원저

Inhibitory effect of *Ulmus davidiana Planch* extracts on bone resorption mediated by processing of cathepsin K in cultured mouse osteoclasts

Park Jun-sung, Kim Kyung-ho, Jo Hyun-seog, Kim Kap-sung and Hwang Min-seob

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

Abstract

Objective : Ulmus davidiana Planch (Ulmaceae) has long been known to have anti-inflammatory in the traditional Korean medicine. UD has been reported as a good enhancer for bone healing.

Methods : In this experiment, we investigate the Inhibitory effects of UD on bone resorption using the bone cells culture. Different concentrations of crude extract of UD were added to mouse bone cells culture. The mitochondria activity of the bone cells after exposure was determined by colorimetric MTT assay. It was demonstrated that UD has potential effects on bone cells culture without any cytotoxicity. The most effective concentration of UD on bone cells were 100 ㎍/㎖. Cathepsin K (Cat K) is the major cysteine protease expressed in osteoclasts and is thought to play a key role in matrix degradation during bone resorption.

Results : When mouse long bone cells including osteoclasts and osteoblast were treated with the PI3-Kinase inhibitor, wortmannin (WT), WT prevented the osteoclast-mediated intracellular processing of Cat K. Similarly, treatment of osteoclasts-containing long bone cells with UD extracts prevented the intracellular maturation of Cat K, suggesting that UD may disrupt the intracellular trafficking of pro Cat K. This is similar to that of WT. Since secreted proenzymes have the potential to reenter the cell via mannose-6-phosphate (M6P) receptor, to prevent this possibility, we tested WT and UD in the absence or presence of M6P. Inhibition of Cat K processing by WT or UD was observed in a dose-dependent manner. Furthermore, the addition of M6P resulted in enhanced potency of WT and UD.

Conclusion : UD dose-dependently inhibited in vitro bone resorption with a potency similar to that observed for inhibition of Cat K processing.

Key words : Ulmus davidiana Planch (Ulmaceae), Osteoporosis, bone resorption, cathepsin

· **Acceptance** : 2005. 1. 13. · **Adjustment** : 2005. 3. 12. · **Adoption** : 2005. 3. 17.

· **Corresponding author** : Hwang Min-seob, Department of Acupuncture & Moxibustion, Dongguk University Oriental Hospital, Yonggang-dong #357, Kyungju-si, Kyungbook, Korea
Tel. 82-54-770-1266 E-mail : acup99@hanmail.net

I. Introduction

Since a large decrease in bone mass occurs in the postmenopause state, women are vulnerable to the osteoporosis known as postmenopausal osteoporosis[1]. Several medications have been reported to be effective for curing osteoporosis based upon the results obtained using these animal models. Non-steroidal substances, such as bisphosphonates, calcitonin[2], calcium products[2-3] and ipriflavone[4] are clinically employed as effective medications. Traditional medicine has been reevaluated by clinicians[5], because these medicines have fewer side effects and because they are more suitable for long-term use as compared to the chemically synthesized medicines. It has been suggested that the effectiveness of Korean medicines on low back pain seems to due to their efficacies in curing osteoporosis[6]. For example, some formula have been used in treating ovary function failure, or the low back pain during the climacteric period after oophorectomies because of malignant tumors[6-7]. However, no data are reported as to the recovery of bone mass by any of these Korean medicines. Because the need for safer and effective anti-inflammatory drug, there is a resurgence of interest in herbal medicines as an alternative source of drugs for intractable diseases such as rheumatoid arthritis[8].

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 traditional Korean medicine for the treatment of oedema, mastitis, gastric cancer, and inflammation[9-10]. As a part of our search for new biologically active substances from traditional medicines, we evaluated whether extracts of U. davidiana stem barks (UD) could modulate the induction of RA in mice. UD water extract has been developed on the basis of the known function of the herb, as described in the literature of traditional Chinese and Korean medicine[11-12]. UD has also been used for protection against degeneration of cartilage and regeneration of damaged tissue[13]. However, little is still known about the mode of action of this traditional medication on bone metabolism.

The structure of the protein included a 15-amino acid presignal, a 99-aa preprotein, and a 215-aa mature enzyme with two potential N-glycosylation sites[14]. Mouse Cat K mRNA is selectively expressed in osteoclast[15-19].

In this article, we investigate the biological effects of UD via the in vitro bone cell culture and the Cat K processing in bone cells and the anti-bone resorption activity of UD based on its inhibitory effects on cathepsin activity.

In a preliminary study, the effectiveness of UD has been confirmed on osteoporosis, where UD prevented the progression of bone loss induced by ovariectomy in rats[16].

For further study, we have extended the present investigation for the anti-bone resorption activity of UD based on its inhibitory effects on cathepsin activity. UD also showed the similar effects as WT on inhibition of pro Cat K processing. These observations are consistent with other previous results[20-21].

These suggest that UD can induce the mistargeting of acid hydrolases to lysosomal vesicles. Moreover, the addition of mannose-6-phosphate (M6P) resulted in enhanced potency of UD suggesting that M6P may prevent the reuptake and delivery of secreted proenzyme to the lysosomes for activation via M6P receptors. In conclusion, we describe inhibition of the processing of Cat K

and bone resorption in cultured mice osteoclasts by UD.

II. Materials and methods

1. Reagents

3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide [MTT], Calphostin C was obtained from Biomol (Plymouth, PA, USA). Wortmannin (WT), mannose-6-phosphate (M6P), and the cysteine protease inhibitor, E64, were purchased from Sigma Co. (St. Louis, MO, USA). Recombinant human M-CSF (rhM-CSF) was kindly provided by Morinaga Milk Co. (Kanagawa, Japan). Recombinant mouse G-CSF and recombinant mouse GM-CSF were purchased from R&D Systems (Minneapolis, MN, USA). Purified recombinant mouse Escherichia coli-expressed Cat K were generated in-house by RT-PCR as will be described else where [Kim et al. unpublished data]. All reagents were purchased from Wako Chemicals (Osaka, Japan). Tissue culture media and reagents, Fetal bovine serum (FBS) were from Gibco-BRL (Bethesda, MD, USA) or Jeil Biotech Services Co. (Daegu, Korea). Z-Leu-Arg-4-MNA was obtained from Bachem Co., (King of Prussia, PA). Five-to eight-week-old male ddY mice and newborn ddY mice were purchased from the KRIBB.

