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Potential anti-osteoporotic activity of low-molecular weight hyaluronan by attenuation of osteoclast cell differentiation and function in vitro

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ABSTRACT

Due to some severe side effects or lack of efficacy of currently used synthetic drugs, such as bisphosphonates (BPs), the search for new therapeutic agents that can more effectively prevent and treat osteoporosis (OP) has been an increasingly important topic of research. In this study, the low-molecular weight hyaluronan (LMW-HA, 50 kDa) produced by enzymatic degradation of high-molecular weight hyaluronan (HMW-HA, 1922 kDa) from *Streptococcus zooepidemicus* was evaluated in vitro for its anti-osteoclastogenic potentials using RAW 264.7 murine macrophage cells. LMW-HA (25–200 μg/ml) dose-dependently inhibited the receptor activator of NF-κB ligand (RANKL)-induced tartrate-resistance acid phosphatase (TRAP) activity and the formation of multinucleated osteoclasts. Western blot analysis showed that LMW-HA reduced the RANKL-induced expression of tumor necrosis factor receptor-associated factor 6 (TRAF6), gelsolin and c-Src-proline-rich tyrosine kinase 2 suggesting that it could inhibit actin ring formation of osteoclast cells. In addition, LMW-HA inhibited the bone resorption activity of osteoclastic cells by dose-dependently attenuating the RANKL-induced expression of carbonic anhydrase II and integrin β3. RT-PCR analysis showed that LMW-HA dose-dependently decreased the expression of osteoclast-specific genes, such as matrix metalloproteinase 9 (MMP-9) and cathepsin K, suggesting that it has potential to inhibit the differentiation of osteoclastic cells. Taken collectively, these results suggested that LMW-HA (50 kDa) has significant anti-osteoporotic activity in vitro and may be used as a potent functional ingredient in health beneficial foods or as a therapeutic agent to prevent or treat OP.

1. Introduction

Osteoporosis (OP) is caused by an imbalance between osteoblastic bone formation and osteoclastic bone resorption during bone remodelling processes, and it is a very serious disease, especially in elderly people [1]. Several compounds and therapies, including bisphosphonates (BPs), calcium, vitamin D, calcitonin, parathyroid hormone, and hormone replacement therapy (HRT), have been widely used in the modern clinical practice for the prevention and treatment of OP [2]. However, some severe side effects or lack of efficacy of currently used synthetic drugs have been reported. Although BPs are the most effective anti-resorptive drugs currently available, it has been reported that they cause serious side effects, such as osteonecrosis of the jaw and renal failure [3]. Therefore, searching for new therapeutic agents that can more effectively prevent and treat OP has been an increasingly important topic of research [2].

Hyaluronan or hyaluronic acid (HA) is a linear glycosaminoglycan polymer with a high-molecular mass ranging from approximately 2×10^5 to 10×10^6, and it is comprised of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid. In the human body, HA is found abundantly in bone marrow and connective tissues, and it is also found in the skin, vitreous humour of the eye, cartilage, and umbilical cord tissues. HA is also an essential component of the extracellular matrix (ECM). The functions of HA vary from structural functions to the regulation of several cellular responses, including proliferation, differentiation, motility, adhesion and gene expression [4,5]. HA is also present as a mucoid capsule of certain microbial strains, including streptococci species [6]. Due to its unique moisturising retention ability, viscoelasticity and biocompatibility, HA has wide applications in

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the cosmetic, biomedical, and food industries as an effective agent for wound healing, osteoarthritis treatment, and drug delivery [7,8]. For industrial application purposes, HA was produced traditionally by extraction from rooster comes, and it is now produced mainly by microbial fermentation [9]. However, despite the potential health beneficial features of HA as an industrially important biomaterial, its high-molecular size and low bioavailability limits its applicability to various industries. For example, it has been reported that low-molecular weight HA (LMW-HA, 50 kDa) penetrates skin much better than high-molecular weight HA (HMW-HA, 800 kDa) resulting in stronger gene regulatory activity than the larger HA [10]. In this regard, we have tried to produce lower-molecular weight HA by enzymatic degradation of microbial HMW-HA. We isolated a microbial strain and identified as a strain of *Vibrio splindidus* BST-398 (KCTC 11899) that can degrade HMW-HA (1922 kDa), produced from the fermentation broth of *Streptococcus zooepidemicus*, into a smaller sized HA (LMW-HA, 50 kDa) [11].

