2007. Vol. 28. No. 4. *158-167*
Korean Journal of Oriental Medicine

Original Article

Effects of *Uncaria rhynchophylla* Extracts on Differentiation and Bone Mineralized Formation in Human Osteoblast-like SaOS-2 cells

Jeong-Eun Huh[1], Yong-Hyeon Baek[2], Do-Young Choi[2], Jae-Dong Lee[2], Dong-Suk Park[2]

[1]Oriental Medicine Research Center for Bone & Joint Disease, KyungHee University.
[2]Department of Acupuncture & Moxibustion, College of Oriental Medicine, KyungHee University.

Background & Objective : *Uncaria rhynchophylla* is traditional medicine herb used for enhancing body resistance against various diseases. The aim of this study was to identify if *Uncaria rhynchophylla* extracts induce osteogenic activity in human osteoblast-like SaOS-2 cells.

Methods : The osteogenic activity of *Uncaria rhynchophylla* was evaluated on cell proliferation assay by WST-8, and osteoblast-specific genes, such as VEGF, type I collagen (Col I), osteocalcin (OCN), and osteopontin (OPN) by RT-PCR analysis and ELISA assay in osteoblasts-like SaOS-2 cells. Bone mineralization was stained with Alizalin red method.

Results : *Uncaria rhynchophylla* had significantly increased cell proliferation at a dose dependent manner in human osteoblast-like SaOS-2 cells. *Uncaria rhynchophylla* markedly increased alkaline phosphatase (ALP), vascular endothelial growth factor (VEGF) mRNA expression at 7 days and dose dependently increased ALP activity and VEGF secretion in human osteoblast-like SaOS-2 cells. Also, *Uncaria rhynchophylla* time-dependently increased type I collagen (Col I), osteopontin (OPN), and osteocalcin (OCN) mRNA in SaOS-2 cells. Extracellular accumulation of proteins such as Col I and OCN was maximal increased by *Uncaria rhynchophylla* at 10 ㎍/ml. Also, *Uncaria rhynchophylla* significantly induced mineralization in the culture of SaOS-2 cells.

Conclusion : This study showed that *Uncaria rhynchophylla* had enhanced proliferation, ALP activity, VEGF, bone matrix proteins such as OCN, OPN, and Col I, and mineralization in SaOS-2 cells. These results propose that *Uncaria rhynchophylla* can play an important role in osteoblastic bone formation, osteogenesis, and may possibly lead to the development of bone-forming drugs.

Key Words : *Uncaria rhynchophylla*, ALP activity; VEGF; OCN; OPN; Col I; Mineralization

Introduction

Bone modeling and remodeling are essential for development, maturation, maintenance, and repair of bones. The differentiation of osteoblasts are included in these events and are controlled by various local growth factors and cytokines produced in bone as well as by systemic hormones.

Osteoblasts, which arise from mesenchymal stem cell precursors, undergo differentiation in response to a number of factors including bone morphogenic proteins (BMPs), transforming growth factor (TGF), insulin-like growth factor I (IGF-1), vascular endothelial growth factor

・received : 1 December 2007
・received in revised from : 3 December 2007
・accepted : 13 December 2007
・Correspondence to : Dong-Suk Park
Department of Acupunture, College of Oriental medicine, KyungHee 149, Sangildong, Kangdonggu, Seoul, South Korea
(Tel : +82-2-440-7702 / Fax : +82-2-440-6799
E-mail : address: dspark49@yahoo.co.kr)

(VEGF), and glucocorticoids[1-6]. These are important for osteoblastic differentiation and modulate the expression of osteoblast-specific genes. Moreover, several different molecules are associated with deposition and maintenance of mineralized skeletal elements. Once matrix synthesis begins in osteoblast culture models such as primary osteoblast cultures, the cells differentiate as genes encoding osteoblastic markers such as alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OCN). Finally, osteoblasts become embedded in the extracellular matrix consisting mainly of type I collagen (Col I), and matrix mineralization begins as mineral deposits extend along and within collagen fibrils[7,8]. Recently, Maeda et al., reported that statins stimulate expression of bone anabolic factors such as VEGF and BMP-2, and promote osteoblast differentiation and mineralization in MC3T3-E1 cells[9,10].

