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Inhibition of Drynariae Rhizoma extracts on bone resorption mediated by processing of cathepsin K in cultured mouse osteoclasts

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Abstract

In the traditional Korean medicine, Drynariae Rhizoma (DR) [*Drynaria fortunei* (kunze) J. Sm] has been reported as a good enhancer for bone healing. In this experiment, we investigate the effects of DR on bone resorption using the bone cells culture. Different concentrations of crude extract of DR 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 DR has potential effects on the bone cells culture without any cytotoxicity. The most effective concentration of DR on bone cells was 100 μg/ml. On the other hand, 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. In this study, Mouse long bone cells including osteoclasts and osteoblast were treated with the PI3-kinase inhibitor, wortmannin (WT), and a specific inhibitor of protein kinase C (PKC), calphostin C. Although WT prevented the osteoclast-mediated intracellular processing of Cat K, calphostin C did not. Similarly, treatment of osteoclasts-containing long bone cells with Drynariae Rhizoma (DR) extracts prevented the intracellular maturation of Cat K, suggesting that DR 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 DR in the absence or presence of M6P. Inhibition of Cat K processing by WT or DR was observed in a dose-dependent manner. Furthermore, the addition of M6P resulted in enhanced potency of WT and DR. DR dose-dependently inhibited in vitro bone resorption with a potency similar to that observed for inhibition of Cat K processing.

Keywords: Drynariae Rhizoma; Bone resorption; Cathepsin; Protease inhibitor; Osteoclast; Biosynthesis; Processing

1. Introduction

Plants used in folk medicine have been accepted as the main sources for drug discovery and devel-

Abbreviations: Cat, cathepsin; DR, Drynariae Rhizoma; WT, wortmannin; PKC, protein kinase C; M6P, mannose-6-phosphate.

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opment. In Korea and China, there is a rich treasury of ethnobotanical knowledge [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 been 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].

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. Nonsteroidal substances, such as bisphosphonates, calcitonin [5], calcium products [5,6] and ipriflavone [7] are clinically employed as effective medications. Traditional medicine has been reevaluated by clinicians [8], 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 oriental medicines on low back pain seems to be due to their efficacies in curing osteoporosis [9]. 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 [9,10]. However, no data are reported as to the recovery of bone mass by any of these oriental 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 [11].

The traditional herbal medicines, Drynariae Rhizoma (DR) [*Drynaria fortunei* (kunze) J. Sm; Gol-Se-Bo in Korean name and Gu-Sui-Bu in Chinese name] was commonly used to manage disorders of orthopedics and had been claimed to have therapeutic effects on bone healing [12]. Specifically, through tissue culture and isotope tracing, it was found that DR injection significantly promoted calcification of the cultivated chick embryo bone primordium, increased alkaline phosphatase activity in the cultivated tissue, and accelerated synthesis of proteoglycan [13]. Liu et al. [14] have also shown that DR has an antioxidant effect on rat osteoblasts from hydrogen peroxide-induced death and may promote bone recovery under similar pathologic conditions. DR should be intensively studied for its possible use in bone diseases. DR is also known to be effective for the treatment of inflammation, hyperlipidemia, arteriosclerosis, rheumatism, and gynecological diseases such as osteoporosis and bone resorption in oriental medicine [15,16].

Bone resorption requires the directional secretion of proteolytic enzymes into the resorption lacunae for the degradation of the organic matrix proteins. Cathepsins (Cats) L, B and K are involved in this process of bone resorption [17]. Of them, cathepsin K (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 has 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 [15,28,29] and encode prepropeptides of 329 to 334 residues. The mouse Cat K gene contains a 990-bp coding for 329 amino acid prepropolypeptide. 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 [25]. Mouse Cat K mRNA is selectively expressed in osteoclast [29–33].

Osteoclasts are specialized bone cells whose primary function is to resorb bone and provide sites for new bone formation. The resorptive phase of bone remodeling is initiated by the attachment of osteoclasts to the bone surface. Furthermore, it has been shown that mature Cat K has proteolytic activity

against several extracellular matrix substrates [34,35], including type I collagen, the most abundant matrix protein in bone. Phosphatidylinoshitol (PI)3-kinase is known to be involved in growth factor signal transduction and vesicular membrane trafficking in a variety of systems. Wortmannin (WT), a PI3-kinase inhibitor, has been previously shown to inhibit Cat K processing in osteoclast and bone resorption both in vitro [36]. 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. It was also known that prostaglandins interact with specific cell surface receptors. Also, prostaglandins mediate hydrolysis of phosphatidylinoshitol 4,5-bisphosphate, with subsequent production of diacylglycerol and inositol 1,4,5-triphosphate. These second messengers activate of protein kinase-C (PKC) and calcium levels increased from intracellular organelles [37].