Several reports have demonstrated that HMW-HA has the potential to antagonise osteoclastogenic activity and bone resorption by suppressing osteoclast differentiation and production of osteoclastogenic cytokines [12,13]. However, there is no available report regarding the anti-osteoporotic and anti-osteoclastogenic activities of LMW-HA (50 kDa). The present study demonstrated that LMW-HA is a potent inhibitor of osteoclastogenesis in RANKL-induced RAW 264.7 cells.

2. Materials and methods

2.1. Materials

Foetal bovine serum (FBS), penicillin–streptomycin, and trypsin–EDTA were purchased from Lonza (Walkersville, MD, USA). 3-(4,5-Dimethylthiazol-yl)-diphenyl tetrazoliumbromide (MTT) was provided by DUCHEPA Biochemie (Haarlem, Netherlands). Minimum essential medium alpha (MEM-α) and Dulbecco's modified eagle's media (DMEM) were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Antibodies against integrin β3 and carbonic anhydrase II were purchased from Cell Signalling Technology (Beverly, MA, USA). The receptor activator of NF-κB ligand (RANKL) was obtained from Peprotech (Rocky Hill, NJ, USA). Low-molecular weight hyaluronan (LMW-HA, 50 kDa) was prepared by enzymatic hydrolysis of high-molecular weight hyaluronan (HMW-HA, 1922 kDa), which was produced by microbial fermentation of *S. zooepidemicus* [14]. The enzyme reaction was performed for 4 h at 30 °C with a portion of culture filtrate of *Vibrio splindidus* BST-398 (KCTC 11899) as a crude enzyme preparation and HMW-HA as the substrate [11]. The chemical composition and molecular size of resulting LMW-HA were determined by size-exclusion column chromatography using a Shodex OHpak column (SB-806HQ, 8.0 × 300 mm, Showa Denko Co., Japan). Experimental details will be published elsewhere.

2.2. RAW 264.7 cell viability and osteoclast differentiation

Murine macrophage RAW 264.7 cells (Mouse leukaemic monocyte macrophage cell line, ATCC® TIB71™) were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM medium (pH 7.4) supplemented with 10% FBS, 2 mM glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C under a humidified condition of 5% CO_2. For osteoclast differentiation, cells were seeded in 24-well plates at a density of 1×10^4 cells/well, and the cells were cultured for 5 days in MEM-α containing 10% FBS and RANKL (50 ng/ml) in the absence or presence of LMW-HA (25–200 μg/ml). Cell culture medium was changed every 2 days. Cell toxicity was determined using a colourimetric assay based on the uptake of MTT by viable cells. After the exposure to various concentrations of LMW-HA for 2 days, cells were treated with 1 mg/ml MTT solution and incubated at 37 °C for 4 h resulting in the formation of blue formazan crystals. Absorbance was measured at 570 nm.

2.3. Measurement of TRAP activity

For measurement of the tartrate-resistance acid phosphatase (TRAP) activity as an osteoclastic marker in RAW 264.7 cells, cells were fixed in 4% formalin solution for 10 min and 95% ethanol for 1 min. Subsequently, the dehydrated cells were incubated in 10 mM citrate buffer (pH 4.6) containing 10 mM sodium tartrate and 5 mM p-nitrophenylphosphate for 1 h. The reaction mixtures were then transferred onto new plates, and an equal volume of 0.1 N NaOH was added. Absorbance was measured at 405 nm by a spectrophotometer, and the TRAP activity was expressed as percent of TRAP activity of untreated control cells. RAW 264.7 cells were fixed in 4% formalin solution for 10 min and stained with a commercial leukocyte acid phosphatase kit (Sigma–Aldrich Chemicals) according to the manufacturer's recommended protocol for TRAP staining. TRAP-positive multinucleated cells were visualised under light microscopy and considered as differentiated osteoclasts.