Uncaria rhynchophylla (UR), mainly distributed in Korea, China and Japan, is a vine or shrub with characteristic peduncle that appear as curved hooks on the side shoots. The dried hooks and stems of this planthave been used as a traditional medicine for the treatment of headache and dizziness due to hypertension, and infantile convulsion and other nervous disorders. UR has been used for suppression of liver hyperfunction, relief of dizziness, treatment of tremors and convulsions, general health, and ischemic heart disease in Oriental medicine. However, these functions have not been scientifically tested and their mechanisms are not known. It is known to have sedative and anti-convulsive effects and has thus been applied in the treatment of epilepsy[11]. Recently, it was reported that the alkaloid fraction of UR protects N-methyl-D-aspartate (NMDA)-induced neuronal cell death by suppressing apoptosis-related genes[12]. However, much more insight into the pharmacological functions and mechanisms of UR are needed, especially as there is no clear experimental evidence supporting its use in the treatment of wound healing and cardiovascular diseases. Recently reported, the hooks and stems from Uncaria sinensis Havil (Rubiaceae) exhibited potent antioxidant activity on free radical and lipid peroxidation[13]. The hooks and stems of Uncaria sinensis have been used as a spasmolytic, an analgesic and a sedative treatment for many symptoms associated with hypertension and cerebrovascular disorders[14]. Oxindole and indole alkaloids, polyoxygenated triterpenes, and quinovic acid glycosides have been isolated form the genus Uncaria, and have been found to possess various pharmacological activities[14,15]. However, at the present time there is no direct experimental evidence of the therapeutic benefit of UR in the treatment of bone healing. Therefore, this study was performed to clarify the effect of UR on the cell proliferation, differentiation, expression of VEGF and bone matrix proteins, and mineralization of human osteoblast-like SaOS-2 cells.

Materials and methods

1. Plant material and preparation of crude extracts

UR was obtained from Kyunghee Oriental Medical Center, following confirmation of Nam-je Kim for quality control. The sprig of UR was incubated in 50 % (v/v) ethanol−water at room temperature for 24 h. The extract was then filtered and concentrated under low pressure using a vacuum rotary evaporator (Eyela, Tokyo, Japan). The remaining residue was lyophilized in a freeze dryer, and stored at −20 ºC. The powder was dissolved in dimethyl sulfoxide

(DMSO) for experimental use, adjusting the final concentration of DMSO in culture medium to below 0.5 %.

2. Cell Culture

Human osteoblast-like SaOS-2 cells were grown in McCoy's 5a Medium supplemented 15 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (Invitrogen Corporation, CA, USA). To maintain exponential growth, the cells were passaged every four days.

3. Measurement of proliferation

The viability of UR was assessed using a cell proliferation assay by WST-8 (Dojindo Lab., Tokyo, Japan). Briefly, the cells were seeded in 96-well plates at a density of 1 ×104 cells/well. After 24 h incubation, the cells were exposed to various concentrations (0.01~1000 ㎍/ml) of UR in a volume of 100 ㎕. After 72 h incubation, 10 ㎕ of WST-8 dye (Dojindo Lab., Toyko, Japan) was added to each well and incubated for 2 h at 37 ℃. The optical density was read at 450 nm in an ELISA plate reader. Results were calculated as the percentage of viable cells in the UR-treated group relative to the 0.5 % DMSO-treated control group.

