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원 저

Astragalus membranaceus promotes differentiation and mineralization in human osteoblast-like SaOS-2 cells

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Abstract

Background & Object : The differentiation of osteoblasts controlled by various growth factors and matrix proteins expression in bone. The aim of this study was to identify the Astragalus membranaceus that may induce the osteogenic activity in human osteoblast-like SaOS-2 cells.

Methods : The osteogenic activity of Astragalus membranaceus were evaluated by WST-8 assay, ALP activity, RT-PCR analysis of VEGF, OCN, OPN, Col I mRNA, and ELISA or colorimetric analysis, and mineralization by Alizarin red staining in SaOS-2 cells.

Results : Astragalus membranaceus had no effect on viability of osteoblastic cells, and dose dependently increased alkaline phosphatase (ALP) activity. Astragalus membranaceus markedly increased mRNA expression for vascular endothelial growth factor (VEGF), osteocalcin (OCN), osteopontin (OPN), and type I collagen (Col I) in SaOS-2 cells. Extracellular accumulation of proteins such as VEGF, and Col I was increased in a dose-dependent manner. Also, Astragalus membranaceus significantly induced mineralization in the culture of SaOS-2 cells.

Conclusion : This study showed that Astragalus membranaceus not affect on viability, but it enhanced ALP activity, VEGF, bone matrix proteins such as OCN, OPN and Col I, and mineralization in SaOS-2 cells. These results propose that Astragalus membranaceus plays an important role in osteoblastic bone formation, and possibly lead to the development of bone-forming drug.

Key words : Astragalus membranaceus ALP activity, VEGF OCN, OPN, Col I, Mineralization

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Ⅰ. Introduction

Bone modeling and remodelingare 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 (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 withdeposition 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].

The roots of Astragalus membranaceus (Huangqi) are among the most popular health-promoting herbs in oriental medicine. In traditional folk medicine, Astragalus membranaceus has been shown to have effects on general health, ischemic heart disease, urination, and as an antinephritis agent. but these functions have not been scientifically tested and their mechanisms are not known. Recently, it was reported that the polysaccharide fraction from Astragalus membranaceus enhanced immune function through activation of B cells and macrophages in mice[11]. Also, Astragalus membranaceus enhance hematopoietic function in mice[12-13]. In addition, it has been demonstrated that Astragalus membranaceus, alone and in combination, prevents bone loss in ovariectomized rats[14]. However, much more insight into the pharmacological functions and mechanisms of Astragalus membranaceus are needed, especially as there is no clear experimental evidence, that Astragalus membranaceus for treatment of bone fracture. Therefore, this study was performed to clarify the roles of Astragalus membranaceus on viability, differentiation, expression of VEGF and bone matrix proteins, and mineralization of human osteoblast-like SaOS-2 cells.

Ⅱ. Materials and methods

1. Preparation of Astragalus membranaceus extract

The root of Astragalus membranaceus was extracted at room temperature in 70% (v/v) ethanol-water for 24 h. The extract was then filtered and concentrated under low pressure using a vacuum rotary evaporator (Eyela, Japan). The remaining residue was lyophilized in a freezing-dryer, and stored at 20℃. The powder was dissolved in dimethyl sulfoxide (DMSO) was used for experiment 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 cytotoxicity

The viability of Astragalus membranaceus 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 x 104cells/well. After 24 h incubation, the cells were exposed to various concentrations of Astragalus membranaceus in a volume of 100 ㎕. After 72 h incubation, 10 ㎕ 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 a percentage of viable cells in Astragalus membranaceus treated group relative to 0.5% DMSO treated group.

