원저

Effects of *Ulmus davidiana Planch* (Ulmaceae) on mineralization, bone morphogenetic protein-2, alkaline phosphatase, type I collagen and collagenase-1 in bone cells

Byun You-seok, Yoon Jong-hwa, Hwang Min-seob, Kim Kap-sung and Jo Hyun-seog

Department of Acupuncture & Moxibustion, Graduate School of Oriental Medicine, Dongguk University

Abstract

유근피가 골세포의 mineralization, bone morphogenetic protein-2, alkaline phosphatase, type I collagen 및 collagenase-1에 미치는 영향

변유석 · 윤종화 · 황민섭 · 김갑성 · 조현석

동국대학교 한의과대학 침구학교실

손상된 조직의 보호와 항염증작용이 있는 것으로 알려진 유근피를 수액추출하여 골다공증의 치료에 응용 가능한가를 실험하였다. 이전의 실험에서 유근피 추출액이 파골세포를 함유한 장골세포의 치료를 통해 Cathepsin K를 억제하는 것을 확인 하였었다.이를 통하여 유근피는 골다공증 치료에서 골재흡수억제제로서의 prodrug의 역할을 할 수 있음을 시사하였다. 본 실험에서는 MC3T3-E1 pre-osteoblastic 세포조직을 이용하여 골화를 유발한 상태에서 유근피가 골아세포의 성장과 감작에 대한 것을 in vitro로 연구하였다. 이 결과 유근피는 용량과 시간의존적으로 ALP의 활동을 향상시킴으로써 강화작용이 있음을확인할 수 있었다. 투여량은 최소치 50㎍/㎖에서 최대치 150㎍/㎖에서 관찰되었다. 100㎍/㎖ UD에서 bone morphogenetic protein-2의 향상을 관찰할 수 있었으며, MC3T3-E1 세포내의 ALP mRNA농도역시 증가하였다. 60㎍/㎖ UD에서 Type I collagen mRNA에 대해서 오랜 배양 기간 동안 약간의 증가를 나타내었으나 15-20일 사이의 배양에서는 급격히 유전자 발현을 억제하는 것으로 나타났다. 이러한 결과는 골아세포의 감작을 통해 유근피가 골대사에 영향을 미침을 시사하는 것이다. 그러므로 추후 연구를 통하여 일반적인 골대사질환에 유근피를 적극적으로 활용할 수 있음을 알 수 있었다.

Key words : Effects of Ulmus davidiana Planch (Ulmaceae) on mineralization, bone morphogenetic protein-2, alkaline phosphatase, type I collagen and collagenase-1 in bone cells

· **접수** : 2005년 1월 13일 · **수정** : 2005년 5월 14일 · **채택** : 2005년 5월 14일
· **교신저자** : 조현석, 경기도 성남시 분당구 수내동 87-2 동국대학교 분당한방병원
Tel. 031-710-3746 E-mail : youclickme@naver.com

Ⅰ. Introduction

The MC3T3-E1 pre-osteoblastic cell line is a well-accepted model of osteogenesis in vitro[1]. Growth and differentiation factors contained in the culture medium stimulate these cells to undergo a developmental sequence that includes proliferation of undifferentiated precursors of osteoblasts which subsequently differentiate into post-mitotic osteoblasts capable of expressing the osteogenic phenotype. During the proliferative phase, these cells undergo DNA synthesis and cell division resulting in a rapid increase in cell number until the cultures become confluent. At this juncture, proliferation is downregulated and increased expression of the osteogenic phenotype is observed indicating presence of mature osteoblasts. Osteoblasts produce alkaline phosphatase, process procollagen to collagen, and deposit extracellular matrix containing additional proteins (e.g. osteopontin, bone sialoprotein and osteocalsin) on the substrate, which is subsequently mineralized.