4. Assay of ALP activity

Time response effect of UR on alkaline phosphatase mRNA expression was measured by RT-PCR analysis in human osteoblast-like SaOS-2 cells. Cells were treated with vehicle or UR at specified concentration for the time periods indicated. The cells were seeded in 48-well plates at a density of 1 × 105 cells/well. After 24 h incubation, the cells were treated with 0.1 ug/ml for 3, 7, 14 days. Dose response effects of UR on alkaline phosphatase activity was measured by colorimetric analysis SaOS-2 cells. The cells were seeded in 96-well plates at a density of 1 × 104 cells/well. After 24 h incubation, the cells were exposed to various concentrations (0.01~100 ㎍/ml) of UR for 7 days. Cells were lysed with 0.1 % Triton X-100, sonicated, and then centrifuged at 14,000 rpm for 20 min at 4 ℃. ALP activity was assayed with a p-Nitrophenyl phosphate liquid substrate system (Sigma-Aldrich Co., MO, USA). Supernatants were incubated with reaction solution in the dark for approximately 30 min at room temperature. The reaction was stopped with 0.1N NaOH, and the absorbance was read at 405 nm. A standard curve was prepared with p-nitrophenol (Sigma-Aldrich Co., MO, USA). Each value was normalized to the protein concentration.

5. RT-PCR analysis of Gene Expression

RNA was prepared with TrizolR reagent (Invitrogen Corporation, CA, USA). Reverse transcription of 1 ㎍ of total RNA was carried out for 60 min at 42 ℃ and then 15 min at 72 ℃, using the system for RT-PCR (TaKaRa Biotechnology, Seoul, Korea), which contained RT buffer, oligo(dT)12 mer, 10 mM dNTP, 0.1M dithiothreitol, reverse transcriptase, and RNAse inhibitor. PCR using primers to unique sequences in each cDNA was carried out in a volume of 10 ㎕ of reaction mixture for PCR (as supplied by TaKaRa, Korea), supplemented with 2.5 units of TaKaRa TaqTM1.5 mM each dNTP, and PCR buffer. 20 pmol of each primer used. Amplification reactions were performed using the following primers and protocol: VEGF: forward, 5'-CTGTGCAGGCTGCTGTAACG-3' reverse, 5'-GTTCCCGAAACCCTGAGGAG-3' OPN: forward, 5'- CAGCCATGAATTTCACAGCC-3' reverse, 5'-GGGAGTTTCCATGAAGCCAC-3'

OCN: forward, 5'-CATGAGAGCCCTCACA-3' reverse, 5'-AGAGCGACACCCTAGAC-3' Col I: forward, 5'-TGACCTCAAGATGTGCCACT-3' reverse, 5'-GGGAGTT TCCATGAAGCCAC-3' and b-actin: forwad, 5'-CCATCATGAAGTGT-GACGTG-3' reverse, 5'-ACATCTGCTGGAA-GGTGGAC-3'. An equal volume from each PCR wasanalyzed by 1.8 % agarose gel electrophoresis, and ethidium bromide-stained PCR products were evaluated. Marker gene expression was normalized to b-actin expression in each sample. Signal intensity was quantified with the Gel Doc EQ (BIO-RAD Laboratories, Milan, Italy).

6. Determination of VEGF, Type I collagen and osteocalcin

SaOS-2 cells were treated with UR for the periods indicated. Conditioned media were prepared from three-day cultures to 7 days or 14 days, and then stored at −70 ºC for the immun-oassay of VEGF, type I collage and osteocalcin. VEGF levels in cultured supernatant were assayed using a quantitative sandwich enzyme linked immunosorbent assay (ELISA) kit (R&D Systems Inc., MN, USA). Type I collagen levels were determined Sircol collagen assay (Biocolor Ltd., Valley business center, Northern Ireland). Samples react with SiriusRed dye containing with sulphonic acid for 30 min at room temperature. The reaction mixture was centrifuged, lysed and then measured at 540 nm. Osteocalcin levels in cultured supernatant were assayed using a quantitative sandwich enzyme linked immunosorbent assay (ELISA) kit (R&D Systems Inc., MN, USA).