2. Plant material and Preparation of herb extract and fractions

The stem barks of U. davidiana were collected from Mt. Phal-gong, Kyungbuk Province, South Korea in May 2002, and identified by Professor Kap-Sung Kim, College of Korean Medicine, Dongguk University, South Korea. The herb had a 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 ㎛ 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 Korean Medical Hospital, Dongguk University, Kyungju city, Kyungbuk, Korea under acquisition number UD-W-57. UD extracts was massproduced as for clinical use, were kindly supplied by the Korean Medical Hospital of Dongguk University (Kyungju, Korea). Fresh stems were dried in a dark, well ventilated place. The voucher specimen (No. UD-W-57) is deposited in the Herbarium of this college.

3. Colorimetric MTT (Tetrazolium) assay for cell viability

For the assay[22], cells were incubated in 96-well plates in the presence of various concentrations of UD. For MTT test of osteoblasts, 1.2 × 104cells/well were added, the cells were cultured for 2 days without treatment to facilitate the attachment of cells and then various concentrations of UD were added. On the other hand, 1.3 × 102 osteoblasts cells/well and 1.52 × 104mononuclear cells/well were added in the MTT test for the mixed-bone cells culture, the bone cells were cultured for 6 days without treatment to facilitate the attachment of cells and differentiation of osteoclasts before adding various concentrations of UD. After various time intervals (1 day, 2 days or 4

days), the supernatant was removed and 100 ㎕ of MTT solution [3-(4,5-dimethylthiazolyl -2)-2,5-diphenyltetrazolium bromide] (Sigma catalog no. M2128, Sigma Co., St. Louis, MO, USA; 1 ㎎/㎖) was added to each well. The plate was incubated at 37℃ for 4 h to allow the formation of formazan crystal. The dark blue crystals were dissolved by acid-isopropanol, then the plate was read on Micro ELISA reader (Molecular Devices, CA, USA).

In order to show that the UD is not cytotoxic at the concentrations used, the effects of various concentrations of UD on bone cell activities were evaluated using MTT assay as described below. Six different concentrations (200 ㎍, 100 ㎍, 50 ㎍, 10 ㎍ and 1.0 ㎍/㎖) were tested for 1 day, 2 days and 4 days. Table 1 shows the effect of various concentrations of UD on different bone cells population measured by MTT assay.

4. Production of a mouse Cat K in E. coli and preparation of Cat K antibody

Sense and antisense oligonucleotide primers based on the previously cloned sequence[23] were synthesized on an Applied Biosystems Instrument (ABI) Model 394 DNA Synthesizer by CoreBio System Co. (Seoul, Korea). cDNA carrying the mouse full Cat K gene was amplified from mouse fetal cDNA library which was prepared in our house laboratory, as described in our previous paper[24]. These primers were used to PCR-amplify a fragment from first-strand cDNA which was generated from fetal mouse brain total RNA by reverse transcription. Sequences for the RT-PCR primers were derived from the previously cloned mouse cathepsin K cDNA sequences (Rantakokko et al., 1996, GenBank/EMBL accession number X94444)[25]. Primers were designed for the mature enzyme size of 215 amino acid residues. The cloned gene was expressed in E. coli carrying a recombinant plasmid pETCatK-1, which was constructed in expression vector, pET3a. The expressed cathepsin K was about 32 kDa with proenzyme (data not shown).

For production of polyclonal Cat K antibody (CHK-2), rabbit was used for immunization of the recombinant Cat K protein. Polyclonal antibody CHK2 was generated against a 34-mer C-terminal peptide, 296Asn-Ser-Trp-Gly- Glu-Ser-Trp-Gly-Asn-Lys-Gly-Tyr-Ala-Leu-Leu-Ala-Arg-Asn-Lys-Asn-Asn-Ala-Cys-Gly-Ile-Thr-Asn-Met-Ala-Ser-Phe-Pro-Lys-Met329, corresponding to the unique sequence in mouse Cat K. This antibody demonstrated similar reactivity to recombinant pro and mature cat K by immunoblot (data not shown).

5. Isolation and preparation of osteoclasts

Osteoclastoma contains a mixture of cells including osteoclasts and stromal cells. Osteoclasts were immunoselected from disaggregated osteoclastoma cells using a MAb (87Meml) to the vitronectin receptor (v3) expressed on osteoclasts[26]. Lysates of unselected cells, v3-positive osteoclasts, and v3-negative stromal cells were analyzed for expression of Cat K by western blot. Primary mouse calvarial cells were also prepared from newborn ddY mice and bone marrow cells from tibia of 5-to 8-week-old ddY mice as described[27]. Calvarial cells of op/op mice were also prepared according to a method previously reported[28]. ing 10% feCalvarial cells (1 × 106 cells) and bone marrow cells (1 × 107cells) were cocultured in a 10 cm culture dish in -Minimal Essential Medium (-MEM)

(Dainippon Pharmaceutical Co., Osaka, Japan) containtal bovine serum (FBS) (Jeil Biotech Services Co., Daegu, Korea). Some cocultures of op/op calvarial cells and normal bone marrow cells were treated with rhM-CSF (100 ng/㎖). After coculture for 7 days (the first coculture), floating cells were removed by washing with -MEM, and the rest of the cells were recovered from the culture dish by thorough pipetting and filtrated through a 40 ㎜ mesh (Falcon, Lincoln Park, NJ). The cells in the filtrate were collected by centrifugation and suspended in a-MEM containing 10% FBS (1 ㎖/10 ㎝ dish). Aliquots of the cell suspension were cytocentrifuged on glass slides and stained with a Wright-Giemsa solution (Sigma, St. Louis, MO). The cell suspension was applied to a Sephadex G-10 column (bed volume, 2 ㎖) (Pharmacia, Uppsala, Sweden) and incubated for 10 min at room temperature. Then cells were recovered from the column by adding -MEM containing 10% FBS, and passed cells were collected. These cells were designated as osteoclast precursors, because most of the cells in this preparation expressed similar lineage markers and differentiated into osteoclastic cells on calvarial cell layers within a short culture period (the second coculture; see Results).