2.4. Bone resorption assay

The bone resorption of the differentiated RAW 264.7 cells was assayed by a bone resorption assay kit (CosMo Bio, Tokyo, Japan). RAW 264.7 cells were suspended in phenol red-free MEM-α supplemented with 10% FBS, seeded at a density of 1×10^4 cells/well and maintained for 5 days in the absence or presence of RANKL (50 ng/ml) and LMW-HA (25–200 μg/ml). Cells were then washed with 6% NaOCl to measure resorption pit areas of differentiated RAW 264.7 cells. The resorbed areas on the plate were visualised under light microscopy.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared using Trizol (Invitrogen, Carlsbad, CA, USA), and cDNAs were synthesised from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). cDNA (1 μL) was amplified with the indicated specific primers. The primer sequences were designed for mouse genes as follows: MMP-9, 5′-TAC CCG AGT GGA CGC GAC CG-3′ and 5′-CAG GAA GAC GAA GGG GAA GAC GC-3′; cathepsin K, 5′-GCC TAG CGA ACA GAT TCT CAA CAG C-3′ and 5′-TAC CCG CGC CAC TGC TCT CTT-3′; and β-actin, 5′-TGC TGT CCC TGT ATG CCT CT-3′ and 5′-AGG TCT TTA CGG ATG TCA ACG-3′. The PCR assay consisted of 31 cycles of 40 s at 60 °C (MMP-9), 26 cycles of 40 s at 59 °C (cathepsin K), and 30 cycles of 40 s at 55.6 °C (β-actin). The number of cycles for each gene was selected to be in the range of linear amplification through an optimisation experiment. The PCR products were separated on a 2% agarose gel and visualised by ethidium bromide staining. The bands were visualised using a TFX-20 M model-UV transilluminator (Vilber-Lourmat, Marne-la-Vallee, France), and gel photographs were obtained.

2.6. Western blot analysis

Western blot analysis was performed using cell lysates prepared from cultured RAW 264.7 cells. Equal amounts of cell lysates were separated on 6–12% SDS–PAGE gels and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by agitating membranes in a 5% non-fat milk blocking solution for 3 h. The membranes were incubated with antibodies against intergrin

β3, carbonic anhydrase II, tumor necrosis factor receptor-associated factor 6 (TRAF6), c-Src, PYK2, and gelsolin at 4 °C overnight, and they were then washed six times in TBST solution for 5 min. HRP-conjugated immunoglobulins were used as secondary antibodies. Following secondary antibody incubation, the membranes were washed six times in TBST solution for 5 min. The bound antibodies were detected using Supersignal West Pico Chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL) by exposing to Konica X-ray film (Konica, Tokyo, Japan). β-actin or β-tubulin levels were used as loading controls.

2.7. Statistical analysis

The results were expressed as the means ± SEM for each treatment group in each experiment. The statistical analysis was performed using Statistical Analysis Systems software package (SAS Institute, Cary, NC, USA). Significance was determined by one-way analysis of variance followed by Duncan's range test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. LMW-HA suppresses RANKL-induced osteoclast formation in RAW 264.7 cells

To determine whether LMW-HA is detrimental to RAW 264.7 cells, the effect of LMW-HA on the viability of RAW 264.7 cells was examined by MTT assay after treatment with LMW-HA. As shown in Fig. 1A, the viability of RAW 264.7 cells was not influenced by treatment with LMW-HA (25–200 μg/ml) for 24 h, showing that LMW-HA is not toxic to these cells in the range of concentrations tested. To evaluate whether LMW-HA inhibits the differentiation of RAW 264.7 macrophages into multinucleated osteoclasts in response to RANKL treatment, the TRAP activity was measured spectrophotometrically at 405 nm (Fig. 1B). LMW-HA suppressed RANKL-induced TRAP activity in RAW 264.7 macrophages in a dose-dependent manner. This result was further confirmed by TRAP staining as shown in Fig. 1C. While undifferentiated control cells showed no TRAP staining, the control cells (Con) treated with only RANKL (50 ng/ml) showed heavily stained multinucleated regions, indicating that the cells were fully differentiated into osteoclasts. However, the TRAP-positive multinucleated regions started to gradually disappear with increasing concentrations of LMW-HA, demonstrating that LMW-HA inhibited the differentiation of RAW 264.7 cells.