Bone forming osteoblasts arise from mesenchymal stem cell precursors and undergo differentiation in response to a number of factors including the bone morphogenetic protein (BMPs), transforming growth factor(TGF), and glucocorticoids[2]. In addition, several different molecules are associated with the development and maintenance of mineralized skeletal elements. Once matrix synthesis begins in osteoblast culture models such as primary osteoblast cultures and murine nontransformed osteoblast-like cell (MC3T3-E1), the cells differentiate in accordance with gene activation of osteoblast markers such as alkaline phosphatase (ALP), type I collagen and collagenase-1 (MMP-1). Thus, osteoblasts become embedded in extracellular matrix, mainly in collagen fibrils, and then matrix mineralization involves mineral growth on and within collagen fibrils [3-4] .

Since a large decrease in bone mass occurs in the postmenopause state, women are vulnerable to the osteoporosis known as postmenopausal osteoporosis.[5] Several medications have been reported to be effective for curing osteoporosis based upon the results obtained using these animal models from non steroidal sources, such as bisphosphonates[6], calcitonin[7], calcium products[8] and ipriflavone [9]. Traditional medicines have been reevaluated by clinicians[10], Since traditional medicines have fewer side effects and because they are more suitable for long-term use as compared to chemically synthesized medicines, these re-evaluated by clinicians. The need for safer and effective anti-inflammatory drug and the lack of scientific data to support the claims made in ancient literature prompted the present study. Because natural products of plant origin are still a major part of traditional medicinal systems, there is also a resurgence of interest in traditional Chinese medicines in Western nations as an alternative source of UDugs often for intractable diseases such as rheumatoid arthritis[10].

Ulmus davidiana Planch (Ulmaceae) is a deciduous tree, which is widely distributed in Korea. The barks of the stem and the root of this plant have been used in oriental traditional medicine for the treatment of oedema, mastitis, gastric cancer, and inflammation[11-12]. As a part of search for new biologically active substances from traditional medicines, we evaluated whether extracts of U. davidiana stem barks (UD) could modulate the induction of rheumatoid arthritis(RA) in mice. UD water extract has been developed on the basis of known functions of the herb, as described in

the literature of traditional Chinese and Korean medicine[13-14]. UD is known for their functions in maintaining or assisting blood circulation. UD has been used for protection against degeneration of cartilage and regeneration of damaged tissue[15]. UD has long been used by Korean traditional medicine physicians in Korea. However, little is known about the mode of action of this traditional medication on bone metabolism. UD is effective for the treatment of inflammation, hyperlipemia, arteriosclerosis, and gynecological diseases such as osteoporosis and bone resorption according to the traditional Chinese medicinal literature[16]. To treat the osteoporosis, a herbal formula containing UD is used in traditional Chinese prescription[16]. But survey about UD revealed that there is still need for usefulness much scientific evidence to prove its in the treatment of RA and osteoporosis. Thus, in order to evaluate the effectiveness of traditional Chinese medicines on osteoporosis, we examined whether UD could prevent the progression of bone loss induced by ovariectomy in rats[17]. In the previous study[18], it was described that UD extracts were shown to be potent inhibitors of the degradation of denatured collagen by cathepsin K and of bone resorption in an in vitro model. Treatment of the UD extracts to cat K strongly inhibited cat K activity and bone resorption activity, suggesting that cat K is involved in the osteoporosis pathway. The naturally occuring UD which contains phenolic compounds, possesses estrogenic activity.

A sharp decrease in ovarian estrogen production is the predominant cause of the rapid hormone-related bone loss during the first decade after menopause. Osteoporosis associated with estrogen deficiency after menopause is the most common cause of age-related bone loss[19]. Traditional therapeutic agents for post-menopausal osteoporosis has been estrogen, calcitonin and bisphosphonate which inhibit bone resorption. Estrogen replacement therapy, recommended only for women who are at high risk of osteoporosis and without any contraindications, seems to be the most effective method to reduce the rate of postmenopausal bone loss, although some side-effects may be accompanied[20]. Attempts have been made to use a combination of anti-resorptive agents, such as estrogen and bone formation-stimulating agents, such as growth hormone, to treat osteoporosis[21]. However, the potential bone-forming agents available either may have serious side-effects, may not improve bone quality, or may not reduce the susceptibility to fracture. Thus, it would be most helpful to discover a natural dietary substance that minimizes bone loss in postmenopausal women. The phytoestrogens, therefore, are potentially important in the prevention of postmenopausal osteoporosis caused by estrogen deficiency.