In this article, we investigate the biological effects of DR via the in vitro bone cell culture and the Cat K processing in bone cells and the anti-bone resorption activity of DR based on its inhibitory effects on cathepsin activity. In a preliminary study, the effectiveness of DR has been confirmed on osteoporosis, where DR 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 DR based on its inhibitory effects on cathepsin activity. DR also showed the similar effects as WT on inhibition of pro Cat K processing. However, a specific inhibitor of protein kinase C (PKC), calphostin C, did not prevent the osteoclast-mediated intracellular processing of Cat K. These observations are consistent with other previous results [22,38]. These suggest that DR can induce the mistargeting of acid hydrolases to lysosomal vesicles. Moreover, the addition of mannose-6-phosphate (M6P) resulted in enhanced potency of DR suggesting that M6P may prevent the reuptake and delivery of secreted proenzyme to the lysosomes for activation via M6P receptors. These results suggest that PI3-kinase plays a role in the delivery of pro Cat K to lysosomal vesicles for enzyme activation. In conclusion, we describe inhibition of the processing of Cat K and bone resorption in cultured mice osteoclasts by DR.

2. Materials and methods

2.1. 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 (St. Louis, MO, USA). Recombinant human M-CSF (rhM-CSF) was kindly provided by MorinagaMilk (Kanagawa, Japan). Recombinant mouse G-CSF and recombinant mouse GM-CSF werepurchased from R and D Systems (Minneapolis, MN). Purified recombinant mouse *Escherichia coli*-expressed cat K were generated in-house by RT-PCR as will be described elsewhere (Kim et al., unpublished data). All reagents were purchased from Wako (Osaka, Japan). Tissue culture media and reagents, Fetal bovine serum (FBS) were from Gibco-BRL (Bethesda, MD, USA) or Jeil Biotech Services (Daegu, Korea). Z-Leu-Arg-4-MβNA was obtained from Bachem (King of Prussia, PA).

2.2. Animals

Five- to eight-week-old male ddY mice and newborn ddY mice were purchased from the Shizuoka Laboratories Animal Center (Shizuoka, Japan). Male and female heterozygotes (+op) of B6C3 mice were obtained from Jackson Laboratory (Bar Harbor, ME). The op/op homozygotes were radiologically distinguished at birth from the phenotypically normal (+/?) siblings, as described by Felix et al. [39].

2.3. Drynariae Rhizoma (DR) extracts preparation

DR extracts was mass-produced as for clinical use, were kindly supplied by the oriental Medical Hospital (herbarium specimen No. D-37-5) of Dongguk University, College of Oriental Medicine (Kyungju, Korea).

DR (60 g) extracted with 500 ml of boiling water for 3 h. After the extract was centrifuged at 7500 rpm for 30 min, the supernatant was lyophilized. For direct use, the extract solution was stored at 4 °C in aliquots. Depending upon the experimental group, DR was added (at 2% volume in culture medium) to or omitted from flasks.

2.4. Colorimetric MTT (tetrazolium) assay for cell viability

For the assay [40], cells were incubated in 96-well plates in the presence of various concentrations of DR. For MTT test of osteoblasts, 1.2×10^4 cells/well were added, the cells were cultured for 2 days without treatment to facilitate the attachment of cells and then various concentrations of DR were added. On the other hand, 1.3×10^2 osteoblasts cells/well and 1.52×10^4 mononuclear 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 DR. After various time intervals (1, 2 or 4 days), the supernatant was removed and 100 μl of MTT solution [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] (Sigma catalog no. M2128, Sigma; 1 mg/ml) was added to each well. The plate was incubated at 37 °C 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).

2.5. PCR cloning of a mouse Cat K gene-encoding cDNA fragment and expression in E. coli and preparation of rabbit Cat K antibody (CHK-2) using the recombinant Cat K protein as an antigen

Sense and antisense oligonucleotide primers based on the previously cloned sequence [28] were synthesized on an Applied Biosystems Instrument (ABI) Model 394 DNA Synthesizer by CoreBio System (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 [41]. 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 (Ref. [28], GenBank/EMBL accession number X94444). 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).