7. Assay of mineralized matrix formation

SaOS-2 cells were cultured in 24 well plate with medium containing 1 % FBS, 1 % penicillin-streptomycin, 50 μg/ml ascorbic acid, and 10 mM b-glycerophosphate for 14 days after reaching confluence. The cells were fixed with 100 % methanol and stained with Alizalin red method. Mineralzation was observed with an optical microscope (200x magnification).

8. Statistical Analysis

The results were expressed as means ±S.D. calculated from the specified numbers of determination. Comparison of the data was performed using Student's t test. Significance was defined as a p value of < 0.05 %.

Results

1. Effect of *Uncaria rhynchophylla* on proliferation

To investigate the proliferative effect of UR, osteoblast-like SaOS-2 cells were first examined. A range of 0, 0.01, 0.1 10, 100 μg/ml UR was applied to the SaOS-2 cells. UR showed cell proliferation effect in SaOS-2 cells after three days (Fig. 1). The presence of 0.1~100 μg/ml UR caused a significant increase in cell viability compared to control (Fig. 1).

2. Effect of *Uncaria rhynchophylla* on ALP

To ascertain whether UR is capable of affecting osteoblastic cell differentiation, we examined the changes in ALP activity. UR time-dependently increased ALP mRNA expression and activity at 0.1 ug/ml, and maximal effect was reached at 7 days (Fig. 2A). Also, UR dose-dependently increased and maximal effect when cells were incubated with 10 μg/ml UR (Fig. 2B).

3. Effect of *Uncaria rhynchophylla* on VEGF

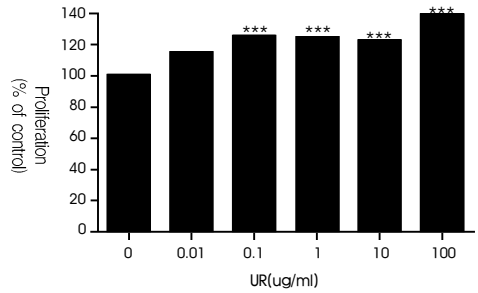
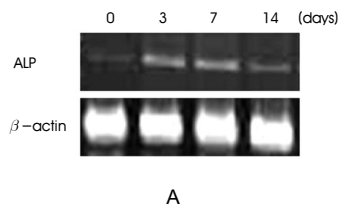


Fig. 1. Effects of *Uncaria rhynchophylla* on proliferationin human osteoblast-like SaOS-2 cells. UR at 0.01, 0.1, 1, 10, 100 ㎍/ml was added to SaOS-2 cells for three days. Cell proliferation was determined by a colorimetric WST-8 assay. Data are expressed as percentage of control. Results are shown as the mean ± SD of three experiments. ***P < 0.001 compared with vehicle-treated control.

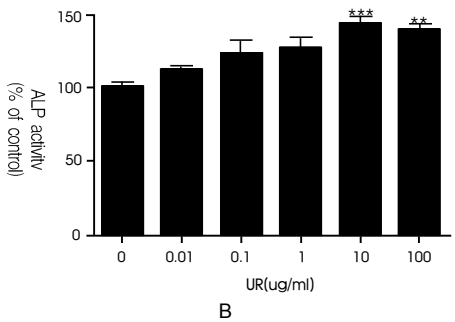
We next tested the effect of UR on mRNA expression and secretion of vascular endothelial growth factor, VEGF, synthesized by SaOS-2 cells at a later stage of culture. Treatment of the cells with UR at 0.1 ㎍/ml increased VEGF mRNA expression at 3, 7 and 14 days of culture (Fig. 3A). Significant increase of VEGF secretion were observed at 7 and 14 days of treatment with 0.1 ㎍/ml UR, secretion markedly increased at 7 days, subsequent slightly increased until the end of the culture (Fig. 3B). Also, UR at 0.1~100 ㎍/ml increased VEGF production at 7 days of culture in SaOS-2 cells (Fig. 3C).