In this study, we investigated whether UD regulates the differentiation and function of osteoblasts using nontransformed osteoblasts (MC3T3-E1) and rat bone marrow cells. The results suggest that UD enhance bone formation through the induction of BMP-2 and ALP and by the accumulation of bone matrix proteins such as type I collagen.

Ⅱ. Materials and methods

1. Materials

1) Plant material

UD were massproduced for clinical use & were kindly supplied by Oriental Medical

Hospital of Dongguk University (Kyungju, Korea). For new substance, the stem barks of U. davidiana were collected from Korea in May 2002, and identified by Professor Kap-Sung Kim, College of Oriental Medicine, Dongguk University, Korea. Fresh stems were dried in a dark, well ventilated place. The voucher specimen (No. UD-W-57) is deposited in the Herbarium of the Dongguk University.

2. Methods

1) Preparation of herb extract and fractions

UD was purchased from a market specializing in herbs (Kyungju herb market, Kyungju, Korea). The herb had moisture content of <10% by weight, and was air-dried. Air-dried barks (totalling 70 g dry weight) were mixed, minced with a grinder, and extracted by storing in 1 litre of boiling water for 3 hours. The supernatant was filtered with 10 μm cartridge paper and ethanol was removed by rotary evaporation (Eyela, Tokyo, Japan), and concentrated extracts were freeze-dried. This process generally produced 15 g of brown powder. A voucher specimen has been deposited at the Kyungju Oriental Medical Hospital, Dongguk University, Kyungju, Korea under acquisition number UD-13.

2) Cell culture

MC3T3-E1 cells, a clonal pre-osteoblasttic cell line derived from newborn mouse calvaria, were grown in (MEM (ICN Pharmaceuticals, Inc., Aurora, OH) with 10% fetal calf serum (FCS). After the cells reached confluence, they were cultured in differentiation medium [(MEM containing 50 (g/ml of the phosphate ester of ascorbic acid (Wako Pure Chemical Co., Osaka, Japan) and 10 mM (-glycerophosphate (Sigma Chemical Co., St. Louis, MO)] for 2 to 25 days in the absence or presence of various doses of UD dissolved in distilled water.

Mouse bone marrow cells were prepared according to the method of Maniatopoulos et al.[22]. Femurs were aseptically removed from 6-week-old male Sprague-Dawley mice (KCTC, Korea) after they were killed under ether anesthesia. The bone marrow cavity was flushed out with (MEM medium supplemented with 10% FCS. After 4 days in primary culture, the bone marrow stromal cells were subcultured in standard medium ((MEM containing 0.2 mM phosphate ester of ascorbic acid, 1 mM (-glycerophosphate, 10% FCS, 10-8 M dexamethasone, and ethanol vehicle or UD).

3) Northern blot analysis

Total RNA was extracted and Northern blot analysis was performed as described[23]. Probes such as BMP-2 and ALP were prepared by RT-PCR. The message intensity was assayed using a Molecular Imager FX (BioRad, Hercules, CA), The mRNA abundance was corrected for the cyclophilin mRNA concentrations.

4) Measurement of ALP activity and mineralization

The cultures in 24-well plates were rinsed with PBS. The cells were sonicated in 0.1 M Tris buffer (pH 7.2) containing 0.1% Triton-X-100. The ALP activity was measured with an (ALP) B-test kit (Wako Chemical Co.). The culture was briefly rinsed with PBS followed by fixation (ice-cold 70% ethanol, 1 h) and then stained for 10 min with 40 mM Alizarin red-S (AR-S) (pH 4.2). AR-S staining was released from the cell matrix by incubation in 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0), for 15 min.

