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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

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