6. Mouse osteoblast culture

Explants of mouse calvarial bone were cultured and the cells obtained have been routinely characterized and shown to express an osteoblast-like phenotype in culture. The population released during the last three digestions was highly enriched in cells expressing two markers of the osteoblast lineage, alkaline phosphatase (ALP) and osteocalcin[29]. Cells released by collagenase digestions were washed and grown to confluent in 75㎠ culture flasks (Falcon) in (DMEM) supplemented with antibiotics (penicillin and streptomycin) and 10% fetal calf serum (FCS; Gibco, BRL, Bethesda, MD, USA). Incubations were carried out at 37℃ in a humidified atmosphere of 5% CO2/95% air; the medium was changed every 2-3 days. Cells were grown to confluence at 37℃ and cultured in duplicate or triplicate wells for an additional 24 h in serum-free medium supplemented with Polymixin B sulfate to prevent endotoxin effects prior to treatment.

7. Preparation of fetal mouse long bone organ tissue culture system containing both of osteoclast and osteoblasts

The fetal mouse long bone organ tissue culture system was based on that described by Raisz[30]. Mouse long bone preparation was also described in our paper[31]. The method for disaggregating osteoclasts from fresh long bone organ tissue and their subsequent use for in vitro assays has been described in detail by Raisz[32-33]. Briefly, cell suspensions derived from collagenase digestion were washed and osteoclasts enriched by negative selection using magnetic beads coated with a murine antimouse HLA-DR MAb. The isolated cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and resuspended in RPMI media containing 10% FBS (Jeil-Biotech Service, Co. Ltd., Taegu, Korea). The cells were then plated on either bone slices or on plastic tissue culture dishes and incubated at 37℃ prior to treatment.

8. Biosynthetic radiolabeling of targent proteins by 35S-isotope

Osteoclast containing cultures were washed with DPBS containing 5 ㎎/㎖ bovine serum

albumin (BSA) and cultured in cysteine/methionine-free α-MEM containing HEPES (pH 7.2) for 60 mins at 37℃. The medium was removed and replaced with cysteine/methionine-free α-MEM containing 250 μCi/mL Trans [35S]-Label (1000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA, USA) for 30 min at 37℃. Cultures were washed three times with ice-cold DPBS and resuspended in RPMI media containing 5% fetal bovine serum (FBS) and 20 μM HEPES (pH 7.4) in the presence or absence of test agents and incubated for the indicated times.

9. Sample collection and preparation

Cell cultures were lysed with 500 ㎕ of 50 μM Tris-HCl (pH 7.4), containing 150 μM NaCl, 1% Triton X-100, 1 μM EDTA, 1 μM phenylmethysulfonylflouride (PMSF), 50 μM leupeptin, 50 μM E-64, and 1 μM pepstatin for 10 mins at 4℃. Cultures were scraped with a rubber policeman and lysates were centrifuged for 5 min at 20,000 × g at 4℃ and supernatants transferred to new tubes. Culture supernatants were collected and centrifuged to remove cell debris and transferred to tubes containing protease inhibitors as described for the lysis buffer.

10. Immunoprecipitation, SDS- polyacrylamide gel electrophoresis and immunoblot analysis

Cell lysate and media samples (450 ㎕) were incubated with 5 ㎕ of polyclonal antibody CHK-2 (0.5 ㎎/mL stock), which recognizes both the pro and mature enzyme, followed by addition of 20 ㎕ of protein A agarose and placed on a rotator overnight at 4℃. Precipitates were centrifuged and washed twice with lysis buffer and once with lysis buffer without Triton X-100. Precipitated proteins were resuspended in 30 ㎕ of 4 × sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer without 2-mercaptoethanol (nonreducing) and boiled for 5 min, as described by Laemmli. Samples were applied to 12% SDS-PAGE gels and run at 30 ㎃/gel. Protein determination of the samples was carried out as described by Lowry et al[34].

For western blot analysis, proteins were transferred onto nitrocellulose membrane, blocked with 5% skim milk in PBST, and probed with a polyclonal anti-Cat K antibody (1: 1000) in PBST containing 0.1% BSA for 2 h. The membrane was washed three times for 15 min with PBST and developed by ECL (Amersham Pharmacia Biotech., Upssala, Sweden). For quantitaitive analysis of radiolabeld Cat K, gels were fixed, dried, and exposed to a phosphor screen, and then scanned using a phosphorimager analyzer (Fuji Co. Ltd., Tokyo, Japan).

11. Zymography of Cat K activity

An activity staining technique using zymography was performed using a suitably modified azo-coupling procedure derived from the cathepsin assay described by Rieman et al.[35] and the matrix metalloproteinase assay described by our group[36]. Bone cells were grown for two days after seeding 105 cells and rinsed with phosphate buffered saline (PBS). The cultured cells were washed twice with PBS, homogenized in sample buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% NaN3, 100 ㎎/㎖ PMSF and 1% Triton X-100. The cell lysates was centrifuged at 10,000 ×G for 10 min at 4℃, and the supernatant was collected and stored at -70℃. The cell homogenates were resolved in 10% polyacrylamide gels containing 100 μM

phosphate buffer (pH 5.3), 15 μM synthetic cathepsin K/L substrate (Z-Leu-Arg-4-MNA) (Bachem, King of Prussia, PA) and 2.0 μM ethylene-diamine tetraacetic acid (EDTA). After electrophoresis, the gels were washed for 1 hour in 2.5% (v/v) Triton X-100 to remove SDS and then incubated for 24 hours at 37℃ in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl and 2.5 mM CaCl2 to allow proteolysis of the synthetic cathepsin K/L substrate Z-Leu-Arg-4-MNA substrate. After incubation, the gels were post-coupled for 30 mins using 0.2 ㎎/㎖ Fast Blue BB (Sigma), and then rinsed in PBS. The gels were finally rinsed with 100 μM copper sulfate for 30 min. The final reaction product is a deep red band corresponding to activity and molecular weights were estimated by reference to prestained SDS-PAGE markers and cathepsin standards (Chemicon, USA).