4. Assay of ALP activity

Cells were treated with various concentration of Astragalus membranaceus for 72 h. Cells were lysed with 0.1% Triton X-100, sonicated, and then centrifuged at 12,000 g for 10 min at 4 ℃. ALP activity was assayed with a commercial kit (Sigma-Aldrich Co., MO, USA). Supernatants were incubated with reaction buffer for 3 min at 37 ℃, 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′-GGGAGTTTCCATG AAGCCAC-3′ OCN: forward, 5′-CATGAGAGCCCTCACA-3′ reverse, 5′-AGAGCGACA C CCTAGAC-3′ Col I: forward, 5′-TGACCTCAAGATGTGCCACT-3′ reverse, 5′-GGGAGTT TCCATGAAGCCAC-3′ and b-actin: forwad, 5′-CCATCATGAAGT GTGACGTG-3′ reverse, 5′-ACATCTGCTGGAA GGTGGAC-3′. An equal volume from each PCR was analyzed 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 and Type I collagen

SaOS-2 cells were treated with Astragalus membranaceus for the period indicated.

Conditioned media prepared from 3 days culture to 14 days, and then stored at 70 ℃for the immunoassay of VEGF. The VEGF was measured by using a commercially available enzyme linked immunosorbant assay (ELISA) kit (R&D Systems Inc., MN, USA). 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. The percentage of recovery was calculated from the peak height of the sample and of the control standard.

7. Assay of mineralized matrix formation

SaOS-2 cells were cultured in 24 well plate with medium containing 1% FBS, 1% penicillin-streptomycin, 50 ㎍/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 quantified by counting with an optical microscope (200X magnification).

8. Statistical Analysis

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

III. Results

1. Effect of Astragalus membranaceus on viability and ALP activity

To investigate the cytotoxic effect of Astragalus membranaceus in osteoblast-like SaOS-2 cells was first examined. A range of 0-100 ㎍/ml Astragalus membranaceus was applied to the SaOS-2 cells. Astragalus membranaceus no cytotoxic effect of SaOS-2 cells for 3 days in a dose-dependent manner (Fig. 1). To ascertain whether Astragalus membranaceus is capable of affecting osteoblastic cell differentiation, we examined the changes in ALP activity. Astragalus membranaceus dose-dependently increased ALP activity for 3 days in osteoblasts, and maximal effect was reached when cells were incubated with 10 ㎍/ml Astragalus membranaceus (Fig. 1).

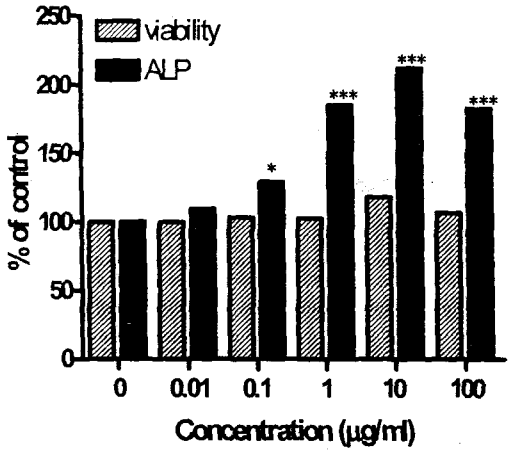

Fig. 1. Effects of Astragalus membranaceus on viability in huama osteoblast-like SaOS-2 cells. Astragalus membranaceus at 0.01, 0.1, 1, 10, 100 ㎍/ml were added to SaOS-2 cells for 3 days. Cell viability was determined by a colorimetric WST-8 assay. ALP activity was measured by using an ALP kit from whole 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.