2.6. 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 (87Mem1) to the vitronectin receptor (v3) expressed on osteoclasts [30]. 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 tibiae of 5- to 8-week-old ddY mice as described [42]. Calvarial cells of op/op mice were also prepared according to a method previously reported [43]. Calvarial cells (1×10^6 cells) and bone marrow cells (1×10^7 cells) were cocultured in a 10-cm culture dish in α-Minimal Essential Medium (α-MEM) (Dainippon Pharmaceutical, Osaka, Japan) containing 10% fetal bovine serum (FBS) (Jeil Biotech Services, Daegu, Korea). Some cocultures of op/op calvarial cells and normal bone marrow cells were treated with rhM-CSF (100 ng/ml). After coculture for 7 days (the first coculture), floating cells were removed by washing with α-MEM, and the rest of the cells were recovered from the culture dish by thorough pipetting and filtrated through a 40 mm mesh (Falcon, Lincoln Park, NJ). The cells in the filtrate were collected by centrifugation and suspended in α-MEM containing 10% FBS (1 ml/10 cm dish). Aliquots of the cell suspension were cytocentrifuged on glass slides and stained with a Wright–Giemsa solution (Sigma). The cell suspension was applied to a Sephadex G-10 column (bed volume, 2 ml) (Pharmacia, Uppsala,

Sweden) and incubated for 10 min at room temperature. Then cells were recovered from the column by adding α-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).

2.7. 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 [44]. Cells released by collagenase digestions were washed and grown to confluent in 75 cm^2 culture flasks (Falcon) in (DMEM) supplemented with antibiotics (penicillin and streptomycin) and 10% fetal calf serum (FCS; Gibco-BRL). Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air; the medium was changed every 2–3 days. Cells were grown to confluence at 37 °C 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.

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

The fetal mouse long bone organ tissue culture system was based on that described by Raiz and Niemann [45]. Mouse long bone preparation was also described in our paper [46].

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 Raiz and Niemann [45] and James et al. [47]. 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, Taegu, Korea). The cells were then plated on either bone slices or on plastic tissue culture dishes and incubated at 37 °C prior to treatment.

2.9. Biosynthetic radiolabeling of target proteins by ^{35}S-isotope

Osteoclast containing cultures were washed with DPBS containing 5 mg/ml bovine serum albumin (BSA) and cultured in cysteine/methionine-free α-MEM containing HEPES (pH 7.2) for 60 min at 37 °C. The medium was removed and replaced with cysteine/methionine-free α-MEM containing 250 μCi/ml Trans [^{35}S]-Label (1000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA, USA) for 30 min at 37 °C. 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.

2.10. Sample collection and preparation

Cell cultures were lysed with 500 μl 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 min at 4 °C. Cultures were scraped with a rubber policeman and lysates were centrifuged for 5 min at 20,000 × g at 4 °C 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.

2.11. Immunoprecipitation, SDS-polyacrylamide gel electrophoresis and immunoblot analysis of the proteins

Cell lysate and media samples (450 μl) were incubated with 5 μl of polyclonal antibody CHK-2 (0.5 mg/ml stock), which recognizes both the pro and mature enzyme, followed by addition of 20 μl of protein A agarose and placed on a rotator overnight at 4 °C. Precipitates were centrifuged and washed twice with lysis buffer and once with lysis buffer

without Triton X-100. Precipitated proteins were resuspended in 30 μl of 4× sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer without 2-mercaptoethanol (nonreducing) and boiled for 5 min, as described by Laemmli [48]. Samples were applied to 12% SDS-PAGE gels and run at 30 mA/gel. Protein determination of the samples was carried out as described by Lowry et al. [49].

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., Uppsala, Sweden). For quantitative analysis of radiolabeld Cat K, gels were fixed, dried, and exposed to a phosphor screen, and then scanned using a phosphorimager analyzer (Fuji, Tokyo, Japan).

2.12. 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. [36] and the matrix metalloproteinase assay described by our group [50].