12. Mouse osteoclast resorption assay

Osteoclast number was adjusted to 2×103cells/㎖ and preincubated with test agent or with vehicle control for 30 min at 37℃ and then seeded onto bovine cortical bone slices (0.5 ㎖/well) in a 48 well plate. Following incubation for 24 h at 37℃, culture supernatants were harvested and resorption was measured using a competitive ELISA, according to the manufacturer's protocol (Osteometer A/S, Rodovre, Denmark). The assay measures carboxyterminal telopeptides of the α1 chain of human type I collagen that are released during the resorption process[34]. The results are expressed as percent inhibition of resorption compared to supernatants derived from untreated osteoclast cultures.

Ⅲ. Results

1. Cytotoxicity of UD on mixed-bone cells and osteoblasts cell population

When mixed-bone cells are cultured with 500 ㎍/㎖ concentrations of UD for 24 h, there is a significant decrease in the mixed-bone cell population, while in the concentration of 100 ㎍/㎖ UD, the population of mixed-bone cells increased significantly and this effect persists till the end of 4 days' culture (Table 1). When the concentration of UD is <100 ㎍/㎖, the effect of UD on the mixed-bone cells disappeared. In this study, we selected the concentration of 100 ㎍/㎖ UD for the evaluation of further bone cell activities. When osteoblasts were cultured with UD for 1 day, the cell population showed significant increase in all the samples ($P<0.05$) except the highest concentration (i.e. 200 ㎍/㎖, $P=0.2210$). As the time interval of culture increases, the effect of increase in osteoblasts population was observed in the lower concentrations of UD. When osteoblasts were cultured with UD for 3 days, the increase in cell population was noted in the samples with the concentration of 0.1 ㎍/㎖ ($P=0.0143$) (Table 1). At the concentration of 100 ㎍/㎖ UD, there was a mild increase of cell population of the osteoblasts at the first day's culture ($P=0.0012$), the effect of increase in cell population disappeared on the 2nd ($P=0.135$) and 4th day's culture ($P=0.0530$) (Table 1).

When mixed-bone cells are cultured with 100 ㎍/㎖ of UD for 24 h, there is a significant increase in the bone cell population ($P=0.001$) and this effect persists till the days' culture ($P=0.001$). At 100 ㎍/㎖ UD, there was a mild increase of cell population of the osteoblasts at the first day's culture ($P=0.035$),

Table 1. Cell Population Changes by Different Concentrations of UD in the Mixed -bone Cells or Osteoblasts culture (n=6)

Treatment	Optical Density					
	Mixed-bone cell			Osteoblasts		
(μg/㎖)	1 day	2 day	4 day	1 day	2 day	4 day
Control	0.25±0.03	0.33±0.03	0.42±0.04	0.12±0.02	0.21±0.01	0.27±0.01
1	0.25±0.01	0.27±0.01	0.39±0.01	0.13±0.01	0.25±0.01	0.27±0.01
10	0.25±0.04	0.35±0.02	0.40±0.02	0.13±0.02	0.25±0.04	0.27±0.02
50	0.24±0.03	0.36±0.05	0.43±0.05	0.15±0.01	0.25±0.02	0.28±0.02
100	0.44±0.05	0.67±0.04	0.73±0.03	0.14±0.02	0.21±0.02	0.26±0.02
200	0.17±0.02	0.13±0.01	0.12±0.01	0.12±0.01	0.14±0.02	0.17±0.02

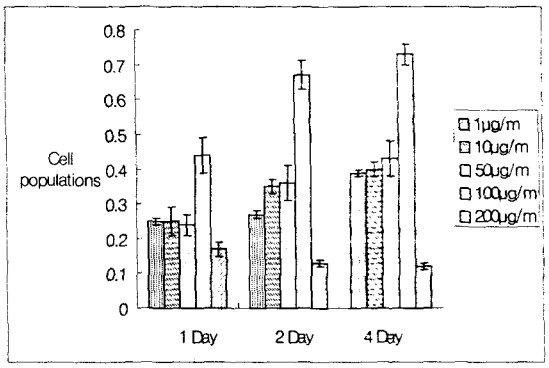

Fig. 1. Cell population changes by different concentrations of UD in the Mixed -bone cells(n=6).

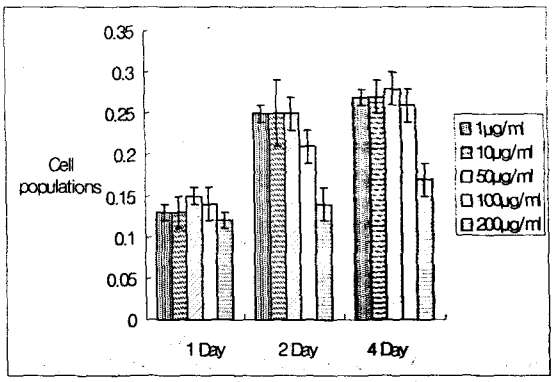

Fig. 2. Cell population changes by different concentrations of UD in the osteoblasts culture (n=6).

the effect of increase in cell population disappeared at the 2nd day (P=0.135) and the 4th day's culture (P=0.0530).

