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Effect of hyaluronan on osteoclast differentiation in mouse bone marrow-derived cells cocultured with primary osteoblasts

Shuitsu Hirota[1], Akiyo Kawamoto[2], Yoshihiro Yoshikawa[3], Takashi Ikeo[3] and Yutaka Komasa[2]

[1]Graduate School of Dentistry (Department of Geriatric Dentistry), [2]Department of Geriatric Dentistry and [3]Department of Biochemistry, 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan

Hyaluronan (HA) is widely used as a disease-modifying drug to reduce pain in patients with osteoarthritis. We investigated whether the commercially available HA product Artz® contributes to bone regeneration. To assess whether exogenous HA influences bone resorption and formation, we examined osteoclast differentiation in mouse bone marrow-derived cells cocultured with primary mouse calvarial osteoblasts, and also analyzed the proliferation and adhesion of these cells. Coculture of osteoblasts and bone marrow cells in the presence of HA resulted in decreased osteoclast differentiation. Cellular proliferation of osteoblasts and bone marrow cells in the presence of HA was significantly greater than in the absence of HA. However, adhesion of bone marrow cells was decreased by HA. The increased proliferation of osteoblasts and decreased number of attached bone marrow cells in the presence of HA lead to an imbalance between the number of these cells, resulting in suppressed osteoclast differentiation. (J Osaka Dent Univ 2015 ; 49(1) : 27–33)

Key words : Hyaluronan ; Coculture ; Osteoclast differentiation

INTRODUCTION

Glycosaminoglycans (GAG) are major components of the extracellular matrix present in many tissues. In bone, hyaluronan (HA) accounts for 4–7% of total GAG.[1] GAGs have both structural and functional roles in the regulation of biological processes including cellular growth, migration, and differentiation.[2–5] GAGs also enhance the biological activity of bone morphogenetic proteins (BMPs).[6] HA hydrogel-delivered BMP-2 precomplexed with dermatan sulfate or heparin can induce bone formation *in vivo.*[7, 8] Furthermore, biglycan regulates osteoclast differentiation through its effect on osteoblasts and their precursors.[5]

HA is an unsulfated polymer of repeating D-glucuronic acid and N-acetylglucosamine disaccharide units[9] which regulates cell proliferation, cell motility, and tissue repair.[10–15] It is a widely used as a disease-modifying drug for reducing pain associated with osteoarthritis.[16] Evidence for the efficacy of HA in reducing pain has been demonstrated in animal experimental models,[17–19] *in vitro* experiments,[20, 21] and clinical trials.[22] Several studies have focused on the possibility that bone regeneration could be induced by the presence of a HA scaffold.[7, 23, 24] For example, HA enhanced BMP-2 induction of osteoblastic differentiation in osteoblast-like cells.[8, 25, 26]

The properties of HA differ depending on the molecular mass of the polymer.[21, 27, 28] Huang *et al.* reported that low molecular mass HA (60 kDa) increased cell growth and osteocalcin mRNA expression, while high molecular mass HA (900 or 2300 kDa) stimulated ALP activity and cell mineralization in rat calvarial osteoblasts.[13] Sasaki *et al.* applied high molecular mass HA (1900 kDa) to bone wounds following bone marrow ablation and observed maintenance of osteoinductive growth factors within the local environment and accelerated new bone formation via mesenchymal cell differentiation within the wound.[29] However, Ariyoshi *et al.* reported that low molecular mass HA ($<$8 kDa) elevated tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cell formation,[30] while high molecular mass HA (2500 kDa) down-regulated differentiation of osteoclast-like cells.[31]

Ideally, a therapeutic biomaterial applied to a bone defect would transiently suppress bone resorption and concurrently increase bone formation. The desirable characteristics of a carrier material in such a biomaterial include biocompatibility, biodegradability, and an osteoconductive capability. We hypothesized that the commercially-available therapeutic Artz® (Seikagaku Kougyo, Osaka, Japan), which is an 800–1200 kDa HA molecule used for the treatment of osteoarthritis, could serve as such a carrier. To assess whether exogenous HA influences bone resorption and formation, we examined osteoclast differentiation in mouse bone marrow-derived cells cocultured with primary mouse calvarial osteoblasts, and measured proliferation and adhesion of each cell type in the presence of HA.