3.2. Inhibitory effects of LMW-HA on bone resorption

The $\alpha_v\beta_3$ integrin regulates cell migration and maintenance of the sealing zone required for effective osteoclastic bone resorption [15], and carbonic anhydrase II is involved in active bone resorption by acidification resulting from production of protons in the cytoplasm and subsequent transport through the ruffled border into the resorption lacuna [16]. Thus, the effects of LMW-HA on the bone resorption activity of osteoclast cells plated on calcium phosphate-coated plates and on the expression levels of integrin β3 and carbonic anhydrase II were examined. As shown in Fig. 2A, the undifferentiated control plate coated with calcium phosphate and cultured for 5 days showed no difference, but the control plate (Con) treated with only RANKL (50 ng/ml) showed large resorption areas. However, LMW-HA (25–200 μg/ml) dose-dependently decreased the resorption area, demonstrating that LMW-HA inhibits osteoclastic bone resorption. This result was further confirmed by measuring the effect of LMW-HA on the RANKL-induced expression of carbonic anhydrase II and integrin β3 by Western blot analysis (Fig. 2B). LMW-HA attenuated the expression of both proteins in a dose-dependent manner. These results suggested that LMW-HA is effective in delaying acidification by carbonic anhydrase II and inhibiting cell migration and attachment, which resulted in suppression of the bone resorption activity of osteoclast cells.

3.3. Effects of LMW-HA on expression of osteoclast-specific genes in RAW 264.7 cells

Functions of cathepsin K and MMP-9 in the regulation of osteoclast differentiation have recently been reported [17,18]. Therefore,

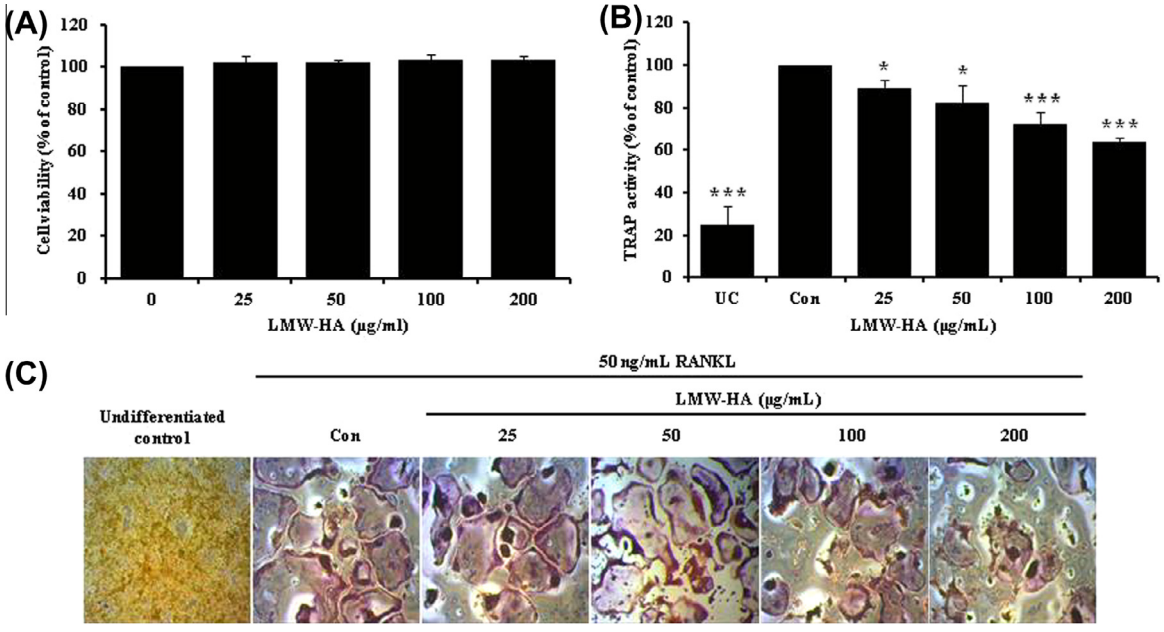

Fig. 1. Effect of LMW-HA on cytotoxicity and TRAP activity in RAW 264.7 cells. Cells were cultured in DMEM containing LMW-HA (0–200 μg/ml) for 24 h and cell viability was measured by MTT assay (A). Cells were cultured with or without LMW-HA (25–200 μg/ml) in the absence or presence of RANKL for 5 days. TRAP activity was measured at 405 nm (B). Cells were stained using a leukocyte acid phosphatase kit and TRAP positive multinucleated osteoclasts were visualised under the light microscopy (C). Values represent the means ± SEM of three separate experiments. $^{*}P < 0.05$ vs Con (control); $^{***}P < 0.001$ vs Con.