2. Effect of Astragalus membranaceus on VEGF expression

We next tested the effect of Astragalus

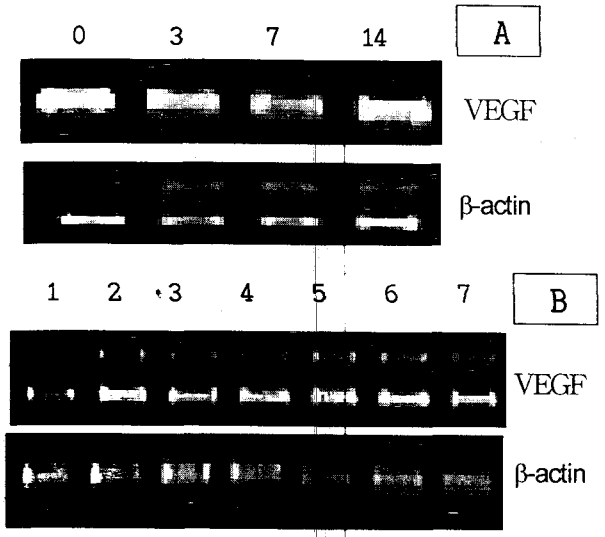

Fig. 2. Effects of Astragalus membranaceus on VEGF mRNA expression in SaOS-2 cells. (A) Time course of VEGF mRNA expression. Cells were treated with vehicle or Astragalus membranaceus at specified concentration for the time periods indicated. Expression of VEGF mRNA was determined RT-PCR analysis. (B) Dose response of VEGF mRNA expression. Cells were treated with vehicle or Astragalus membranaceus (Am) treated with different concentration for 7 days culture.
1; control, 2; 0.01μg/ml Am, 3; 0.1 μg/ml Am, 4; 1 μg /ml Am, 5; 10 μg/ml Am, 6, 100 μg/ml Am, 7; 50 ng/ml VEGF. Each value is expressed as relative level of target gene to b-actin.

membranaceus on expression of growth factor, VEGF, synthesized by SaOS-2 cells at a later stage of culture. Treatment of the cells with Astragalus membranaceus at 1 μg/ml increased VEGF mRNA synthesis at 3, 7 and 14 days of culture (Fig. 2A). Also, Astragalus membranaceus dose-dependently increased VEGF mRNA synthesis at 7 days of culture (Fig. 2B). Significant increase of VEGF secretion were observed at 3, 7 and 14 days of treatment with 1 μg/ml Astragalus membranaceus, secretion markedly increased at 7 days, subsequent slightly increased until the end of the culture (Fig. 3A). Also, Astragalus membranaceus dose-dependently increased VEGF secretion at 7 days of culture in SaOS-2 cells (Fig. 3B).

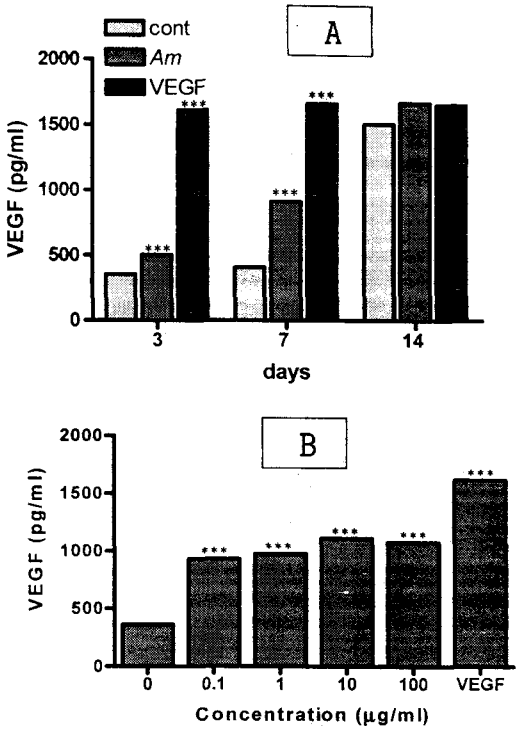

Fig. 3. Effects of Astragalus membranaceus on VEGF protein secretion in SaOS-2 cells. (A) Time course of VEGF protein secretion. Cells were treated with vehicle or Astragalus membranaceus at specified concentration for the time periods indicated. Conditioned media for indicated period were collected, and VEGF concentrations were measured by human VEGF assay kit. (B) Dose response of VEGF protein secretion. Results are shown as the mean ±SD of three experiments.
*P < 0.05 and ***P < 0.001 compared with vehicle-treated control.

3. Effect of Astragalus membranaceus on extracellular matrix proteins expression

Because expression of OCN, OPN, and Col I change during maturation and differentiation of osteoblast, we examined the effect of Astragalus membranaceus on their expression in SaOS-2 cells. Treatment of the cells with 1 μg/ml Astragalus membranaceus for14 days of culture, OCN, OPN, and Col I mRNA was not affected by treatment with Astragalus

membranaceus at 3 and 7 days, but markedly enhanced at 14 days of culture (Fig. 4A). Also, OCN, OPN, and Col I mRNA expression dose-dependently increased at 14 days of culture by Astragalus membranaceus as compared to vehicle treatment cells (Fig. 4B). Col I protein accumulation rarely increased at 7 days, but markedly increased by Astragalus membranaceus at 14 days in a dose-dependent manner (Fig. 5).