MATERIALS AND METHODS

Reagents

HA (ARTZ Dispo® 25 mg, molecular mass : 800–1200 kDa) was purchased from Seikagaku Kogyo, Osaka, Japan. TRAP buffer solution was prepared by mixing 0.1 M sodium acetate and 50 mM tartaric acid (both from Wako Pure Chemicals, Osaka, Japan) in a 1 : 1 ratio, followed by adjustment to pH 5. TRAP staining solution was prepared by mixing 50 mL of TRAP buffer solution, 5 mg of naphthol AS-MX phosphate (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mL of N, N-dimethylformamide (Wako Pure Chemicals), and 25 mg of Fast Red Violet LB salt (Sigma-Aldrich).

Cell culture

Primary osteoblasts were isolated from the calvaria of 1-day-old newborn male Slc : ddY mice. Bone marrow cells were obtained from tibias of the 6-8-week-old mice. To generate osteoclasts, these cells were cocultured in α-modified minimal essential medium (Wako Pure Chemicals) containing 10% fetal bovine serum (FBS ; Equitech-Bio, Kerrville, TX, USA), 100 units/mL penicillin G sodium, 100 μg/mL streptomycin and 292 μg/mL L-glutamine (Invitrogen, Carlsbad, CA, USA) in the presence of 10^{-8} M 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$; Wako Pure Chemicals) and 10^{-6} M prostaglandin E_2 (PGE_2 ; Wako Pure Chemicals) at 37°C in 5% CO_2. Bone marrow cells were also cultured in growth medium with 100 ng/mL receptor activator of nuclear factor-κB ligand (RANKL ; Wako Pure Chemicals) and 10^{-4} U/mL macrophage colony-stimulating factor (M-CSF ; Wako Pure Chemicals) to induct osteoclast differentiation without osteoblast involvement. For PCR analysis, primary osteoblasts were cultured in growth medium with or without $1,25(OH)_2D_3$ and PGE_2 for a day. All experimental protocols involving animals were reviewed and approved by the Animal Committee of Osaka Dental University (Approval no : 23–4–24), and conformed with procedures described in the Guiding Principles for the Use of Laboratory Animals.

TRAP staining

Primary osteoblasts (6.0×10^3, 3.0×10^3, 1.5×10^3, 0.75×10^3, or 0 cells/well) and bone marrow cells (5.0×10^3, 2.5×10^3, 1.25×10^3, 0.625×10^3 cells/well) were cocultured in all combinations in a 96-well plate for 8 days to determine the optimum number of cells for maximal osteoclast differentiation. To investigate the effect of HA on osteoclast differentiation, cells were cocultured in growth medium in the presence of 10^{-8} M $1,25(OH)_2D_3$ and 10^{-6} M PGE_2 with or without 1 mg/mL HA for 8 days. Differentiation of bone marrow cells to osteoclasts occurred after 4 days. Osteoclasts were identified by staining for TRAP activity. Cells were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) and a 1 : 1 acetone : ethanol mixture. Fixed cells were treated with TRAP staining solution for 20 minutes, washed with water, and observed under a light microscope. TRAP-positive multinucleated cells containing at least three nuclei were counted as osteoclasts.

