Hyaluronan inhibits osteoclast differentiation via Toll-like receptor 4

Eun-Ju Chang[1,*], Hyon Jong Kim[1,*], Jeongim Ha[1], Hyung Joon Kim[1], Jiyoon Ryu[1], Kwang-Hyun Park[2], Uh-Hyun Kim[2], Zang Hee Lee[1], Hyun-Man Kim[1], David E. Fisher[3] and Hong-Hee Kim[1,‡]

[1]Department of Cell and Developmental Biology and Brain Korea 21 Program, DRI, Seoul National University, Seoul 110-749, Korea
[2]Department of Biochemistry and Institute of Cardiovascular Research, Chonbuk National University Medical School, Jeonju 561-182, Korea
[3]Division of Pediatric Hematology/Oncology and Melanoma Program in Medical Oncology, Dana-Farber Cancer Institute and Children's Hospital, Harvard Medical School, Boston, MA 02115, USA

*These authors contributed equally to this work
‡Author for correspondence (e-mail: hhbkim@snu.ac.kr)

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Summary

The differentiation of osteoclasts, cells specialized for bone resorption, is governed by two key factors, macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). The extracellular matrix (ECM) is an important factor influencing cell fate. To date, little investigation on the relationship between ECM components and osteoclast differentiation has been documented. In this study, we uncovered a potent anti-osteoclastogenic effect of hyaluronan (HA), an ECM component present in bone marrow and soft connective tissues, in primary mouse and human osteoclast precursor cell cultures. The anti-osteoclastogenic function of HA was dependent on Toll-like receptor 4 (TLR4) but not on CD44. HA inhibited M-CSF-dependent signaling pathways involving Rac, reactive oxygen species and mitogen-activated protein kinases, resulting in suppression of transcription factors AP-1 and MITF that control RANK expression. Furthermore, in an in vivo mouse model of calvarial bone resorption assays HA reduced RANKL-induced bone erosion and osteoclastogenesis. Our results clearly show that HA inhibits osteoclast differentiation through TLR4 by interfering with M-CSF signaling, and point that the interaction between ECM components and innate immune receptors can play an important role in the regulation of bone metabolism.

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Key words: Hyaluronan, Osteoclast, Toll-like receptor, Receptor activator of nuclear factor kappaB, Macrophage colony stimulating factor

Introduction

Osteoclasts are cells specialized for the function of bone catabolism and play, together with osteoblasts, important roles in skeletal development, bone remodeling and fracture healing. These cells are generated from monocyte/macrophage lineage of hematopoietic cells through a sophisticated differentiation program. Macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) (Tanaka et al., 1993) are the two key factors essential for osteoclast differentiation (Takahashi et al., 2002; Teitelbaum, 2000; Lee and Kim, 2003). M-CSF is required for both proliferation and differentiation of osteoclast precursors (Tanaka et al., 1993). This cytokine also stimulates migration and supports survival of osteoclast lineage cells during differentiation (Tanaka et al., 1993; Felix et al., 1994). RANKL plays a crucial role for the commitment to osteoclast lineage and the fusion of committed cells to achieve multinucleated feature of mature osteoclasts. In addition, RANKL is also an important activating and survival factor for mature osteoclasts (Jimi et al., 1999).