2. Expression of Cat K in osteoclasts

This activity was completely inhibited by the addition of E64, a nonselective cysteine protease inhibitor. However, no inhibition was observed using the aspartate protease inhibitor, pepstatin A, or the serine protease inhibitor, phenylmethylsulfonylfluoride (PMSF) (data not shown), indicating that the enzyme was a cysteine protease.

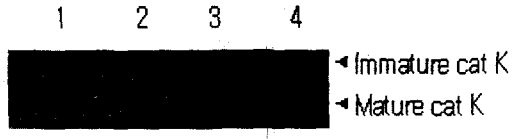

Fig. 3. Differential expression of immature and mature Cat K in cultured mouse osteoclasts and non-osteoclastic cells.

To confirm the specific expression of Cat K in osteoclasts, immunoblot analysis was performed using cell extracts of total bone cells (osteoclast + osteoblast + stromal cells), as well as osteoclast-enriched (vitronectin receptor, αvβ3, -positive osteoclasts) and osteoclast-depleted αvβ3-negative cell mixtures (osteoblasts + stromal cells). As shown in Fig. 1, cat K expression is observed in both the total cell mixtures (lanes 1 and 2) and osteoclast-enriched cell populations (lane 3), but not in the osteoclast-depleted cells (lane 4). Furthermore, the majority of Cat K detected in osteoclast lysates by immunoblot analsys was mature enzyme, which was preosteolytically processed.

Osteoclasts were isolated from calvarial and fetal long bone cells as described in Methods Section. Lysates of total bone cells (lane 1, control without treatment; lane 2, 10 μg), osteoclast-enriched (lane 3, 10 μg) or osteoclast-depleted cells (lane 4, 10 μg) were electrophoresed on a 12.5% SDS-PAGE gel. The gel was subjected to the immunoblot

analysis using an anti-Cat K polyclonal antibody. Immature Cat K (37 kDa) and mature Cat K (27 kDa) are distinguished.

3. UD extract inhibits Cat K processing and bone resorption

As shown in Fig. 2, following a 30 min pulse (T=0, lane 1), only the pro form of Cat K was detected in the osteoclast cell lysate. After a 4 h chase in the absence of WT and UD, significant intracellular processing of immature Cat K were observed (lane 4 and lane 5). However, treatment of osteoclasts with WT and UD inhibited the intracellular maturation of Cat K (lane 3 for WT and lane 2 for UD).

It is well known that secreted proenzymes have the potential to reenter the cell via M6P receptors, and can be delivered to the lysosome for proteolytic processing and modification. Preventing this reentrance and delivery of proenzyme into lysosome by M6P receptor is important for inhibition and blocking of pro Cat K processing, and finally preventing bone resorption caused by processed Cat K mature enzyme. Therefore, we tested WT, calphostin C and UD at various doses in the absence or presence of M6P (5 μM). As shown in Table 2, inhibition of Cat K processing by WT or UD alone was dose-dependent with an IC50 of 1.2 μM and 87.5 μg/mℓ, respectively. WT inhibited the bone resorption via Cat K processing, while calphostin C, a specific inhibitor of PKC, did not affect the Cat K processing and bone resorption by osteoclasts. Furthermore, the presence of M6P resulted in enhanced potency of WT (IC50 = 0.25 μM) and UD (IC50 = 40 μg/mℓ) (Table 3).

Osteoclasts were pulse radiolabeled and

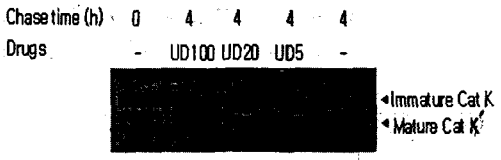

Fig. 4. UD inhibit Cat K processing from proenzyme form to mature enzyme form.

chased in the presence or absence of WT (1 μM) or UD (100 μg/mℓ). Cells and media were harvested at 0 and 4 h after chase and Cat K was immunoprecipitated using polyclonal antibody CHK-2 and analyzed by SDS-PAGE. Immature Cat K and mature Cat K are shown.

Table 2. Inhibition of Cat K processing by WT, UD and Calphostin C in The Absence of M6P

		Labeled mature Cat K (arbitrary units)	Processing (% inhibition)
Wortmannin(μM)	0	8.5±0.4	0
	0.1	8.1±0.5	0.01±0.0
	0.5	6.6±0.4	12.6±1.2
	1.0	4.4±0.5	49.3±3.4
UD(μg/mℓ)	0	9.3±0.5	0
	10	8.1±0.4	9.4±0.8
	50	6.2±0.3	36.7±3.5
	100	4.0±0.2	57.6±4.5
Calphostin C(μM)	0	8.4±0.3	0
	0.01	8.4±0.6	0.1±0.01
	0.5	8.3±0.4	0.1±0.01
	1.0	8.3±0.3	0.1±0.01

Pulse-radiolabeled osteoclasts were chased with increasing doses of WT and UD in the absence of M6P (5 μM) for 4 h. Cells extracts were immunoprecipitated with an anti-Cat K antibody. Cat K was quantified using a phosphorimager and results are expressed as percent inhibition of processing. M6P alone had no effect on Cat K processing. Radiolabeled Cat K was expressed as total mature Cat K processed. Data are expressed as the mean ±SE values (n=5).

Table 3. Inhibition of Cat K Processing in The Presence of M6P by WT, UD and Calphostin C

		Labeled mature Cat K (arbitrary units)	Processing (% inhibition)
Wortmannin(μM)	0	8.4±0.2	0
	0.01	7.1±0.3	16.7±1.4
	0.1	4.1±0.5	53.4±5.6
	0.3	3.7±0.4	53.5±3.7
UD (μg/mℓ)	0	8.4±0.5	0
	10	6.8±0.6	13.4±1.5
	50	4.0±0.2	41.2±2.3
	100	3.8±0.3	54.5±3.5
Calphostin C(μM)	0	8.4±0.6	0
	0.01	8.36±0.7	0.2±0.0
	0.2	8.36±0.8	0.2±0.01
	1.0	8.38±0.9	0.1±0.01

Pulse-radiolabeled osteoclasts were chased with increasing doses of WT, calphostin C and UD in the presence of M6P (5 μM) for 4 h. Cells extracts were immunoprecipitated with an anti-cathepsin K antibody. Cat K was quantitated, as shown Table 2. M6P alone had no effect on Cat K processing. Radiolabeled Cat K was expressed as total mature Cat K processed. Data are expressed as the mean ±SE values for both the immature and mature Cat K bands from phosphoimager analysis for each incubation condition (n=6).