4. Effect of UR on extracellular matrix proteins

Because expression of OCN, OPN, and Col I change during maturation and differentiation of osteoblast, we examined the effect of UR on their expression in SaOS-2 cells. Treatment of the cells with 0.1 ㎍/ml UR for 14 days of culture, Col I , OPN, and OCN mRNA expression



A



B

Fig. 2. Effects of *Uncaria rhynchophylla* on alkaline phosphatase in human osteoblast-like SaOS-2 cells. (A) Time course of alkaline phosphatase mRNA expression. Cells were treated with vehicle or UR at 0.01, 0.1, 1, 10, 100 ㎍/ml for the time periods indicated. Expression of ALP mRNA was determined RT-PCR analysis. (B) ALP activity was measured usingan ALP kit from whole cell extracts. Data are expressed as percentage of control. Results are shown as the mean ± SD of three experiments. **P < 0.05 and ***P< 0.001 compared with vehicle-treated control.

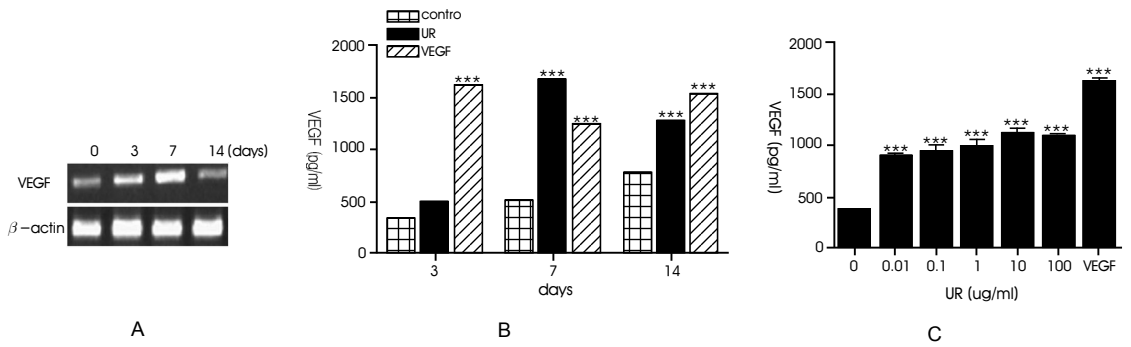


Fig. 3. Effects of *Uncaria rhynchophylla* on Vascular endothelial growth factor (VEGF) in SaOS-2 cells. (A) Time course of VEGF mRNA expression. Cells were treated with vehicle or UR at specified concentration for the time periods indicated. Expression of VEGF mRNA was determined RT-PCR analysis. (B) Time course of VEGF production. Cells were treated with vehicle and UR at specified concentration 0.1 ㎍/ml for the time periods indicated. (C) Dose response of VEGF production. Cells were treated with vehicle or UR treated with indicated concentration for 7 days culture. Results are shown as the mean ±SD of three experiments. ***P< 0.001 compared with vehicle-treated control.

was not affected by treatment with UR at 3 days, but markedly enhanced at 14 days of culture (Fig. 4A). Also, Col I and OCN production significantly increased at 14 days of culture by UR was compared to vehicle treatment cells (Fig. 4B). Col I and OCN protein accumulation significantly increased by 10 ㎍/ml UR at 14 days (Fig. 4C).

5. Effect of *Uncaria rhynchophylla* on mineralized nodule formation

We finally tested the effect of UR on osteoblast differentiation as evidenced by mineralization. Calcified tissue formation was clearly observed after 14 days of culture by UR at a dose dependent manner (Fig. 5).