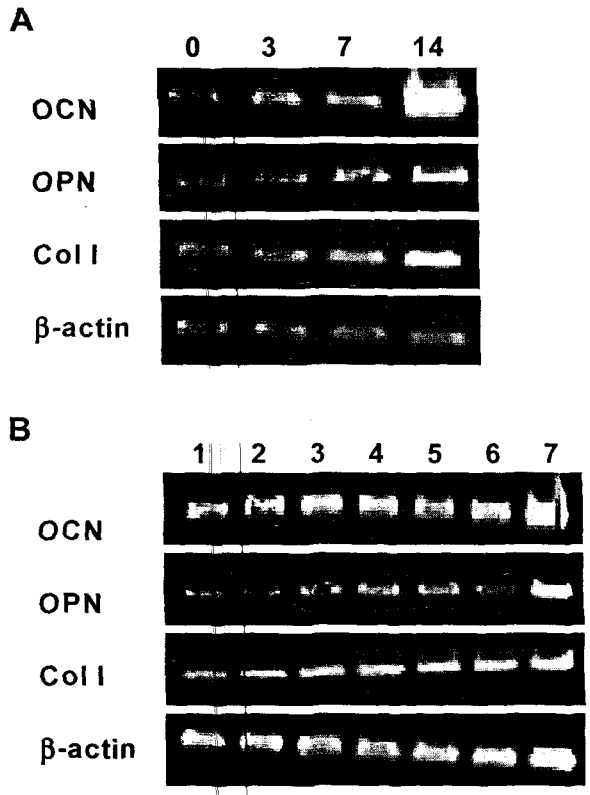

Fig. 4. Effects of Astragalus membranaceus on the expression of OCN, OPN, and Col I mRNA in SaOS-2 cells. (A) Time course of OCN, OPN, and Col I mRNA expression. Cells were treated with vehicle or Astragalus membranaceus at specified concentration for the time periods indicated. (B) Dose response of OCN, OPN, and Col I mRNA expression. Cells were treated with vehicle or Astragalus membranaceus at different concentration for 14 days culture.

1; control, 2; 0.01μg/ml Am, 3; 0.1 μg/ml Am, 4; 1 μg /ml Am, 5; 10 μg/ml Am, 6, 100 μg/ml Am, 7; 50 ng/ml VEGF. Each value is expressed as relative level of target gene to b-actin.

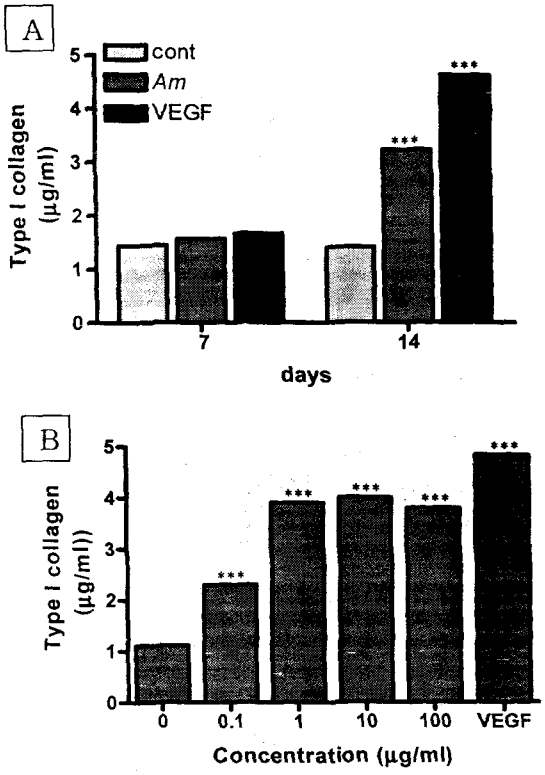

Fig. 5. Effects of Astragalus membranaceus on type I collagen protein secretion in SaOS-2 cells. (A) Time course of type I collagen protein secretion. Cells were treated with vehicle or Astragalus membranaceus at specified concentration for the time periods indicated. Conditioned media for indicated period were collected, and type I collagen were measured by colorimetric assay kit. (B) Dose response of type I collagen protein secretion. Results are shown as the mean ±SD of three experiments.