4. Dose-dependent inhibition of in vitro bone resorption by UD

WT has previously been shown to inhibit bone resorption; therefore, we determined the ability of WT, calphostin C and UD to prevent in vitro bone resorption under similar conditions. WT and UD dose-dependently inhibited in vitro bone resorption (Table 4) with a potency similar to that observed inhibition of Cat K processing. Furthermore, the same result was obtained in UD treatment as shown in Table 4. However, no any

Table 4. Inhibition of in vitro bone resorption by WT, UD and Calphostin C

		Resorption (% inhibition)
Wortmannin (μM)	0	0
	0.1	12.3±1.5
	0.2	57.3±6.3
	1.0	61.2±5.6
UD(μg/mℓ)	0	0
	10	7.4±1.0
	50	47.5±4.6
	100	63.5±4.7
Calphostin C(μM)	0	0
	0.01	0.3±0.01
	0.2	0.3±0.01
	1.0	0.4±0.02

inhibitory effect was observed when treated with calphostin C.

Osteoclasts were cultured on bone particles and treated with WT, calphostin C and UD for 24 h. Osteoclastic resorption was measured as described in the Materials and Methods. The results are expressed as percent inhibition of resorption compared to supernatants derived from osteoclasts cultured in the absence of inhibitors. Data are expressed as the mean ±SE values (n=5).

Ⅳ. Discussion

In Korea, there is a rich treasury of ethnobotanical knowledge and over the past decade[37-38]. During our field studies, we have coincided following Korean and herbal remedy claimed to be used in the treatment of rheumatism, bone resorption and related inflammatory diseases. The aim of this study is to investigate the antibone resorption activity of UD by using an in vitro screening method. A literature survey on the plant UD revealed that there is no scientific evidence of its usefulness in the treatment of RA and

osteoporosis. Therefore, the need for safer and effective anti-inflammatory drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study. There are several reports that demonstrated an improvement in clinical association with the use of traditional Korean medicine in the treatment of fractures[39]. Traditional Korean medicines may offer advantages over the longer term over synthetic agent medication[8]. Initial studies showed that herbal medicines that have traditionally been effective for the gynecological diseases[9-10] may also be administered for the prevention of osteoporosis.

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 Korean 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 Korean region, and their safety and efficacy are well established through a long history of human use, but their use still lacks scientific support[40]. Although the barks of UD stem and root have been used in Korean 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. 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, we examined the relationships between bone cells and this specific herbal medicine UD, which may contribute to the possible justification for the clinical application in the treatment of bone disease. We have also found that UD is non-cytotoxic and the addition of UD into the culture medium will affect the cell population of the bone cells. When the concentration of UD is <100 ㎍/㎖, the effect of UD on the mixed-bone cells disappeared (Table 1). Therefore, we selected the concentration of 100 ㎍/㎖ UD for the further evaluation. Although 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, little is known on mechanisms how does the osteoclasts deliver to catalytically active enzyme to bone resorption sites. Thus, we have further examined the biosynthesis, and processing of Cat K using cultured mouse osteoclasts 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[41]. The enhanced potency of M6P on WT and UD effects was observed on processing by bone osteoclasts (Table 3). Rieman et al.[36] demonstrated that Cat K is not normally secreted or found as a proenzyme in the media, but rather distributed selectively at the ruffled border membrane of activated osteoclasts. The exception was when WT was present; in this case the osteoclasts secreted pro Cat K into the medium. The inhibition of its reuptake by M6P can be expected, as the enhancement of M6P on WT and UD effects was observed in this study. Furthermore, the fact that UD also shows an enhancement in the presence of M6P may indicate that UD operates through a similar mechanism as that

for WT. It is, therefore, suggested that UD shows additive effects with WT on blocking Cat K processing. 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[42]. 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.

PI3-kinase is known to be involved in growth factor signal transduction and vesicular membrane trafficking in a variety of systems. WT, a PI3-kinase inhibitor, has been previously shown to inhibit a number of osteoclast functions, including cell attachment and spreading[43], ruffled border formation[44] and bone resorption both in vitro and in vivo[18]. WT inhibited in a dose dependent manner Cat K processing in mouse osteoclasts, and eventually resulted in the secretion of unprocessed pro Cat K into the culture media. Furthermore, UD also showed the similar effects as WT on inhibition of pro Cat K processing. These observations are consistent with other previous results[22,38]. The inhibition of bone resorption by WT and UD occured at concentrations consistent with the inhibition of Cat K processing. Therefore, WT and UD may exert its antiresorptive effects on osteoclasts, in part by reducing lysosomal pools of catalytically active Cat K at least. The majority of Cat K detected in osteoclast lysates by immunoblot analysis was mature enzyme, which was preoteolyically processed. Treatment of osteoclasts with WT and UD inhibited the intracellular maturation of Cat K, suggesting that WT and UD may inhibit the intracellular trafficking of pro-Cat K. However, when mouse total bone cells were treated with calphostin C, a specific inhibitor of PKC, no effect was observed, indicating that calphostin C did not affect osteoclast-mediated Cat K processing and maturation signaling. These results also strongly suggested that Cat K processing and maturation in osteoclasts might be mediated by cAMP-dependent PKA pathway in mouse osteoclast cells.