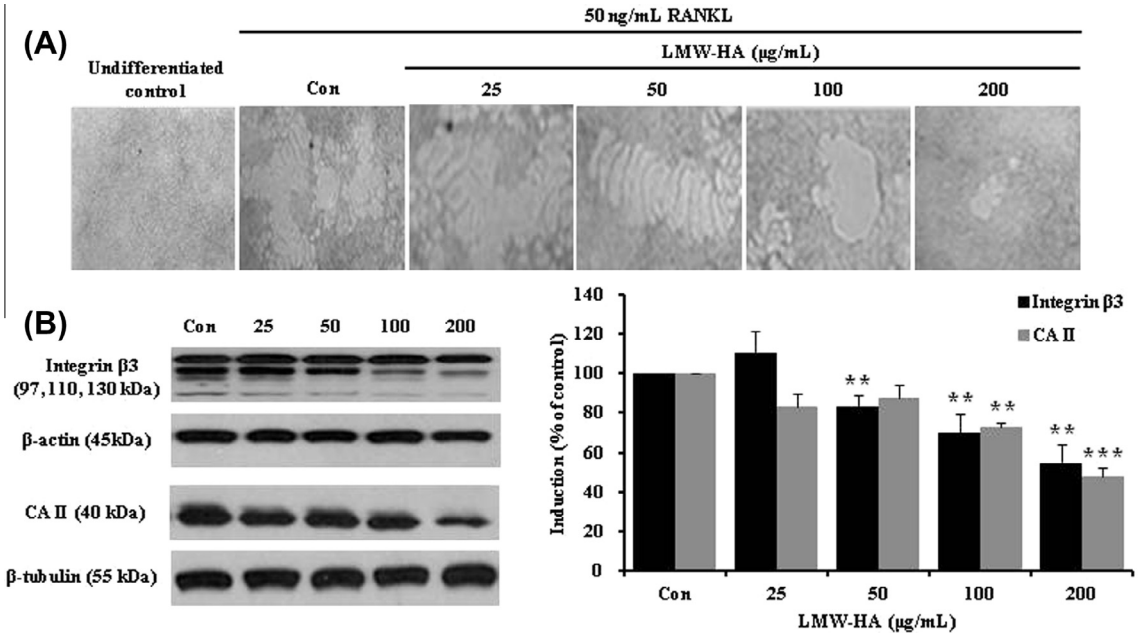

Fig. 2. Effect of LMW-HA on RANKL-induced bone resorption (A) and protein expressions of carbonic anhydrase II and integrin β3 (B) in RAW 264.7 cells. Cells were treated with or without LMW-HA (25–200 μg/ml) and cultured in the absence or presence of RANKL (50 ng/ml) for 5 days. The osteoclast bone resorption was assayed with a commercially available bone resorption kit (A). Cell lysates were subjected to SDS–PAGE and Western blot analysis against carbonic anhydrase II and integrin β3 (B). Each value was normalised to β-actin or β-tubulin and expressed as means ± SEM of three separate experiments. $^{**}P < 0.01$ vs Con (control); $^{***}P < 0.001$ vs Con.

the mRNA expression levels of these genes were analysed to clarify molecular mechanisms underlying the inhibitory activity of LMW-HA on RANKL-induced osteoclast differentiation. The mRNA levels of cathepsin K and MMP-9 were determined after differentiation induction in RAW 264.7 cells in the presence of LMW-HA. The results indicated that LMW-HA significantly suppressed the expression levels of these genes compared to the control (Con) treated with RANKL only (Fig. 3).

3.4. Disturbance of cytoskeletal organisation in RANKL-induced osteoclasts by LMW-HA

It has been known that TRAF6-c-Src-PYK2 signalling pathway plays a vital role in cytoskeletal organisation by forming actin [19–22]. Thus, the effects of LMW-HA on the expression levels of these proteins or their activities were determined by Western blot analysis. The treatment of cells with LMW-HA (25–200 μg/ml) for 5 days significantly repressed the RANKL-induced protein expression of TRAF6 (Fig. 4A) and dose-dependently inhibited the phosphorylation of c-Src (Fig. 4B) and PYK2 (Fig. 4C). LMW-HA also significantly attenuated the expression of gelsolin (Fig. 4D), which is involved in the osteoclast activation-required cell adhesion as well as extensive actin filament assembly and disassembly. These results suggested that LMW-HA inhibits the bone resorption activity of osteoclast cells by inhibiting cytoskeletal organisation and osteoclast function through attenuation of c-Src kinase activity and PYK2 activation as well as by suppressing gelsolin expression.