Cell proliferation

Osteoblasts and bone marrow cells were seeded in 96-well plates containing growth medium. Following attachment of cells, wells were washed with PBS and growth medium with or without 1 mg/mL HA was added. After 3 days, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was conducted according to manufacturer's protocol to determine the number of viable cells. Absorbance at 490–650 nm was determined using a microplate reader (SpectraMax Plus 384 ; Molecular Devices, Sunnyvale, CA, USA)

mRNA expression

Total RNA was isolated from cultured cells using the illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Little Chalfont, UK). The quantity and purity of extracted RNA was determined spectrophotometrically at 260 and 280 nm. One microgram of total RNA was reverse-transcribed into cDNA using the High Capacity RNA-to-cDNA Master Mix including random hexamers and oligo (dT) primers (Applied Biosystems, Foster, CA, USA). Relative levels of mRNA expression were measured by quantitative real-time PCR using predesigned and preformulated gene-specific primer and probe sets for osteoclast differentiation factor molecules (TaqManQ Gene Expression Assays ; Applied Biosystems).

Analysis was performed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems) as per the manufacturer's protocol. Relative gene expression levels in multiplex reactions were quantified using the comparative Ct method by normalizing the amount of target to endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Probes for detecting cDNA encoding RANKL, osteoprotegerin (OPG), and GAPDH were designed and synthesized by Applied Biosystems according to gene sequences registered in the Celera Data Base. The assay IDs for TaqMan Gene Expression Assay probes are : RANKL, Mm00441908_mL ; OPG, Mm 00435452_mL ; and GAPDH, 4352932E.

Cell adhesion

Bone marrow cells (5.0×10^3 cells/well) were seeded in growth medium in 96-well plates. After 1 hour incubation, unattached cells were removed by rinsing with PBS. The total number of cells adherent to the plate was quantified using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Absorbance was measured at 490–650 nm using a microplate reader.

Statistical analysis

Statistical differences were determined using the Student's t-test. All data are expressed as the mean and standard deviation (SD). A p-value of less than 5% was regarded as significant.

RESULTS

Primary osteoblasts and bone marrow cells were cocultured to determine the optimum number of cells for maximal osteoclast differentiation. The combination of 3.0×10^3 osteoblasts and 5.0×10^3 bone marrow cells was selected for subsequent experiments (Fig. 1). To investigate the effect of HA on osteoclast differentiation, primary osteoblasts and bone marrow cells were cocultured in the presence of $1,25(OH)_2D_3$ and PGE_2 with or without HA. The number of TRAP-positive multinucleated cells was significantly decreased when the cells were treated with HA (Fig. 2).

As HA treatment suppressed osteoclast differentia-

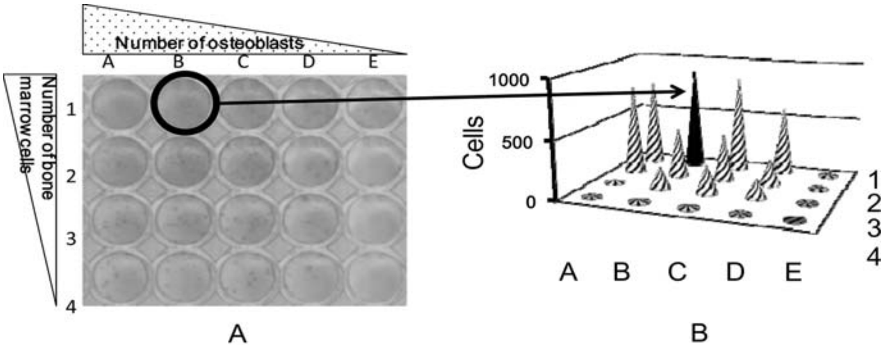

Fig. 1 Primary osteoblasts and bone marrow cells cocultured and stained for TRAP (A) and the number of TRAP-positive multinucleated cells scored (B).