The AR-S concentration was determined by measuring the absorbance at 562 nm[24].

5) Statistical analysis

All values were expressed at the mean (SEM of 4 or 5 measurements. Statistical analysis was performed using ANOVA followed by Fisher's protected least significant differences (Stat View 4.02, Abacus Concepts, Inc., Berkeley, CA). A $P<0.05$ value was considered significant (*$P<0.05$ and ** $P<0.01$ on the figure)

Ⅲ. Results

1. Stimulative effect on ALP activity of UD in MC3T3-E1 cells.

We examined the effects of UD on ALP activity and mineralization by MC3T3-E1 cells. The ALP activity increased during differentiation, reaching a maximum at day 10 and maintaining the plateau level by day 25.

UD increased the enzyme activity between 10 and 20 days in culture in a dose dependent manner. The increase in the activity was significant between UD concentration of 5 - 50 (g/ml and maxima at 20 (g/ml between 10 and 20 days in culture (Fig. 1).

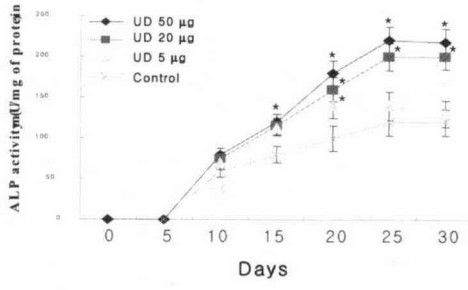

Fig. 1. Stimulative effect on ALP activity of UD in MC3T3-E1 cells

Cells in 24-well plates were treated with vehicle or graded doses (10 - 150 (g/ml) of the UD for the indicated time period. Each pont represents the mean (SEM of five determinations. *Significantly different from vehicle control ($p < 0.01$).

2. Effect of UD on the mineralizatio of extracellular matrix by MC3T3-E1 cells

Mineralization by mineralization by MC3T3-E1 cells occured in a time-dependent manner after 15 days in culture. In addition, UD stimulated the mineralization by the cells between 15 and 25 days in culture in a dose-dependent manner. Maximal and significant effects were observed at a concentration of 20 and 10 (g/ml UD, respectively (Fig. 2).

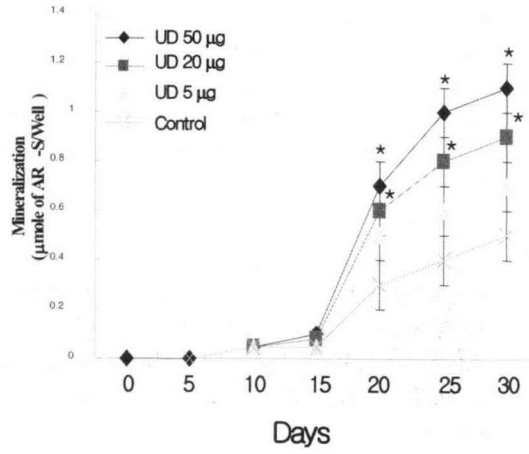

Fig. 2. Effect of UD on the mineralization of extracellular matrix by MC3T3-E1 cells

Cells were treated with vehicle or the UD for the indicated time period. AR-S staining was performed for the demonstration of mineralized nodule formation at days 15 and 20. AR-S was then eluted from the matrix and measured by spectrophotometry at 562 nm. The data are expressed as the mean (SEM of five determinations. *Significantly different from vehicle control ($p < 0.01$).

3. Effect of UD on the mineralization of extracellular matrix by rat bone marrow cells

We confirmed the promoting effects of UD on mineralization using primary cultures of rat

bone marrow stromal cells (Fig. 3). The addition of 10 (g/ml UD significantly stimulated the mineralization between 5 and 10 days in culture. Maximal effect was obtained at 20 (g/ml UD.