***P < 0.001 compared with vehicle-treated control.

4. Effect of Astragalus membranaceus on mineralized nodle formation

We finally tested the effect of Astragalus membranaceus on osteoblast differentiation as evidenced by mineralization. Calcified tissue formation was clearly observed after 14 days of culture by Astragalus membranaceus (Fig. 6A), respectively, and the amount of mineralization was increased in a dose dependent manner (Fig. 6B).

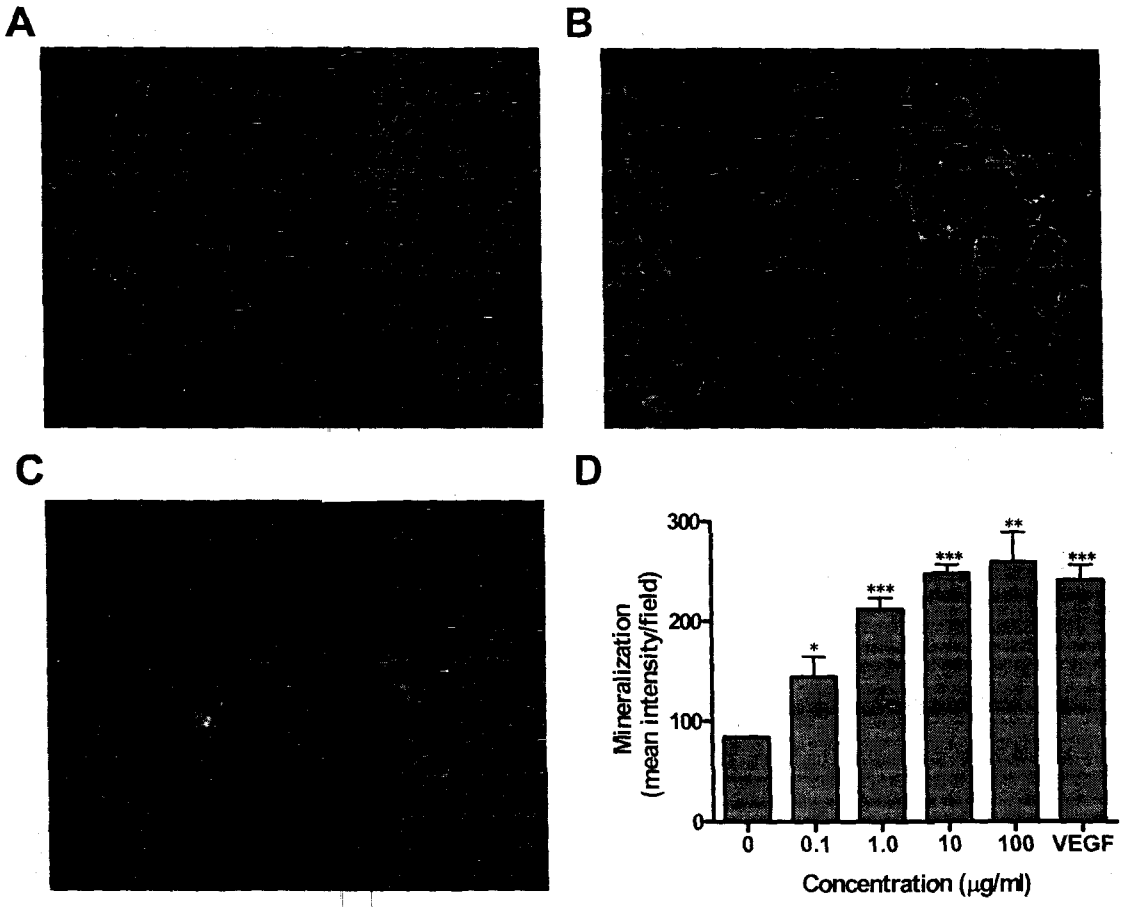

Fig. 6. Effects of Astragalus membranaceus on mineralization in SaOS-2 cells. Cell layers stained with Alizarin red were destained, observed under the microscope, and photographed (magnification, 200). A; control, B; 1 μg/ml Astragalus membranaceus, C; 50 ng/ml VEGF, D; Mineralization was quantified field in at least threefields after each experiment, and result was expressed as the mean intensity.
*P < 0.05, **P < 0.05, and ***P< 0.0001 compared with vehicle-treated control.

Ⅳ. Discussion

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

The present study, we determined whether Astragalus membranaceus extract has cytotoxicity, because many therapeutic agents have been side effect. Thus more effective agents with little toxicity and good solubility are required. Astragalus membranaceus not affect on viability of SaOS-2 cells This result indicate that Astragalus membranaceus is non-toxic on osteoblastic cells, which suggests the possibility of reducing side effects (Fig. 1). Up-regulation of ALP, an enzymeserving as a

marker of osteoblast differentiation, occurs at the middle stage of differentiation[15]. Astragalus membranaceus significantly increased ALP activity at a dose dependent manner. Therefore, Astragalus membranaceus stimulates osteoblastic activity at least in part by enhancing synthesis of ALP (Fig. 1).

During the past decade, investigation of VEGF has focused largely on regulation of skeletal growth. Recently reported that VEGF was a much more potent inducer of osteoblast differentiation on amolar basis than BMP-2 [2,16-17]. 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 Astragalus membranaceus 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 (Figs. 2 and[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 Puerariae radix treated cell at 14 days (Figs. 2 and 3). These finding suggest that enhanced VEGF production by osteoblasts is involved importantly in osteoblast differentiation and mineralization response to Astragalus membranaceus.