Bone cells were grown for 2 days after seeding 10^5 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% NaN_3, 100 mg/ml PMSF and 1% Triton X-100. The cell lysates was centrifuged at 10,000 × *g* for 10 min at 4 °C, and the supernatant was collected and stored at − 70 °C. 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-MβNA) (Bachem) and 2.0 βM ethylene-diamine tetraacetic acid (EDTA). After electrophoresis, the gels were washed for 1 h in 2.5% (v/v) Triton X-100 to remove SDS and then incubated for 24 h at 37 °C in 50 mM Tris–HCl (pH 7.5), 200 mM NaCl and 2.5 mM $CaCl_2$ to allow proteolysis of the synthetic cathepsin K/L substrate Z-Leu-Arg-4-MβNA substrate. After incubation, the gels were post-coupled for 30 min using 0.2 mg/ml 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).

2.13. Mouse osteoclast resorption assay

Osteoclast number was adjusted to 2×10^3 cells/ml and preincubated with test agent or with vehicle control for 30 min at 37 °C and then seeded onto bovine cortical bone slices (0.5 ml/well) in a 48 well plate. Following incubation for 24 h at 37 °C, culture supernatants were harvested and resorption was measured using a competitive ELISA, according to the manufacturer's protocol (Osteometer, 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.

3. Results

3.1. Cytotoxicity of DR on mixed-bone cells and osteoblasts cell population

In order to show that the DR is not cytotoxic at the concentrations used, the effects of various concentrations of DR on bone cell activities were evaluated using MTT assay as described below. Six different concentrations (500, 100, 50, 5, 1, and 0.1 μg/ml) were tested for 1, 2 and 4 days. Table 1 shows the effect of various concentrations of DR on different bone cells population measured by MTT assay. When mixed-bone cells are cultured with 500 μg/ml concentrations of DR for 24 h, there is a significant decrease in the mixed-bone cell population, while in the concentration of 100 μg/ml DR, 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 DR is < 100 μg/ml, the effect of DR on the mixed-bone cells disappeared. In this study, we selected the concentration of 100 μg/ml DR for the evaluation of further bone cell activities. When osteoblasts were cultured with DR for 1 day, the cell population showed significant increase in all the samples ($P < 0.05$) except the highest concentra-

Table 1
Changes in the cell population after adding various concentrations of DR into the bone cells or osteoblasts culture ($n=8$)

Treatment (μg/ml)	Optical density					
	Mixed-bone cells			Osteoblasts		
	1 day	2 days	4 days	1 day	2 days	4 days
Control	0.23 ± 0.04	0.33 ± 0.03	0.42 ± 0.03	0.12 ± 0.02	0.21 ± 0.01	0.27 ± 0.03
500	0.16 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.13 ± 0.02	0.17 ± 0.01
100	0.54 ± 0.05	0.72 ± 0.06	0.76 ± 0.04	0.14 ± 0.02	0.20 ± 0.02	0.26 ± 0.02
50	0.21 ± 0.03	0.35 ± 0.04	0.41 ± 0.04	0.15 ± 0.02	0.24 ± 0.02	0.27 ± 0.03
5	0.23 ± 0.03	0.32 ± 0.03	0.38 ± 0.04	0.15 ± 0.02	0.24 ± 0.03	0.27 ± 0.02
1	0.23 ± 0.02	0.25 ± 0.02	0.37 ± 0.03	0.14 ± 0.01	0.24 ± 0.02	0.27 ± 0.04
0.1	0.24 ± 0.03	0.25 ± 0.03	0.38 ± 0.04	0.14 ± 0.02	0.23 ± 0.01	0.26 ± 0.03

When mixed-bone cells are cultured with 100 μg/ml concentrations of DR for 24 h, there is a significant increase in the bone cell population ($P=0.0001$) and this effect persists till the end of 4 days' culture ($P=0.0001$). At the concentration of 100 μg/ml DR, there was a mild increase of cell population of the osteoblasts at the first day's culture ($P=0.0053$), the effect of increase in cell population disappeared at the second day ($P=0.1740$) and the fourth day's culture ($P=0.0710$).

tion (i.e. 500 μg/ml, $P=0.3640$). As the time interval of culture increases, the effect of increase in osteoblasts population was observed in the lower concentrations of DR. When osteoblasts were cultured with DR for 4 days, the increase in cell population was noted in the samples with the concentration of 0.1 μg/ml ($P=0.0265$) (Table 1). At the concentration of 100 μg/ml DR, there was a mild increase of cell population of the osteoblasts at the first day's culture ($P=0.0053$), the effect of increase in cell population disappeared on the second day ($P=0.1740$) and the fourth day's culture ($P=0.0710$) (Table 1).