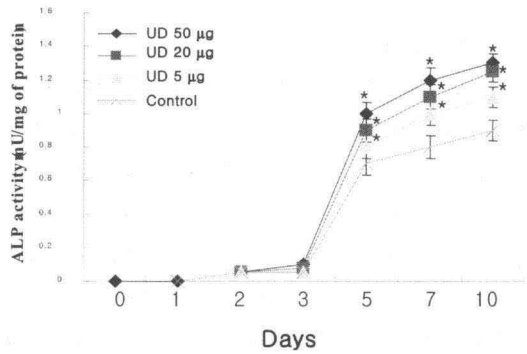

Fig. 3. Effect of UD on the mineralization of extracellular matrix by rat bone marrow

cells Cells were treated with vehicle or the UD (10 - 150 (g/ml) for the indicated time period. Mineralization was determined by AR-S. The data are expressed as the mean (SEM of five determinations. *Significantly different from vehicle control a each time point ($p < 0.01$).

4. Effects of UD on the gene expression of osteoblastic differentiation makers such as BMP-2, ALP, type I collagen and collagenase-1 in MC3T3-E1 cells.

To determine a mechanism underlying promotion of mineralization by UD, the gene expression of markers of osteoblastic differentiation was studied using MS3T3-E1 cells in absence or presence of the UD. Northern blot analysis showed that 20 (g/ml UD increased BMP-2 and ALP mRNA expression at day 10 and 15 in culture. A reduction in collagenase-1 mRNA in response to the UD was observed at culture day 15 and 20 in MC3T3-E1 cells, whereas the abundance of Collagenase-I mRNA did not change (data not shown).

The changes in the mRNA abundance of these with regard to the culture period were quantitatively monitored in the absence and presence (20 (g/ml) of UD. There was a significant increase in BMP-2 mRNA early during the culture period (day 10 and 15), and the ALP mRNA increased during 10 to 20 days in culture (Fig. 4 and 5). We also assessed the involvement of UD on collagen accumulation in the extracellular matrix. The expression of type I collagen mRNA expression did not change throughout the culture period but MC3T3-E1 cells was markedly increased the accumulation of collagenase-1 mRNA transcript in long-term culture (15 top 20 days), as shown in Fig. 6 and 7. UD slightly increased type I collagen mRNA early during culture (2 to 10 days), whereas collagenase-1 mRNA expression was markedly inhibited by the addition of 50 (g/ml UD during 15 and 20 days in culture (Fig. 6 and 7).

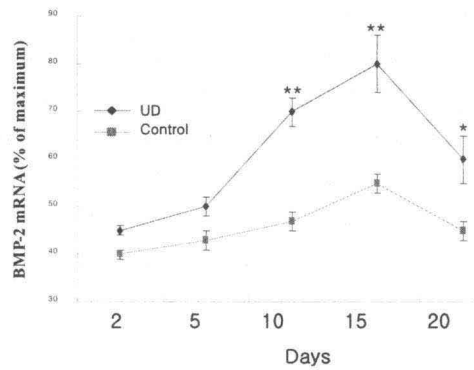

Fig. 4. Effects of UD on the gene expression of osteoblastic differentiation makers BMP-2 in MC3T3-E1 cells.

Cells were cultured for the indicated time period in the absence (O) and presence (●) of UD (100 (g/ml). Northern blot analysis was performed. The mRNA concentrations were determined densitometorically and normalized to that of β-actin mRNA. The data are expressed as the mean (SEM of four determinations. * $P<0.05$; ** $P<0.01$ Significantly different from vehicle control a each time point.

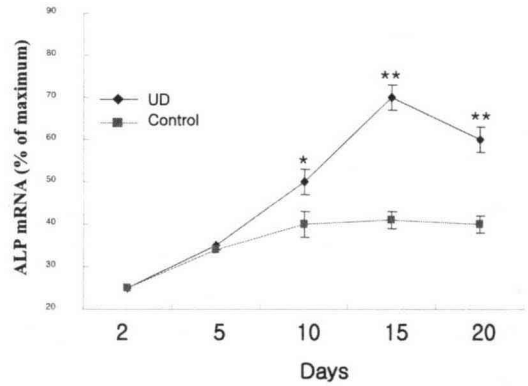

Fig. 5. Effects of UD on the gene expression of osteoblastic differentiation makers ALP in MC3T3-E1 cells.