V. Conclusion

Plants used in folk medicine have been accepted as the main sources for drug discovery and development. In Korea and China, there is a rich treasury of ethnobotanical knowledges[1]. Several drugs isolated from plants have been also prepared in the salt form in recent years, but herbal medication, developed in the ancient tradition, continued to be widely used in Chinese populations[2]. The increasing popularity of traditional herbal medicine and/or natural products has also produced fear about their toxicity and uncertainty about their ingredients. In the Western world, medicinal herbs are becoming increasingly popular and important in the public and scientific communities, but they have met with skepticism from much of the medical community. Until the safety, efficacy, mechanism of action, and toxicity determination as well as clinical trials have been scientifically evaluated, many western health care experts are hesitant to embrace their use[3-4].

Bone resorption requires the directional

secretion of proteolytic enzymes into the resorption lacunae for the degradation of the organic matrix proteins. Cathepsin(Cat)s L, B and K are involved in this process of bone resorption[17]. Of them, Cat K is the key enzyme involved in the resorptive process[18-19] and Cat K belongs to the papain superfamily of lysosomal cystein proteases. Cat K was first identified as a cysteine protease in osteoclasts and have been implicated in various pathological settings, such as rheumatoid arthritis[20], tumor invasion[21] metastasis[22], inflammation[23], and osteoporosis[24]. Cathesin K (EC 3.4.22.38) efficiently cleaves peptide bonds in various proteins including collagen, elastin, and gelatin[25]. Cat K cDNA have been cloned from rabbit osteoclasts[26], human osteoclastoma[27] and mouse[28-29] and encode prepropeptides of 329 to 334 residues. The mouse Cat K gene contains a 990-bp coding for 329 amino acid prepropolypeptide.

In summary, these results strongly suggested that UD could be useful for preventing both postmenopausal osteoporosis and osteoporosis associated with the ovary function failure. Mature and catalytically active Cat K is produced in bone resorption and that UD and WT prevented this processing of the enzyme and bone resorption. An understanding of the factors that inhibit the activation and trafficking of Cat K in osteoclasts should provide insight for treatment of bone resorption. Our results suggest that the effect of UD in the inhibition of inflammatory diseases may be partially associated with the down-regulation of IL-1. UD has great potential as an alternative to these treatments, and has no adverse effects. UD can be given orally, and it inhibits disease progression by both controlling inflammatory proteins and protecting cartilage. UD warrants further investigation, including preclinical and clinical studies. We are now in progress to isolate r active molecules, as have tried[45].

VI. References

1. Ettinger B, Genant HK, Cann CE. Postmenopausal bone loss is prevented by treatment with low-dosage estrogen with calcium. Ann Intern Med 1987;106:40-45.
2. Kalu DN. The ovariectomized rat model of postmenopausal bone loss. Bone Miner 1991;15:175-192.
3. Morris HA, Need AG, Horowitz M, O'Leughlin PD, Nordin BEC. Calcium absorption in normal and osteoporotic postmenopausal women. Calcif Tissue Int 1991;48:240-243.
4. Agnusdei D, Comporeaale A, Zacchei F, Gennari C, Baroni M, Costi D, Biondi M, Passeri M, Ciacca A, Sbrenna C, Falsettini E, Ventura A. Effects of ipriflavone on bone mass and bone remodeling in patients with established postmenopausal osteoporosis. Curr Ther Res 1992;51:82-91.
5. Xiu RJ. Microcirculation and traditional Chinese medicine. JAMA 1988;269:1755-1757.
6. Koyama T. Osteoporosis with Kampo treatment (in Japanese). Sanfujinka Chiryo 1991;63:203-207.
7. Terasawa K. Kampo: Japanese Oriental medicine, K. K. Standard McIntyre, Tokyo, Japan, 1993.
8. Phillipson JD, Anderson LA. Ethnopharmacology and western medicine. J. Ethnopharmacol. 1989;25:61-72.
9. Lee, S.J., 1996. Korean Folk Medicine, Monographs Series No. 3. Publishing Center of Seoul National University, Seoul, South Korea, pp39.

10. Jun CD, Pae HO, Kim YC, Jeong SJ, Yoo JC, Lee EJ, Choi BM, Chae SW, Park RK, Chung HT. Inhibition of nitric oxide synthesis by butanol fraction of the methanol extract of Ulmus davidiana in murine macrophages. J Ethnopharmacol. 1998. 62; 129-135.
11. Shizhen L. (1590 A.D.). Ben Cao Gang Mu [Compendium of materia medica]. Peking: Zhong Guo Yi Xue, 1978.
12. Hocheol Kim. Herbalogy. Seoul: Young Lim Sa, 2000.
13. Heo Jun. (1613 A.D.). Dong Yi Bao Jian [The precious mirror of oriental medicine]. Seoul: Namsangdang, 1999.
14. Gelb BD, Moissoglu K, Zhang J, Martgnetti, JA, Bromme D, Desnick RJ. Cathepsin K: isolation and characterization of the murine cDNA and genomic sequence, the homologue of the human pycnodysostosis gene. Biochemical and Molecular Medicine 1996;59:200-206.
15. Li YP, Chen W. Characterization of mouse cathepsin K gene, the gene promoter, and the gene expression. J Bone Miner Res 1999;14:487-499.
16. Bromme D, Okamoto K, Wang BB, Biroc S. Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. J Biol Chem 1996;271:2126-2132.
17. Drake FH, Dodds FA, James IE, Connor JR, DeBouck C, Richardson S, Lee-Rykaczewski E, Coleman L, Rieman D, Barthlow R, Hastings G, Gowen M. Cathepsin K, but not B, L or S is abundantly expressed in human osteoclasts. J Biol Chem 1996;271: 12511-12516.
18. Inaoka T, Bilbe G, Ishibashi O, Tezuka K, Kumegawa M,Kokubo T. Molecular cloning of human cDNA for cathepsin K. Novel cysteine proteinase predominantly expressed in bone. Biochem Biophys Res Commun 1995;206:89-96.
19. Tezuka K, Tezuka Y, Maejima A, Sato T, Nemoto K, Kamioka H, Hakeda Y, Kumegawa M. Molecular cloning of a possible cysteine proteinase predominantly expressed in osteoclasts. J Biol Chem 1994;269: 1100-1109.
20. Chambers AF, Colella R, Denhardt DR, Wilson SM. Increased expression of cathepsins L and B and decreased activity of their inhibitors in metastatic, ras-transformed NIH 3T3 cells. Mol Carcin 1992;5:238-245.
21. Delaisse JM, Eeckout Y, Vaes G. In vivo and in vitro evidence for the involvement of cysteine proteinases in bone resorption. Biochem Biophys Res Commun 1984;12; 441-447.
22. Rantakokko J, Aro HT, Savontaus M, Vuorio E. Mouse cathepsin KL cDNA cloning and predominant expression of the gene in osteoclasts, and in some hypertrophying chondrocytes during mouse development. FEBS Lett 1996;393:307-313.
23. Rantakokko J, Aro HT, Savontaus M, Vuorio E. Mouse cathepsin KL cDNA cloning and predominant expression of the gene in osteoclasts, and in some hypertrophying chondrocytes during mouse development. FEBS Lett 1996;393:307-313.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227: 680-685.
25. Yagel S, Warner AH, Nellans HN, Lala PK, Waghorne C, Denhardt DT. Suppression by cathepsin L inhibitors of the invasion of the amnion membranes by murine cancer cells. Cancer Res 1989;49: 553-557.

