carbohydrate Research, 333*, 281–293.

Ducy, P., Schinke, T., & Karsenty, G. (2000). The osteoblast: A sophisticated fibroblast under central surveillance. *Science, 289*, 1501–1504.

Eichner, A., Brock, J., Heldin, C. H., & Souchelnytskyi, S. (2002). Bone morphogenetic protein-7 (OP1) and transforming growth factor-beta1 modulate 1, 25(OH)2-vitamin D3-induced differentiation of human osteoblasts. *Experimental Cell Research, 275*, 132–142.

Hofer, A. M., Fasolato, C., & Pozzan, T. (1998). Capacitative Ca^{2+} entry is closely linked to the filling state of internal Ca^{2+} stores: A study using simultaneous measurements of I_{CRAC} and intraluminal $[Ca^{2+}]$. *Journal of Cell Biology, 140*, 325–334.

Hogan, B. (1996). Bone morphogenetic proteins in development. *Current Opinion in Genetics & Development, 6*, 432–438.

Kim, M. M., Eresha, M., Niranjan, R., & Kim, S. K. (2007). Glucosamine sulfate promotes osteoblastic differentiation of MG-63 cells via anti-inflammatory effect. *Bioorganic & Medicinal Chemistry Letters, 17*, 1938–1942.

Kuo, P. L., Hsu, Y. L., Chang, C. H., & Chang, J. K. (2005). Osthole-mediated cell differentiation through bone morphogenetic protein-2/p38 and extracellular signal-regulated kinase 1/2 pathway in human osteoblast cells. *Journal of Pharmacology and Experimental Therapeutics, 314*, 1290–1299.

Li, I. W., Cheifetz, S., McCulloch, C. A., Sampath, K. T., & Sodek, J. (1996). Effects of osteogenic protein-1 (OP-1, BMP-7) on bone matrix protein expression by fetal rat calvarial cells are differentiation stage specific. *Journal of Cell Physiology, 169*, 115–125.

Maruyama, H., Tamauchi, H., Iizuka, M., & Nakano, T. (2006). The role of NK cells in antitumor activity of dietary fucoidan from *Undaria pinnatifida* sporophylls (Mekabu). *Planta Medica, 72*, 1415–1417.

Mourao, P. A. (2004). Use of sulfated fucans as anticoagulant and antithrombotic agents: Future perspectives. *Current Pharmaceutical Design, 10*, 967–981.

Nagahata, M., Nakaoka, R., Teramoto, A, Abe, K., & Tsuchiya, T. (2005). The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes. *Biomaterials, 26*, 5138–5144.

Onishi, T., Ishidou, Y., Nagamine, T., Yone, K., Imamura, T., Kato, M., Sampath, T. K., & Sakou, T. (1998). Distinct and overlapping patterns of localization of bone morphogenetic protein (BMP) family members and a BMP type II receptor during fracture healing in rats. *Bone, 22*, 605–612.

Painter, T. (1983). Algal polysaccharides. *The polysaccharides* (Vol. 2, pp. 195–285). New York: Academic Press.

Percival, E. (1967). Chemistry and enzymology of marine algae polysaccharides (pp. 53–71). New York: Academic Press.

Rodan, G. A., & Martin, T. J. (2000). Therapeutic approaches to bone diseases. *Science, 289*, 1508–1514.

Sykaras, N., & Opperman, L. A. (2003). Bone morphogenetic proteins (BMPs): How do they function and what can they offer the clinician? *Journal of Oral Science, 45*, 57–73.

Teitelbaum, S. L. (2000). Bone resorption by osteoclasts. *Science, 289*, 1504–1508.

Thompson, K. D., & Dragar, C. (2004). Antiviral activity of *Undaria pinnatifida* against herpes simplex virus. *Phytotherapy Research, 18*, 551–555.

Urist, M. R. (1965). Bone: Formation by autoinduction. *Science, 150*, 893–899.

Wang, E. A., Rosen, V., Cordes, P., Hewick, R. M., D'Alessandro, J. S., Baudy, M., Cordes, P., Harada, T., Israel, D., Hewick, R. M., Kerns, K. M., LaPan, P., Luxenberg, D. P., McQuaid, D., Moutsatsos, K., Nave, J., & Wozney, J. M. (1990). Recombinant human bone morphogenetic protein induces bone formation. In Proceeding of the National Academy of Science USA, (Vol. 87, pp. 2220–2224).

Xiao, Y., Haase, H., Young, W. G., & Bartold, P. M. (2004). Development and transplantation of a mineralized matrix formed by osteoblasts in vitro for bone regeneration. *Cell Transplant, 13*, 15–25.