3.2. Specific expression of Cat K in osteoclasts was elucidated by Cat K-recognizing polyclonal antibodies

To determine whether the osteoclast Cat K enzyme was catalytically active, an activity staining technique using zymography was performed on total bone cells including osteoclast and osteoblsts using a synthetic cathepsin K/L substrate (Z-Leu-Arg-4-MβNA). 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.

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, α vβ 3-positive osteoclasts) and osteoclast-depleted α vβ 3-negative cell mixtures (osteoblasts + stromal cells). As shown in Fig. 1, cat K expression is observed in both the total

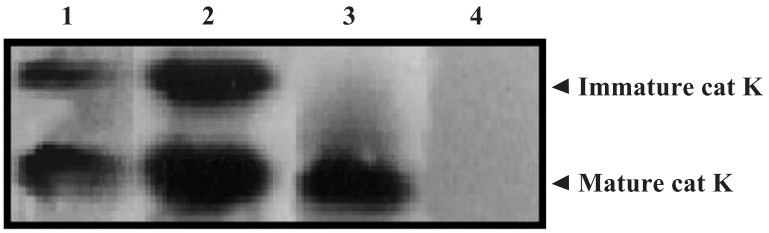

Fig. 1. Differential expression of immature and mature Cat K in cultured mouse osteoclasts and non-osteoclastic cells as confirmed by Western blot analysis. Osteoclasts were immunologically isolated from calvarial and fetal long bone cells using magnetic beads coated with an anti-vitronectin receptor monoclonal antibody as described in Section 2. 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 by their mobilities as indicated.

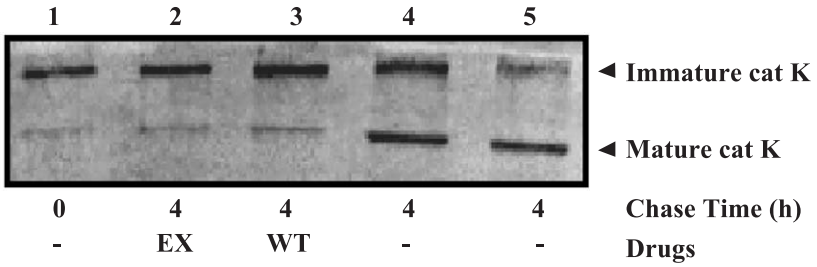

Fig. 2. Drynariae Rhizoma inhibit Cat K processing from immature proenzyme form to mature enzyme form. Osteoclasts were pulse radiolabeled and chased for the indicated times in the presence or absence of WT (1 μM) or DR (100 μg/ml). Cells and media were harvested at 0 and 4 h after chase and Cat K was immunoprecipitated using polyclonal antibody CHK-2 and analyzed by SDS-PAGE. Immature Cat K and mature Cat K are shown. EX indicates DR.

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 preoteolyically processed.

3.3. Wortmannin and Drynariae Rhizoma extract inhibits Cat K processing and bone resorption

The role of PI3-kinase in Cat K processing was investigated using the PI3-kinase inhibitor, WT and also using DR. Osteoclasts were pulse-labeled and pulse-chased in the presence or absence of WT (1 μM) and DR (100 μg/ml) for 4 h and the radiolabeled Cat K was analyzed by immunoprecipitation and SDS-PAGE.

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 DR, significant intracellular processing of immature Cat K were observed (lanes 4 and 5). However, treatment of osteoclasts with WT and DR inhibited the intracellular maturation of Cat K (lane 3 for WT and lane 2 for DR).