Cells were cultured for the indicated time period in the absence (O) and presence (●) of UD (100 (g/ml). Northern blot analysis was performed. The mRNA concentrations were determined densitometorically and normalized to that of β-actin mRNA. The data are expressed as the mean (SEM of four determinations. * P<0.05; ** P<0.01 Significantly different from vehicle control a each time point.

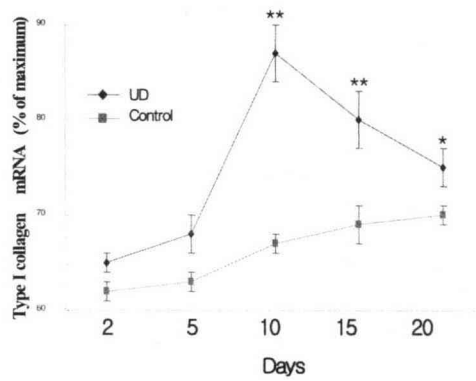

Fig. 6. Effects of UD on the gene expression of osteoblastic differentiation makers type I collagen RNA in MC3T3-E1 cells.

Cells were cultured for the indicated time period in the absence (O) and presence (●) of UD (100 (g/ml). Northern blot analysis was performed. The mRNA concentrations were determined densitometorically and normalized to that of β-actin mRNA. The data are expressed as the mean (SEM of four determinations. * P<0.05; ** P<0.01 Significantly different from vehicle control a each time point. A) BMP-2 mRNA. B) ALP mRNA. C) Type I collagen mRNA. D) Collagense-1 mRNA

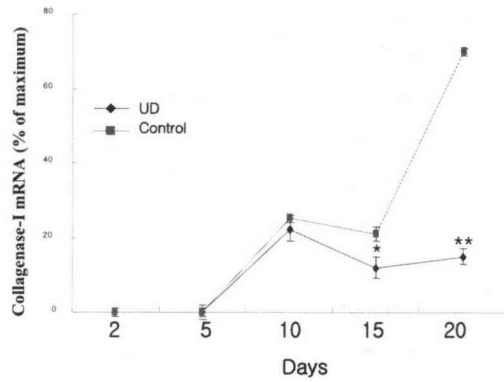

Fig. 7. Effects of UD on the gene expression of osteoblastic differentiation makers collagenase-1 mRNA in MC3T3-E1 cells.

Cells were cultured for the indicated time period in the absence (O) and presence (●) of UD (100 (g/ml). Northern blot analysis was performed. The mRNA concentrations were determined densitometorically and normalized to that of β-actin mRNA. The data are expressed as the mean (SEM of four determinations. * P<0.05; ** P<0.01 Significantly different from vehicle control a each time point.

Ⅳ. Discussion

It was reported that cat K is a cysteine protease abundantly and selectively expressed in human osteoclasts, and is thought to have an integral role in bone resorption. However, little is known on mechanisms how does the osteoclasts deliver to catalytically active enzyme to bone resorption sites. Thus, we examined the biosynthesis, and processing of cat K using cultured mouse osteocalsts derived from fetal long bone cells. Although the nature of the glycosylation is presently unknown, the modification of high-mannose oligosaccharides present on many proenzymes facilitates intracellular targeting to the lysosomal traffic via M6P receptors[25].