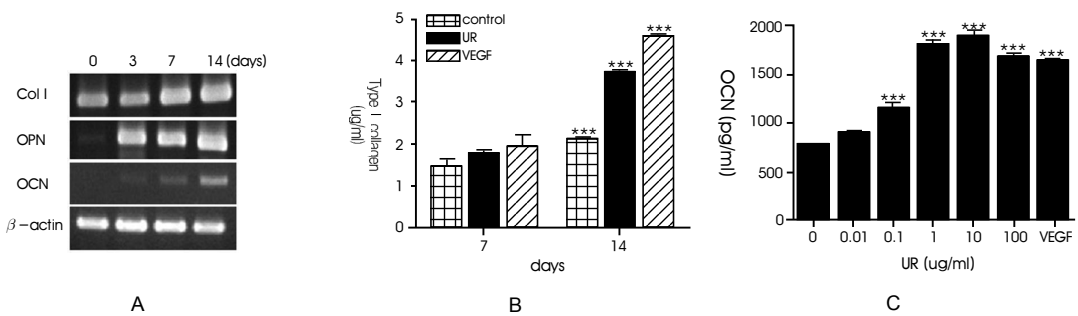


Fig. 4. Effects of *Uncaria rhynchophylla* on Col I, OPN, OCN mRNA expression and secretion in SaOS-2 cells. (A) Time course of mRNA expression. (B) Time course of Col I and OCN secretion. Cells were treated with vehicle and UR at specified concentration (0.1 ㎍/ml) for the time periods indicated. (C) Dose response of Col I, OCN production. Cells were treated with vehicle or UR treated with different concentration for 14 days culture. Results are shown as the mean ±SD of three experiments. *P< 0.05, **P< 0.01, and ***P< 0.001 compared with vehicle-treated control.

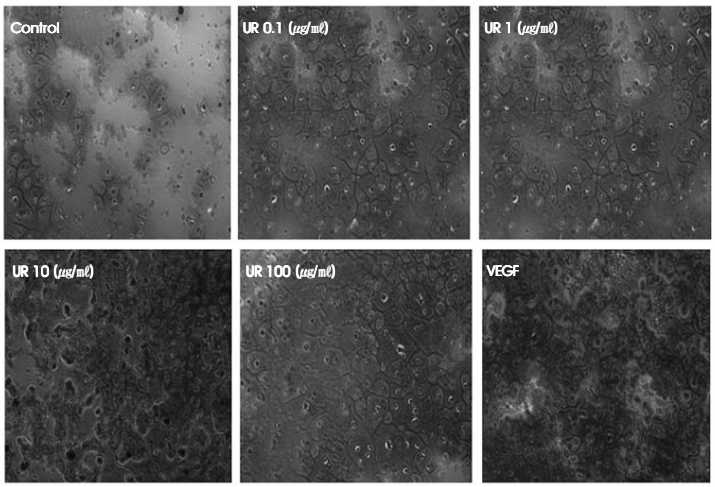


Fig. 5. Effects of *Uncaria rhynchophylla* on mineralization in SaOS-2 cells. Cells exposed vehicle (control), 0.1, 1, 10 100 ㎍/ml UR, and 50 ng/ml VEGF for 14 days. Cell layers stained with Alizarin red were destained, observed under the microscope, and photographed (magnification, ×200).

Discussion

We demonstrated that UR potently induced osteoblast differentiation markers such as ALP, VEGF, OCN, OPN, Col I, and mineralization in SaOS-2 cells. This is the first demonstration that UR regulates much of the tightly linked control between maturation and differentiation in SaOS-2 cells through increased synthesis and secretion of growth factor and matrix proteins, and ultimately stimulates mineralization.

Importantly, we determined whether UR extracts was cytotoxic, because many therapeutic agents have been shown to possess severe side effects. Thus, more effective agents with little toxicity and good solubility are required. UR had effect on the proliferation of SaOS-2 cells. This result indicates that UR extracts is nontoxic to osteoblastic cells, which suggests the possibility for reducing side effects (Fig. 1).

Up-regulation of ALP, an enzyme serving as a marker of osteoblast differentiation, occurs at the middle stage of differentiation[15,16]. UR significantly increased ALP activity in a dose-dependent manner. Therefore, UR stimulates osteoblastic activity at least in part by enhancing synthesis of ALP (Fig. 2). ALP may be one such gene activated. The upregulated differentiation of osteoblasts need not lead to the formation of bone, as these doses exert inhibitory effect on osteoblast proliferation which would eventually result in impaired bone matrix synthesis and ultimately in reduced bone mass and fracture. The data on ALP activity during 48 and 72 hrs exposure to varying doses of ethanol depict completely different picture[17].