3.4. No effect of calphostin C, a specific inhibitor of protein kinase C (PKC), on Cat K processing and maturation

Furthermore, to determine whether Cat K processing and maturation involves protein kinase C (PKC) signaling, mouse total bone cells were pulse radiolabeled and chased for 0, 1 and 4 h in the presence or absence of 0.2 μM calphostin, a specific inhibitor of PKC [51]. Cat K was immunoprecipitated with polyclonal antibody CHK-2 on SDS-PAGE. Interestingly, no effect of calphostin C on Cat K processing and maturation by osteoclasts was observed in the mouse total bone cells including both osteoblasts and osteo-

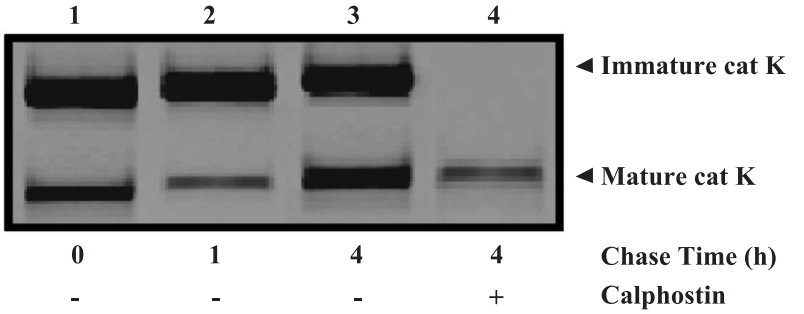

Fig. 3. Effect of calphostin C on Cat K processing and maturation by osteoclasts in the mouse total bone cells including both osteoblasts and osteoclasts. Osteoclasts were pulse radiolabeled and chased for the indicated times in the presence or absence of PKC inhibitor Calphostin C (0.2 μM). Cells and media were harvested at 0, 1 and 4 h after chase and Cat K was immunoprecipitated with polyclonal antibody CHK-2 and analyzed by SDS-PAGE. Immature Cat K and mature Cat K are indicated on the right of blot.

Table 2
Dose-dependent inhibition of Cat K processing by WT, calphostin C and Drynariae Rhizoma in the absence of M6P

Wortmannin (μM)				DR (μg/ml)				Calphostin C (μM)			
0	0.1	0.2	1.0	0	10	30	100	0	0.01	0.2	1.0
Labeled mature Cat K (arbitrary units)											
8.4 ± 0.2	8.3 ± 0.7	7.3 ± 0.6	4.2 ± 0.3	8.4 ± 0.4	8.4 ± 0.3	7.4 ± 0.3	4.8 ± 0.6	8.4 ± 0.5	8.38 ± 0.4	8.36 ± 0.5	8.3 ± 0.5
Processing (% inhibition)											
0	0.01 ± 0.0	13.2 ± 1.4	50.3 ± 7.4	0	0	11.6 ± 1.6	43.4 ± 6.4	0	0.1 ± 0.01	0.2 ± 0.01	0.1 ± 0.01

Pulse-radiolabeled osteoclasts were chased with increasing doses of WT and DR in the absence of M6P (5 μM) for 4 h. Cells extracts were immunoprecipitated with an anti-cathepsin K antibody as described. 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 ± S.E. values for both the immature and mature Cat K bands from phosphoimager analysis for each incubation condition ($n = 5$).

clasts (Fig. 3), indicating that calphostin C did not affect to osteoclast-mediated Cat K processing and maturation signaling. These results also strongly suggested that Cat K processing and maturation in osteoclasts is mediated by cAMP-dependent PKA pathway in mouse osteoclast cells.

3.5. Wortmannin and DR inhibit Cat K processing, where calphostin C, a specific inhibitor of protein kinase C (PKC) had no effect

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 resoprion caused by processed Cat K mature enzyme.

Therefore, we tested WT, calphostin C and DR 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 DR alone was dose-dependent with an IC_{50} of 1.0 μM and 134 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 ($IC_{50} = 0.25$ μM) and DR ($IC_{50} = 30$ μg/ml) (Table 3).

3.6. Dose-dependent inhibition of in vitro bone resorption by WT, calphostin C and DR

WT has previously been shown to inhibit bone resorption; therefore, we determined the ability of WT, calphostin C and DR to prevent in vitro bone

Table 3
Dose-dependent inhibition of cathepsin K processing in the presence of M6P by Wortmannin, calphostin C and Drynariae Rhizoma

WT (μM)				DR (μg/ml)				Calphostin C (0.2 μM)			
0	0.01	0.1	0.3	0	10	30	100	0	0.01	0.2	1.0
Labeled mature Cat K (arbitrary units)											
8.4 ± 0.2	7.1 ± 0.3	4.1 ± 0.5	3.7 ± 0.4	8.4 ± 0.5	7.4 ± 0.5	4.3 ± 0.3	4.0 ± 0.5	8.4 ± 0.6	8.36 ± 0.7	8.36 ± 0.8	8.38 ± 0.9
Processing (% inhibition)											
0	15.5 ± 2.1	50.9 ± 6.7	55.7 ± 6.4	0	11.6 ± 2.3	49.2 ± 5.1	52.4 ± 4.9	0	0.2 ± 0.01	0.2 ± 0.01	0.1 ± 0.01