Several reports have speculated that, during

the bone resorption, osteoclasts directionally secrete cysteine proteases as proenzymes into the bone resorption sites and that activation occurs extracellularly in this acidic milieu[26]. Therefore, we examined cat K processing in nonadherent osteoclasts, which are devoid of an extracellular acidic compartment. Pro cat K was not detected in the media of these osteoclast cells, indicating that the osteoclasts could proteolytically modify the enzyme and active cat K is processed intracellularly and the resulting mature enzyme is released in a catalytically active form

UD is an extract developed to have therapeutic effects in inflammatory diseases involving cartilage destruction, such as RA. According to published work that is well accepted by the traditional oriental medicine community, UD was formulated to facilitate blood circulation as well as to reduce anti-inflammatory activity. The UD have been used for hundreds of years in this oriental region, and their safety and efficacy are well established through a long history of human use, but their use still lacks scientific support [15-17]. Although the barks of UD stem and root have been used in oriental traditional medicine for inflammatory diseases, the action mechanisms of this species are not nearly understood. It may be important to understand how this plant extract performs anti-inflammatory action in vivo.

A our recent study[27] reported that pharmacologic doses of UD stimulated in vitro and iv vivo bone formation in rodents and increased newbone volume under calvarial culture in vitro. In our preliminary study, to evaluate the role of UD on inflammatory diseases, we studied the effect of the water extract of UD on the production of collagen-induced RA in rats in vivo. Our results showed that the UD clearly reduced this inflammatory disease in a dose-dependent manner. In this study, it was found that relatively low doses of UD induced osteoblast differentiation and merkedly increased mineralization in osteoblastic cells. Cells of the osteoblastic lineage express BMP-2, BMP-4 and BMP-6, which concomitant with glucocorticoids induce osteoblastic cell differentiation and enhance the function of the osteoblasts[28-29]. After reaching confluency, the MC3T3-E1 cells began an ordered program of multilayering and expression of BMP-2 mRNA, followed by ALP mRNA and its activity.

UD would increase the accumulation of type I collagen by the inhibition of collagenase-1 expression, and elevated collagen content results in the increased mineralization. UD stimulated the expression of BMP-2 early during stage of the culture, whereas the abundance of BMP-4 mRNA did not change (not shown). These results indicte that enhanced expression of BMP-2 mRNA by UD is a trigger of osteoblast differentiation such as mineralized nodule formation. The UD thereafter induced the production of ALP, an important enzyme for mineralization by osteoblasts. The data presented in this study show that UD warrants further investigation, including preclinical and clinical studies. We are now in progress to isolate active molecules, as have tried[19].

V. References

1. Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. (1992) Distinct proliferative and differentiated stages of mouse MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J. Bone