During the past decade, investigation of VEGF has focused largely on the regulation of skeletal growth. Recently, VEGF was reported to be a more potent inducer of osteoblast differentiation on a molar basis than BMP-27.[16]. In another study, inactivation the VEGF gene was shown to inhibit endochondral bone formation via inhibition of angiogenesis[18]. Also,

it has been reported short-term treatment with statins stimulated gene expression and protein synthesis for VEGF in MC3T3-E1 cells[10]. We found that UR stimulated osteoblastic differentiation in osteoblast cell, and increased VEGF mRNA and protein secretion start at 3 days of culture, but VEGF secretion no more increase under condition of long-term cultureat 14 days (Fig. 3). Although production of VEGF per cell may be stored at high levels, most of VEGF protein may be broken down in the cells. Furthermore, long-term cultured osteoblasts in mineralized, or osteocytes, would not secrete of bioactive proteins such as VEGF, which were saturated in the microenvironment. Based on these reasons, we explained the results that VEGF accumulation in the medium not increased despite of increased expression of the mRNA by UR treated cell at 14 days (Fig 3). These finding suggest that enhanced VEGF production by osteoblasts is involved importantly in osteoblast differentiation and mineralization response to UR.

We next focused on determining theresponse of SaOS-2 cells to UR in terms of extracellular matrix protein expression. Col I and OCN is a later marker of osteoblast differentiation that is closely related to osteoblast maturation[7,15]. We demonstrated increased Col, OPN, OCN mRNA expression in response to UR at 7-14 days of SaOS-2 cell culture (Fig. 4A). In addition, OPN is a bone matrix protein secreted by osteoblasts, and regarded as the last in a chronologic sequence of markers of osteoblast differentiation. OPN expression is enhanced by hormones, cytokines, and regulates mineral growth *in vitro* and *in vivo*[19,20]. In our experiments, UR moderately increased OPN mRNA expression in a dose-dependent manner at 14 days of culture (Fig. 4A). Osteoblasts abundantly synthesize and secrete Col I and OCN, a major bone matrix constituent and extracellular macromolecule in osteoblast cultures. UR markedly increased Col I and OCN secretion at 7-14 days of SaOS-2 cell culture, and maximal increased at 10 μg/ml UR for 14 days (Figs. 4B, 4C). It is likely that VEGF stimulated by UR induces the matrix protein at a late stage of the culture.

Finally, we found that UR induced mineralized nodule formation at 14 days of SaOS-2 cell culture (Fig. 5). This resultsupports the hypothesis that UR promotes osteoblast differentiation *in vitro*, through increased synthesis and secretion of growth factor and matrix proteins.

In this study, we have investigated the effects of UR on osteoblast-like differentiation, and our findings show that UR has an effect on cell viability and regulates cellular differentiation in SaOS-2 cells. However, the mechanisms for gene expression are complex and the results of this study only begin to clarify these mechanisms. Thus, further investigation is required to isolate the active constituents and develop new therapeutics. These results suggest that UR can play an important role in osteoblastic bone formation through up-regulation of ALP activity, VEGF, OCN, OPN, Col I expression and mineralization, and possibly lead to the development of bone healing and osteogenesis drugs.