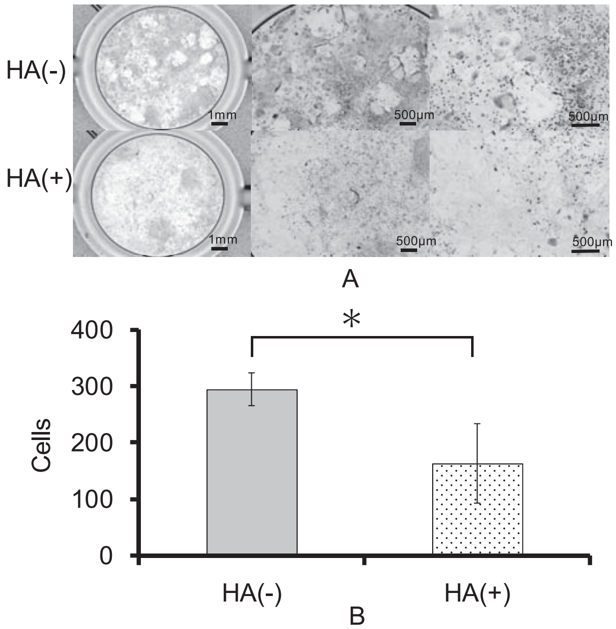

Fig. 2 Primary osteoblasts and bone marrow cells cocultured with or without HA, and stained for TRAP (A) and the number of TRAP-positive multinucleated cells scored (*$p<.05$) (B).

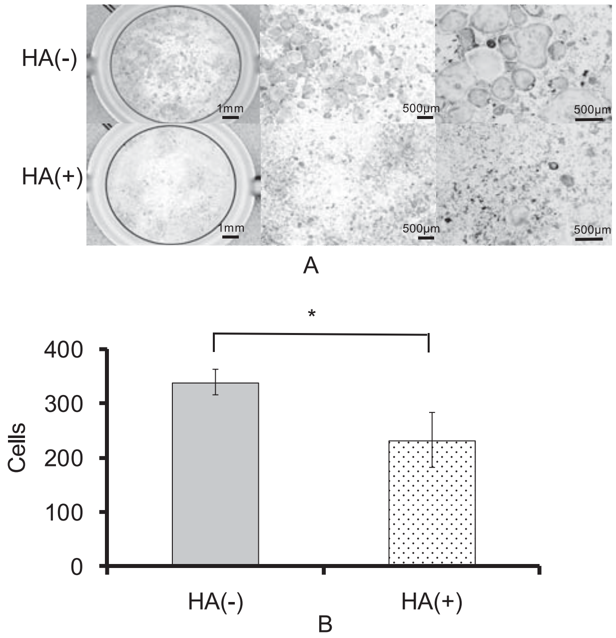

Fig. 4 Bone marrow cells cultured in media containing RANKL and M-CSF with or without HA, and stained for TRAP (A) and the number of TRAP-positive multinucleated cells scored (*$p<.05$) (B).