4. Discussion

Bone remodelling is a tightly regulated process to repair skeletal damage and replace old bones with new ones. The old bone is removed by osteoclasts, which is sequentially followed by the formation of mineralised bone matrix by osteoblasts [1,23]. The excessive bone resorption over bone formation leads to developing skeletal diseases, such as osteoporosis [23]. Interfering RANKL pro-

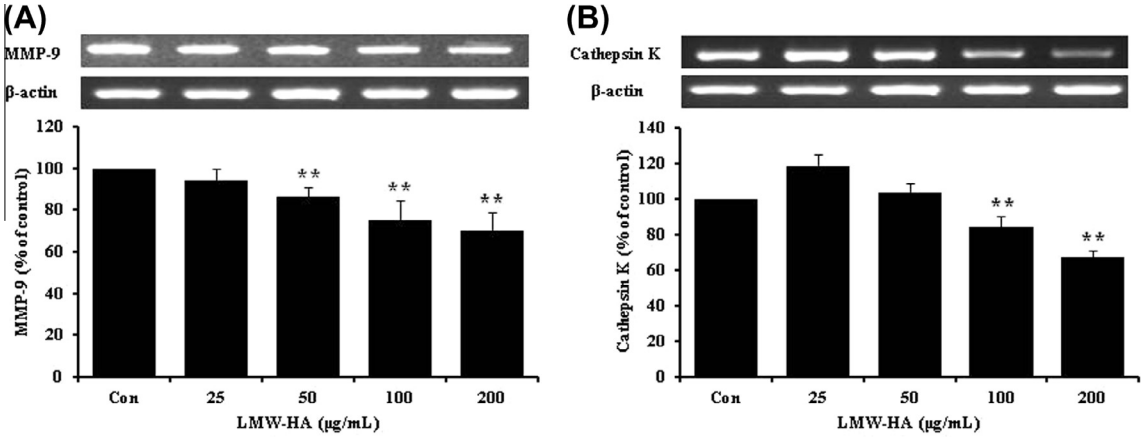

Fig. 3. Effects of LMW-HA on the expression of RANKL-induced MMP-9 (A) and cathepsin K (B) in RAW 264.7 cells. Cells were treated with or without LMW-HA (25–200 μg/ml) and cultured in the absence or presence of RANKL (50 ng/ml) for 5 days. mRNA expression levels of the indicated gene were determined by RT-PCR. Values represent the means ± SEM of three separate experiments. $^{**}P < 0.01$ vs Con (control).