Acknowledgements

This study was supported by a grant of the Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of Korea (B030008)

References

1. McCarthy, T.L., Centrella, M., Canalis, E..

Regulatory effects of insulin-like growth factors I and on bone collagen synthesis in rat calvarial cultures. Endocrinology. 1989; 124:301-309.
2. Midy, V., Plouet, J.. Vasculotropin/vascular endothelial growth factor induces differentiation in cultured osteoblasts. Biochemical Biophysics Research Communication. 1994; 199:380-386.
3. Hughes, F.J., Collyer, J., Stanfield, M., Goodman, S.A.. The effects of bone morphogenic protein-2, -4, and 6 on differentiation of rat osteoblast cells *in vitro*. Endocrinology. 1995; 136:2671-2677.
4. Goad, D.L., Rubin, J., Wang, H., Tashjian, A.H., Jr., Patterson, C.. Enhanced expression ofvascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinology. 1996; 137:2262-2268.
5. Gerber, H.P., Vu, T.H., Ryan, A.M., Kowalski, J., Werb, Z., Ferrara, N.. VEGF couples hypertropic cartilage remodeling, and angiogenesis during endochondral bone formation. Nature Medicine. 1999; 5:623-628.
6. Spelsberg, T.C., Subramaniam, M., Riggs, B.L., Khosla, S.. The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. Molecular Endocrinology. 1999; 13:819-828.
7. Franceschi, R.T., and Iyer, B.S.. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. Journal of Bone Mineral Research. 1992; 7:235-246.
8. Lian, J.B., Stein, G.S., Boskey, A.L.. Bone formation: Maturation and functional activists osteoblast linease cells. In Favus, M.J., editor. Primer on the metabolicbone diseases and disorders of mineral metabolism. 5th edition Washington DC: The American Society for Bone and Mineral Research. 2003 13-28.
9. Maeda, T., Matsunuma, A., Kawane, T., Horiuchi, N.. Simvastatin promotes osteoblast differentiation and mineralization in MC3T3-E1 cells. Biochemical Biophysics Research Communication. 2001; 280:874-877.
10. Maeda, T., Kawane, T., Horiuchi, N. Statins augument vascular endothelial growth factor expression in osteoblastic cells via inhibition of protein prenylation. Endocrinology. 2003; 144:681-692.
11. Shao, B.M., Xu, W., Dai, H., Tu, P., Li, Z., Gao, X.M.. A study on the immune receptors for polysaccharides from the roots of Astragalus membranaceus, a chinese medicinal herb. Biochemical and Biophysical Research Communications. 2004; 320:1103-1111.
12. Zhu, X., Zhu, B.. Effect of AMI on proliferative cycle phase of bone marrow in mice. Zhong Yao Cai. 2000; 23:625-627.
13. Zhu, X., Zhu, B.. Effect of Astragalus membranaceus injection on megakeryo hematopoiesis in anemic mice. Hua Xi Yi Ke Da Xue Xue Bao. 2001; 32:590-592.
14. Kim, C., Ha, H., Lee, J.H., Kim, J.S., Song, K., Park, S.W.. Herbal extract prevents bone loss in ovariectomized rats. Archives of Pharmacology and Research. 2003; 26:917-924.
15. Aubin, j.E., Liu, F., Malaval, L., Gupta, A.K.. Osteoblast and chondtroblast differentiation. Bone. 1995; 17:77S-83S.
16. Deckers, M.M., Karperien,M., van der Bent, C., Yamashita, T., Papapoulos, S.E., Lowik, C.W.. Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. Endocrinology. 2000; 141:1667-1674.
17. Furumatsu, T., Shen, Z.N., Kawai, A., Nishida, K., Manabe, H., Oohashi, T., Inoue, H., Ninomiya, Y.. Vascular endothelial

growth factor principally acts as the main angiogenic factor in the early stage of human osteoblastogenesis. Journal of Biochemisty (Tokyo). 2003; 133:633-639.

18. Maes C, Carmeliet P, Moermans K, Stockmans I, Smets N, Collen D, Bouillon R, Carmeliet G.. Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Mechanism of Developement. 2003 111:61-73.
19. Hunter, G.K., Hauschka, P.V., Poole, A.R., Rosenberg, L.C., Goldberg, H.A.. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. Biochemical Journal. 1996; 317:59-64.
20. Klein, R.F., Fausti, K.A., Carlos, A.S., 1996. Ethanol inhibits human osteoblastic cell proliferation. Alcohol Clin. Exp. Res. 20, 572-578.