26. Votta BJ, Levy MA, Badger A, Dodds RA, James IE, Thompson S, Bossard MJ, Carr T, Connor JR, Tomaszek JA, Szewczuk L, Drake FH, Veber D, Gowen M. Peptide aldehyde inhibitors of cathepsin K inhibit bone resorption both in vivo and in vitro. J Bone Miner Res 1997;12:1396-1406.
27. Tsurukai T, Takahashi N, Jimi E, Nakamura I, Udagawa N, Nogimori K, Tamura M, Suda T. Isolation and characterization of osteoclast precursors that differentiate into osteoclasts on calvarial cells within a short period of time. J Cell Physiol 1998177:26-35.
28. Takahashi N, Udagawa N, Akatsu T, Tanaka H, Shionome M, Suda T. Role of colony-stimulating factors in osteoclast development. J Bone Miner Res 1991;6: 977-985.
29. Evans DB, Bunning RAD, Russell RGG. The effects of recombinant human interleukin-1 on cellular proliferation and the production of prostaglandin E2, plasminogen activator, osteocalcin and alkaline phosphatase by osteoblast-like cells derived from human bone. Biochem Biophys Res Comm 1990;166:208-216.
30. Raiz LG, Niemann I. Effect of phosphate, calcium and magnesium on bone resorption and hormonal responses in tissue culture. Endocrinology 1969;85:446-452.
31. Hong HT, Kim HJ, Kim DW, Lee YC, ParK YG, Kim HM, Choo YK, Kim CH. Inhibotory effect of a Korean traditional medicine, Honghwain- Jahage (water extracts of Carthamus tinctorius L. seed and Homminis placenta) on interleukin-1-mediated bone resorption. J Ethnopharm 2002;43:11-16.
32. Iwata Y, Mort JS, Tateishi H, Lee ER. Macrophage cathepsin L, a factor in the erosion of subchondral bone in rheumatoid arthritis. Arthritis Rheum 1997;40:499-509.
33. James IE, Lark MW, Zembryki D, Lee-Rykaczewski E, Hwang SM, Tomaszek TA, Belfiore P, Gowen M. Development and characterization of a human in vitro resorption assay: Demonstration of utility using novel antiresorptive agents. J Bone Miner Res 1999;14:1562-1569.
34. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-275.
35. Rieman DJ, Mcclung HA, Dodds RA, Hwang SM, Lark MW, Holmes S, James IE, Drake FH and Gowen M. Biosynthesis and processing of cathepsin K in cultured human osteoblasts. Bone 2001;28:282-289.
36. Chung TW, Moon SK, Lee YC, Kim JG, Ko JH, Kim CH. Enhanced expression of matrix metalloproteinase-9 by hepatitis B virus infection in liver cells. Arch Biochem Biophys 2002;408:147-154.
37. Kim HM, An C.-S, Jung K-Y, Choo Y-K, Park J-K, Nam S-Y. Rehmannia Glutinosa inhibits tumor necrosis factor-alpha and interleukin-1 secretion from mouse astrocytes. Pharmacol. Res. 1999; 40: 171-6.
38. Hong H.T Effects of Yukmijihangtang-Jahage extracts on cellular regulation of bone cell and function. Thesis of Ph.D. Dongguk University, Kyungju, Korea. 1998.
39. Huang HF, You JS. The use of Chinese herbal medicine on experimental fracture healing. Am J Chin Med 1997;25:351-356.
40. Won K.C Chinese medicine encyclopedia. Seoul: DaeSung,1992.
41. Nakajima Y, Pfeffer SR. Phosphoinositol 3-kinase is not required for recycling of mannose-6-phosphate receptors from late endosomes to the trans-Golgi network. Mol Cell Biol 1997;8:582-677.

42. Delaisse JM, Eeckout Y, Vaes G. Inhibition of bone resorption in culture by inhibitors of thiol proteinases. Biochem J 1980;192: 365-368.
43. Lakkakorpi PT, Wesolowski G, Zimolo Z, Rodan GA, Rodan SB. Phosphatidylinositol 3-kinase association with the osteoclast cytoskeleton, and its involvement in osteoclast attachment and spreading. Exp Cell Res 1997;237:296-306.
44. Nakamura I, Sasaki T, Tanaka S, Takahashi N, Jimi E, Kurokawa T, Kita Y, Ihara S, Suda T, Fukui Y,.PI3 kinase is involved in ruffled border formation in osteoclasts. J Cell Physiol 1997;172: 230-239.
45. Kim JP, Kim WG, Koshino H, Jung J, Yoo ID. Sesquiterpene O-naphthoquinones from the root bark of Ulmus davidiana. Phytochemistry. 1996; 43, 425-430.