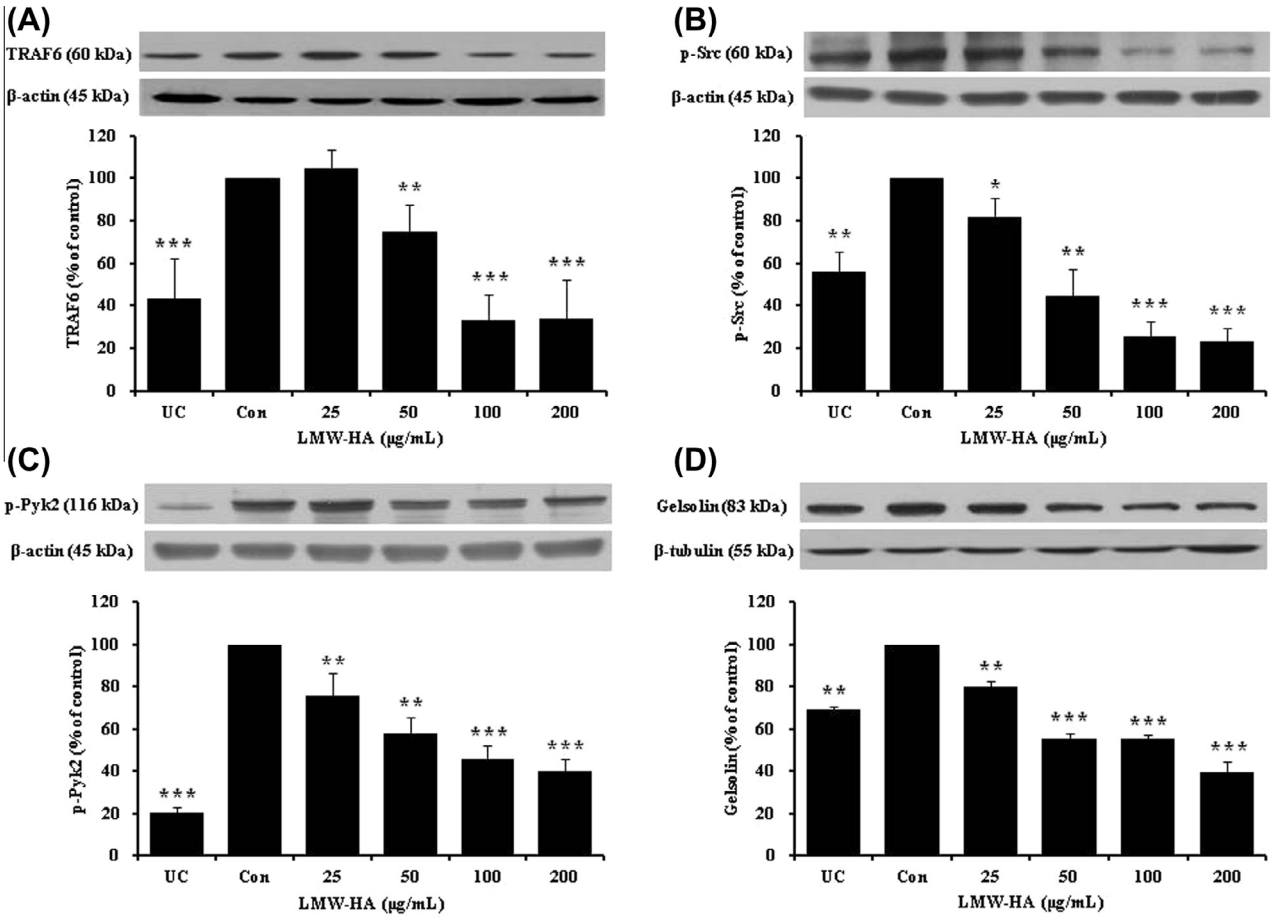

Fig. 4. Effect of LMW-HA on TRAF6 expression (A), p-Src activation (B), PYK2 activation (C), and gelsolin (D). RAW 264.7 cells were treated with or without LMW-HA (25–200 μg/ml) and cultured in the absence or presence of RANKL (50 ng/ml) for 5 days. Cell lysates were subjected to SDS–PAGE and Western blot analysis against TRAF6, p-Src, p-PYK2, and gelsolin. Each value was normalized to β-actin or β-tubulin and expressed as means ± SEM of three separate experiments. $^{**}P < 0.01$ vs Con (control); $^{***}P < 0.001$ vs Con.

duction of osteoblasts or antagonising RANKL binding to its receptor, RNAK, of osteoclasts might prevent osteoclasts from excessive differentiation. Impairing the RANK signalling pathway could be a therapeutic target for pathological bone loss diseases [24]. It has been previously reported that HA shows an anti-osteoporotic activity by enhancing osteoblast differentiation induced by BMP-2 [25]. However, in our preliminary study with LMW-HA, we couldn't find any beneficial effect in osteoblast differentiation. LMW-HA did not change mRNA expressions of osteoblast differentiation marker such as osteocalcin, collagen type-1, osteopontin and alkaline phosphatase in differentiating osteoblast (data not shown). Therefore, this study was aimed to investigate if LMW-HA interferes with the RANKL actions responsible for osteoclastogenesis. Interestingly, the results of this study showed that LMW-HA significantly retarded osteoclast differentiation of RAW 264.7 macrophages by diminishing TRAP activity without any detectable level of cytotoxicities at the concentrations tested (25–200 μg/ml). HA-induced cytotoxicities have not been reported in cell lines or in humans [12,13,26].