We next focused on determining response of SaOS-2 cells to Astragalus membranaceus in terms of extracellular matrix protein expression. OCN is a later marker of osteoblast differentiation that is related closely to osteoblast maturation[7,15]. We demonstrated that increased OCN mRNA expression in response to Astragalus membranaceus at 14 days of SaOS-2 cell culture (Fig. 4). Also, OPN is bone matrix protein secreted by osteoblasts, and regarded as the last in a chronologic sequence of markers of osteoblast differentiation. Expression of OPN is enhanced by hormones, cytokines, and regulates mineral growth in vitro and in vivo[19]. In our results, Astragalus membranaceus moderately increased OPN mRNA expression in a dose-dependent manner at 14 days culture (Fig. 4). Osteoblasts abundantlysynthesize and secrete Col I, a major bone matrix constituent and extracellular macromolecule in osteoblast cultures. Astragalus membranaceus markedly increased Col I mRMA expression, and proteins at 14 days of SaOS-2 cell culture (Figs. 4 and 5). It is likely that VEGF stimulated by Astragalus membranaceus induces the matrix protein at late stage of the culture.

Finally, we found that Astragalus membranaceus showed mineralization nodule formation at 14 days of SaOS-2 cell culture (Fig. 6). This result is supported that Astragalus membranaceus promotes osteoblastdifferentiation in vitro, through increase synthesis and secretion of growth factor and matrix proteins.

V. Conclusion

In this study, we have investigated the effects of Astragalus membranaceus on osteoblast-like differentiation, and findings show that Astragalus membranaceus may not

affect cell viability and regulates of cellular differentiation in SaOS-2 cells. However, the mechanisms for the gene expression are complex and these results of this study only begin to clarify mechanisms. Thus, further investigation is requiredfor the isolated active constituents to be developed as new therapeutics. These results suggest that Astragalus membranaceus playsan important role in osteoblastic bone formation through up-regulation of ALP activity, VEGF, OCN, OPN, Col I expression and mineralization, and possibly lead to development of bone healing and osteoporesis drug.

VI. Acknowledgement

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