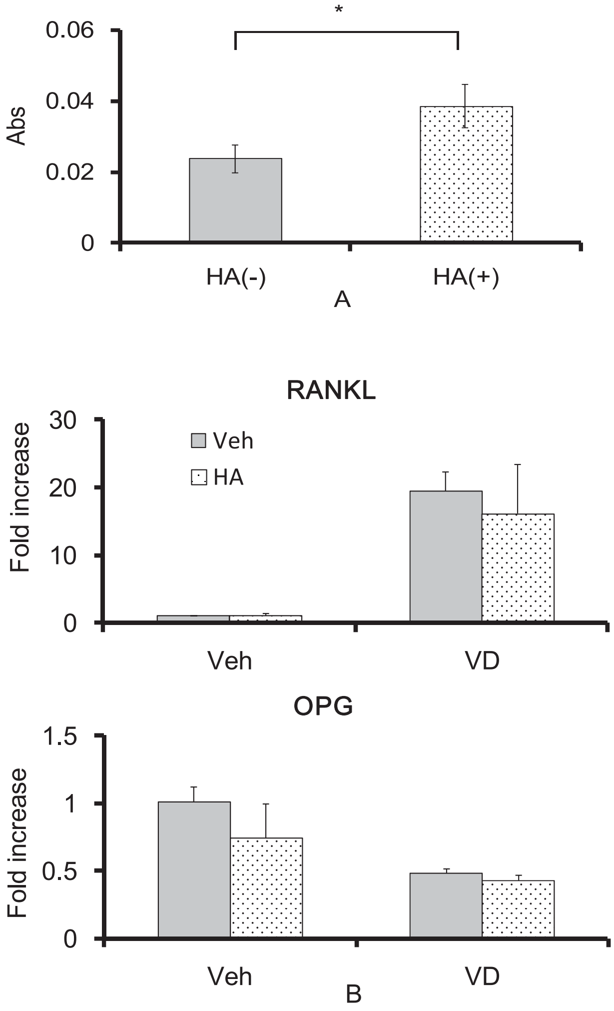

Fig. 3 Proliferation of primary osteoblasts treated with HA (*$p<.05$) (A) and mRNA expression of RANKL and OPG in primary osteoblasts with or without HA (B).

tion under coculture conditions, we next examined the effect of HA on primary osteoblasts and bone marrow cells separately. The number of mouse calvarial osteoblasts was markedly increased in the presence of HA compared with the control (Fig. 3 A). Furthermore, the mRNA expression levels of the osteoclast differentiation-inducing factor RANKL and the decoy RANKL receptor OPG were assessed in mouse calvarial osteoblasts treated with HA. No significant difference in RANKL or OPG expression was identified between cells cultured with or without HA (Fig. 3 B).

We next cultured bone marrow cells alone with or without HA to eliminate the influence of osteoblasts. Significantly fewer osteoclasts had differentiated in the presence of HA (Fig. 4). The effects of HA on cell proliferation and attachment in bone marrow cells

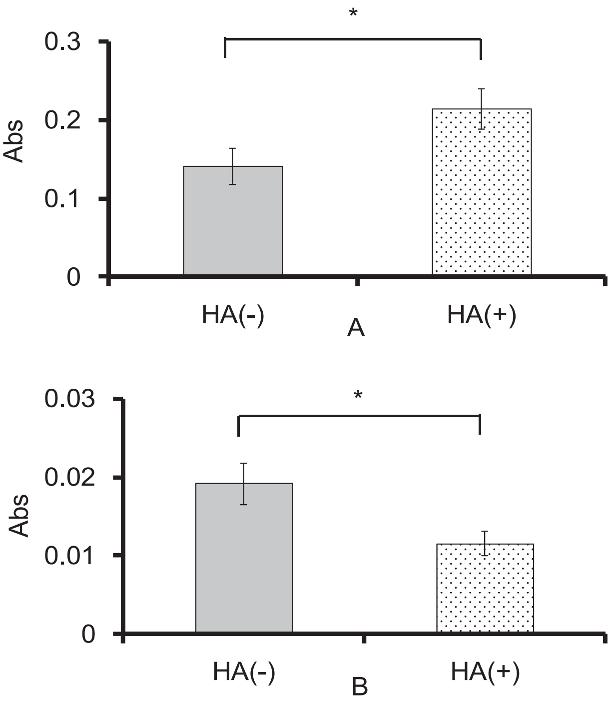

Fig. 5 Proliferation of bone marrow cells treated with HA (A) and cell adhesion of bone marrow cells treated with HA (*$p < .05$) (B).

were then evaluated. Bone marrow cell proliferation increased significantly when cells were incubated with HA (Fig. 5 A). Furthermore, the number of bone marrow cells attached to culture plates 1 hour after seeding decreased when cells were treated with HA compared with the control (Fig. 5 B).