Miner. Res. 7, 683-689.
2. Spelsberg T. C. Subramaniam M. Riggs B. L. Khosla S. (1999) The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. Mol. Endocrinol. 13, 819-828.
3. Lian J. B. Stein G. S. Canalis E. Robey P. G. Boskey A. L. (1999) Bone formation: osteoblast lineage cells, growth factors, matrix proteins, and the mineralization process Primer on the Metaboric Bone Diseases and Disorders of Mineral Metabolism, (M. J. Favus, Ed.) 14-29 Lippincott Williams and Wilkins, Philadelphia.
4. Franceschi R. T. Iyer B. S. (1992) Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J. Bone Miner. Res. 7, 235-246.
5. Ettinger B, Genant HK, Cann CE. (1987) Postmenopausal bone loss is prevented by treatment with low-dosage estrogen with calcium. Ann. Intern. Med. 106, 40-45.
6. Kalu DN. (1991) The ovariectomized rat model of postmenopausal bone loss. Bone Miner. 15, 175-192.
7. Morris HA, Need AG, Horowitz M, O'Leughlin PD, Nordin BEC. (1991) Calcium adsorption in normal and osteoporotic postmenopausal women. Calcif. Tissue Int. 48, 240-243.
8. Agnusdei D, Comporeaale A, Zacchei F, Gennari C, Baroni M, Costi D, Biondi M, Passeri M, Ciacca A, Sbrenna C, Falsettini E, Ventura A. (1992) Effects of ipriflavone on bone mass and bone remodeling in patients with established postmenopausal osteoporosis. Curr. Ther. Res. 51, 82-91.
9. Xiu RJ. (1988) Microcirculation and traditional Chinese medicine. JAMA 269, 1755-1757.
10. Phillipson, J.D. and Anderson, L. A. (1989) Ethnopharmacology and western medicine. J. Ethnopharmacol. 25, 61-72.
11. Lee, S.J., 1996. Korean Folk Medicine, Monographs Series No. 3. Publishing Center of Seoul National University, Seoul, South Korea, 39 pp.
12. Jun CD, Pae HO, Kim YC, Jeong SJ, Yoo JC, Lee EJ, Choi BM, Chae SW, Park RK, Chung HT. 1998. Inhibition of nitric oxide synthesis by butanol fraction of the methanol extract of Ulmus davidiana in murine macrophages. J Ethnopharmacol. 62, 129-135.
13. Shizhen L. (1590 A.D.). Bon Cho Gang Mok [Compendium of materia medica]. Peking: ChungKuK ChungEuiHak, 1978.
14. Hocheol Kim. Herbalogy. Seoul: Young Lim Sa, 2000.
15. Heo Jun. (1613 A.D.). Dong Ui Bo Gam [The precious mirror of oriental medicine]. Seoul: Namsangdang, 1999.
16. Shi, X.C. Chinese-English terminology of traditional chinese medicine. Hunan Science Publishing Co. Chian, pp71-98. 1983.
17. Sang yong Gil, Suppressive effects of a water extract of Ulmus davidiana Planch (Ulmaceae) on collagen-induced arthritis in mice ms Thesis, Dongguk University Graduate School, Kyungju, Korea, 2003.
18. ms Thesis, Dongguk University Graduate School, Kyungju, Korea, 2002.
19. Gruber H.E., Ivey JL, Baylink DL, Mathews M, Nelp WB, Sisom B, and Chestnut CH. (1984) Long-term calcitonin therapy in postmenopausal osteoporosis. Metabolism. 33, 295-303.
20. Genant HK, Baylink DJ, and Gallapher JC. (1989) Estrogens in the prevention of osteoporosis in postmenopausal women. Am. J. Obset. Gynecol. 161, 1842-1846.
21. Tunner CH. (1991) Toward a cure for

osteoporosis: reversal of excessive bone fragility. Osteoporosis Int., 2, 12-19.
22. Maniatopoulos C. Sodek J. Melcher A. H. (1988) Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell Tissue Res. 254, 317-330.
23. Kawane T. Horiuchi N. (1999) Insulin-like growth factor I suppresses parathyroid hormone (PTH)/PTH-related protein receptor expression via a mitogen-activated protein kinase pathway in UMR-106 osteoblast-like cells. Endocrinology 140, 871-879.
24. Stanford C. M. Jacobson P. A. Eanes E. D. Lembke L. A. Midura R. J. (1995) Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). J. Biol. Chem. 270, 9420-9428.
25. Nakajima Y and Pfeffer SR. (1997) Phosphoinositol 3-kinase is not required for recycling of mannose-6-phosphate receptors from late endosomes to the trans-Golgi network. Mol. Cell. Biol. 8, 677-582.
26. Delaisse JM., Y. Eeckout and G. Vaes. (1980) Inhibition of bone resorption in culture by inhibitors of thiol proteinases. Biochem. J. 192, 365-368.
27. Delaisse JM., Y. Eeckout and G. Vaes. (1980) Inhibition of bone resorption in culture by inhibitors of thiol proteinases. Biochem. J. 192, 365-368.
28. Varghese S. Canalis E. (1997) Regulation of collagenase-3 by bone morphogenetic protein-2 in bone cell cultures. Endocrinology 138, 1035-1040.
29. Gazzerro E. Gangji V. Canalis E. (1998) Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. J. Clin. Invest. 102, 2106-2114.
30. Kim JP, Kim WG, Koshino H, Jung J, Yoo ID. 1996. Sesquiterpene O-naphthoquinones from the root bark of Ulmus davidiana. Phytochemistry. 43, 425-430.