Pulse-radiolabeled osteoclasts were chased with increasing doses of WT, calphostin C and DR in the presence of M6P (5 μM) for 4 h. Cells extracts were immunoprecipitated with an anti-cathepsin K antibody as described. Cat K was quantitated 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 ± S.E. values for both the immature and mature Cat K bands from phosphoimager analysis for each incubation condition ($n = 5$).

Table 4
Dose-dependent inhibition of in vitro bone resorption by WT, calphostin C and DR

WT (μM)				DR (μg/ml)				Calphostin C (0.2 μM)			
0	0.1	0.2	1.0	0	10	30	100	0	0.01	0.2	1.0
Resorption (% inhibition)											
0	12.3 ± 1.5	57.3 ± 6.3	61.2 ± 5.6	0	9.2 ± 1.2	47.5 ± 3.7	59.3 ± 4.2	0	0.3 ± 0.01	0.3 ± 0.01	0.4 ± 0.02

Osteoclasts were cultured on bone particles and treated with various doses of WT, calphostin C and DR 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 ± S.E. values ($n = 5$).

resorption under similar conditions. WT and DR 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 DR treatment as shown in Table 4. However, no any inhibitory effect was observed when treated with calphostin C.

4. Discussion

There are several reports that demonstrated an improvement in clinical association with the use of traditional Korean medicine in the treatment of fractures [52]. Traditional oriental 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. One of the traditional Korean medicines, Drynariae Rhizoma [*D. fortunei* (kunze) J. Sm] (Gu-Sui-Bu in Chinese name), had been alleged to have therapeutic effects on bone healing [53].

In a previous study for evaluation of the cytotoxic and antioxidant effects of the water extract of DR, Sun et al. [12] and Liu et al. [53] reported that DR is not only non-cytotoxic but also has an anti-oxidative effect on osteoblasts [9,12]. Administration of DR can accelerate the speed of intracellular ALP synthesis by the bone cells. Since a major feature of bone is its continuous remodeling, the molecular constituents of bone are closely related to this process. The osteopontin and osteonectin mRNA expression was significantly down regulated in the bone cells cultured with DR [12]. They demonstrated that DR has potential effects on the bone cells culture and that one of the major effects of DR on the bone cells is probably mediated by its effect on the osteclasts attachment.

In this study, we examined the relationships between bone cells and this specific herbal medicine DR, which may contribute to the possible justification for the clinical application in the treatment of bone disease. We have also found that DR is non-cytotoxic and the addition of DR into the culture medium will affect the cell population of the bone cells. When the concentration of DR is < 100 μg/ml, the effect of DR on the mixed-bone cells disappeared (Table 1). Therefore, we selected the concentration of 100 μg/ml DR 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 the mechanisms of how 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 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 [54]. The enhanced potency of M6P on WT and DR 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 DR effects was observed in this study. Furthermore, the fact that DR also shows an enhancement in the presence of M6P may indicate that DR

operates through a similar mechanism as that for WT. It is, therefore, suggested that DR 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 [55]. 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 [56], ruffled border formation [57] and bone resorption both in vitro and in vivo [18,51]. 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, DR 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 DR occurred at concentrations consistent with the inhibition of Cat K processing. Therefore, WT and DR may exert its antiresorptive effects on osteoclasts, in part by reducing lysosomal pools of catalytically active Cat K at least.

Results on Cat K processing were consistent with the osteoclast-selective expression of Cat K observed by immunolocalization and in situ hybridization as described by Drake et al. [31]. Furthermore, 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 DR inhibited the intracellular maturation of Cat K, suggesting that WT and DR 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 [51], 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.

In summary, these results strongly suggested that DR could be useful for preventing both postmenopausal osteoporosis and osteoporosis associated with the ovary function failure. Our data suggested that mature, catalytically active Cat K is produced in bone resorption and that DR 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. Although the study had limitations and our findings are preliminary, continued and advanced study on the alterations in gene expression of bone cells by DR will provide a basis for understanding the observed bone cell responses to various pharmacological interventions.

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