DISCUSSION

Bone remodeling is necessary for the maintenance of bone tissue. However, an imbalance in this process can cause abnormal bone resorption. Bone resorption is commonly observed in dentistry in the form of periodontal disease, osteoarthritis of the temporomandibular joint, or during orthodontic treatment. Although osteoclast precursor cells are present throughout the body, mature osteoclasts responsible for active bone resorption and osteoblasts required for new bone formation are observed only in bone tissue. Takahashi *et al.* focused on the locations at which these cells can be found, and determined that osteoblasts are involved in regulating the differentiation of osteoclast precursor cells in the spleen.[32] They also reported that $1,25(OH)_2D_3$ and parathyroid hormone produced by osteoblasts play a key role in osteoclast differentiation.[33] This indicates that osteoblast dynamics must also be considered when studying osteoclast differentiation. We therefore examined the influence of HA on osteoclast differentiation and its effects on osteoblasts.

TRAP staining revealed that co-cultures of osteoblasts and bone marrow-derived cells at different cell number ratios yielded different numbers of osteoclasts. Subsequent co-culture of osteoblasts and bone marrow cells at the combination that produced the most osteoclasts was performed in the presence of HA. Here, fewer TRAP-positive multinucleated cells were detected. While the molecular weight of HA used in our study is different, this result is consistent with a report from Ariyoshi *et al.,*[30] who found that high-molecular-mass HA (2500 kDa) inhibited osteoclast differentiation.

We next studied the effects of HA on osteoblast dynamics, as they can influence osteoclast differentiation. Osteoblast proliferation increased in the presence of HA. Quantitative polymerase chain reaction was also used to examine the mRNA expression of several genes implicated in cell proliferation, although no significant differences were observed (data not shown). In vascular smooth muscle cells, the binding of high-molecular-weight HA to CD44 inhibits entry into S phase in response to a strong mitogenic stimulus, while the binding of low-molecular-weight HA to CD44 stimulates G1 phase progression and S phase entry.[34] High-molecular-weight HA bound to CD44 selectively inhibits Rac and Rac-dependent signaling to the cyclin D1 gene, whereas low-molecular-weight HA binding to CD44 selectively stimulates ERK activation and ERK-dependent cyclin D1 gene expression.[35, 36] Although CD44 is thought to be involved in this system, it is possible that these findings reflect differences in CD44 expression or the involvement of other receptors.

Expression of RANKL by osteoblasts plays a key role in osteoclast differentiation. RANKL binding of RANK on the surface of osteoclast precursor cells activates a pathway to promote differentiation of mature osteoclasts. Factors contributing to increased expression of RANKL include aging[37] and inflammation.[38] We observed no significant difference in the expression of RANKL or OPG mRNA by osteoblasts in the presence or absence of HA. In contrast, others have reported that high-molecular-weight HA could promote[39] or inhibit[30] RANKL mRNA expression. Although the molecular weight of HA used in this earlier study was similar to that used in our current investigation, it is possible that the concentrations used were different.

Altered RANKL expression was not responsible for the inhibition of osteoclast differentiation observed following HA treatment. We therefore examined the influence of HA on osteoclast differentiation using bone marrow cells cultured in media containing M-CSF and RANKL to exclude the effect of osteoblasts. HA treatment of osteoclast precursor cells in the absence of osteoblasts yielded reduced numbers of TRAP-positive multinucleated cells. Furthermore, HA enhanced the proliferation of bone marrow cells and impaired their adhesion to culture vessels. Chang *et al.* have reported that the toll-like receptor 4 (TLR-4), and not CD44, was involved in inhibition of osteoclast differentiation in the presence of HA.[40] CD44 was not detected in our present study (data not shown), so we speculate that TLR-4 was involved instead.

In summary, the increased proliferation of osteoblasts and decreased cell attachment by bone marrow cells in the presence of HA leads to an imbalance in the number of these cells, resulting in suppressed osteoclast differentiation. This suggests that application of HA to periodontal tissue as a carrier could positively influence bone tissue regeneration by transiently inhibiting osteoclast differentiation while promoting osteoblast proliferation.

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