Osteoclastic bone resorption involves a series of regulatory phases as follows: migration of osteoclasts to the resorption site, attachment of osteoclasts to the calcified tissue, development of the ruffled border and the clear zone, and secretion of acids by carbonic anhydrase and lysosomal enzymes, such as cathepsin K, and MMP-9, into the space under the ruffled border [27]. The binding of RANKL to RANK triggers the activation of various signalling cascades during osteoclast differentiation and activation. TRAF6 is recruited and trimerised by interaction of RANKL and RANK. In our study, the reduction of TRAF6 expression by LMW-HA weakened osteoclast bone resorption activity. Gelsolin, an actin-binding, severing and capping protein, is a crucial regulator of actin filament assembly and disassembly [19]. Gelsolin is involved in the osteoclast activation requiring cell adhesion and extensive actin cytoskeletal reorganisation [20]. LMW-HA suppressed the RANKL-induced gelsolin expression and formation of actin rings, thereby suggesting that this compound interrupted cytoskeletal reorganisation during osteoclast differentiation through blocking the RANK-TRAF6 pathway. PYK2 is known to physically interact with and activate gelsolin, and the PYK2-gelsolin pathway is involved in regulating actin cytoskeletal organisation in osteoclasts [20]. LMW-HA inhibited the RANKL-induced activation of PYK2, which is required for the formation of actin rings, by decreasing gelsolin binding to actin monomers. c-Src is a ubiquitously expressed non-receptor tyrosine kinase and is involved in the regulation of many cellular events, such as cell adhesion, growth, migration and survival, and plays a unique role in bone metabolism, including actin organisation [21]. c-Src knockout mice have more than a twofold increase in the number of osteoclasts compared to normal mice, and they do not form the ruffled borders of osteoclasts, which implicates that c-Src is related to osteoclast function rather than osteoclast differentiation [22]. Thus, it may be possible that LMW-HA interferes with either cytoskeletal organisation or cellular function of osteoclast cells by suppressing c-Src kinase activity. Accordingly, the activity of TRAP and the induction of cathepsin K and MMP-9 (osteoclastogenic marker proteins) were examined in differentiating osteoclasts. Cathepsin K and

MMP-9 that are localised exclusively in osteoclasts of the bone tissues have been suggested to be involved in the osteoclastic bone resorption process by facilitating the migration of osteoclasts [28]. The present study demonstrated that LMW-HA inhibited RANKL-induced osteoclastogenesis by dampening the secretion of the three osteoclastogenic proteolytic enzymes, namely TRAP, cathepsin K, and MMP-9. In addition, LMW-HA also diminished integrin β3 induction in RANKL-induced osteoclasts. Integrin $\alpha_v\beta_3$, another player in osteoclastogenesis, has been shown to regulate cell migration and maintenance of the sealing zone required for effective osteoclastic bone resorption [15]. On the other hand, the results of this study showed that LMW-HA effectively suppressed the RANKL-induced expression of carbonic anhydrase II. It has been documented that active bone resorption involves acidification resulting from production of protons in the cytoplasm by carbonic anhydrase II and their subsequent transport through the ruffled border into the resorption lacuna by an osteoclast-specific V-ATPase. Therefore, it appeared that LMW-HA abolished the acidification and demineralisation of the bone matrix of sealed lacunae by suppressing RANKL-induced expression of carbonic anhydrase II.

In conclusion, the present study clearly demonstrated that LMW-HA (50 kDa) produced by enzymatic degradation of HMW-HA (1922 kDa) has potential inhibitory activity on RANKL-induced osteoclastogenesis by the down-regulation of various signalling molecules in osteoclast differentiation and bone resorption. LMW-HA also suppressed RANKL-induced cellular expression of gelsolin responsible for actin ring formation. Furthermore, the reduction of RANKL-induced osteoclastic gelsolin expression by LMW-HA was achieved by disturbing the c-Src-PYK2 signalling pathway. LMW-HA dampened the differentiation of macrophage lineage to TRAP-positive multinucleated osteoclasts and suppressed the bone resorption by attenuating the RANKL-induced integrin β3 and carbonic anhydrase II production. LMW-HA also remarkably reduced the expression levels of MMP-9 and cathepsin K, which are specific markers for osteoclast differentiation. Taken collectively, these results strongly suggested that LMW-HA can be used as a potent functional ingredient in health beneficial foods or as a therapeutic agent to prevent or treat OP. Future studies are required to determine if LMW-HA could be equally effective in vivo.

Acknowledgments

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