Hyaluronan (hyaluronic acid, HA) is a glycosaminoglycan polymer of repeating disaccharide units consisted of D-glucuronic acid and N-acetylglucosamine. It is a major component of the extracellular matrix (ECM) and normally present as high molecular mass (several thousand kDa) in mammalian bone marrow and loose connective tissues (Laurent and Fraser, 1992). In the human bone marrow microenvironment, HA is produced by both stromal and hematopoietic cells and detected on sinusoidal endothelium and endosteum as well as stromal ECM (Avigdor et al., 2004). Together with a structural role in the matrix, HA regulates diverse cellular responses including proliferation, differentiation, motility, adhesion and gene expression (Lee and Spicer, 2000). Several HA-binding proteins have been documented and CD44 is the one most extensively studied. This ubiquitous HA receptor has functions in the regulation of ECM turnover and the migration of lymphocytes during inflammation (Stoop et al., 2002). Other less-well characterized HA receptors include RHAMM, LYVE-1, layilin, HARE, stabilin, and Toll-like receptor 4 (TLR4) (Kim et al., 2004). In a manner dependent on TLR4, low molecular mass (LMM)-HA has been shown to activate human dendritic cells and to stimulate endothelial recognition of injury, whereas high molecular mass (HMM)-HA deactivated human monocytes (Termeer et al., 2002).

HA is clinically used for joint and cartilage diseases such as

osteoarthritis (Goldberg and Buckwalter, 2005). Suppression of cytokine-induced expression of matrix metalloproteinases (MMPs) in articular cartilage was suggested to be one of the explanations for the therapeutic efficacy of HA (Julovi et al., 2004). Reduced production of inflammatory mediators PGE_2 and IL-6 by osteoarthritic subchondral osteoblasts may also contribute to the effects of HA (Lajeunesse et al., 2003). In addition, HA was shown to enhance both proliferation and differentiation of rat calvarial osteoblasts (Huang et al., 2003). However, some studies have implicated HA in bone resorption, although its molecular mechanism remains to be elucidated (Prince, 2004). By contrast, little information on the relationship between HA and osteoclast differentiation has been documented. In fact, there have been no reports describing the effects of ECM on osteoclast differentiation.

In the present study, we have investigated the effects of HMM-HA on osteoclastogenesis in mouse primary bone-marrow-derived macrophages and human peripheral blood cells. We found that HMM-HA profoundly inhibits osteoclast differentiation in a manner dependent on TLR4. HMM-HA interferes with M-CSF signaling in osteoclast precursors, causing decreases in the activation of AP-1 and MITF transcription factors and the expression of RANK. Finally, preventive effects of HMM-HA on in vivo bone resorption were observed in calvarial bone erosion assays.

Results

HMM-HA decreases osteoclastogenesis from BMMs but not from RAW264.7 cells

HA is present as a HMM form with an average molecular mass of several thousand kDa under physiological conditions (Takahashi et al., 2004; Dahl et al., 1985). We investigated the effects of HA prepared from human umbilical cord on osteoclastogenesis using two widely used mouse cell models, primary bone marrow-derived macrophage (BMM) and RAW264.7 cells. BMMs differentiate to osteoclasts in the presence of M-CSF and RANKL, whereas RAW264.7 cells form osteoclasts in the presence of RANKL alone. HA profoundly inhibited the generation of $TRAP^+$ multinuclear cells ($TRAP^+$ MNCs; differentiated osteoclasts) from BMMs cultured with RANKL and M-CSF (Fig. 1A,B). About 90% inhibition was observed at a concentration of 2 μg/ml HA (Fig. 1B). By contrast, HA had no effect on osteoclastogenesis of RAW264.7 cells cultured with RANKL alone (Fig. 1C). HA did not cause any toxicity to BMM or RAW264.7 cells at the concentrations used (data not shown), indicating that the anti-osteoclastogenic effect of HA could not be attributed to cytotoxicity. The inhibitory effect of HA on osteoclastogenesis was also observed with human peripheral blood monocytic cells (PBMCs) (Fig. 1D).

Next we investigated whether the HMM property is required for the anti-osteoclastogenic effects of HA. The HA preparation used was treated with hyaluronidase or pronase before being added to the culture. Hyaluronidase treatment, but not pronase treatment, abolished the anti-osteoclastogenic activity of HA (Fig. 2A). We also examined other HA preparations from different sources with known molecular mass. 1300-kDa HA displayed some inhibitory effect on osteoclastogenesis from BMMs (Fig. 2B) with a potency much weaker than the HA preparation in the experiments described

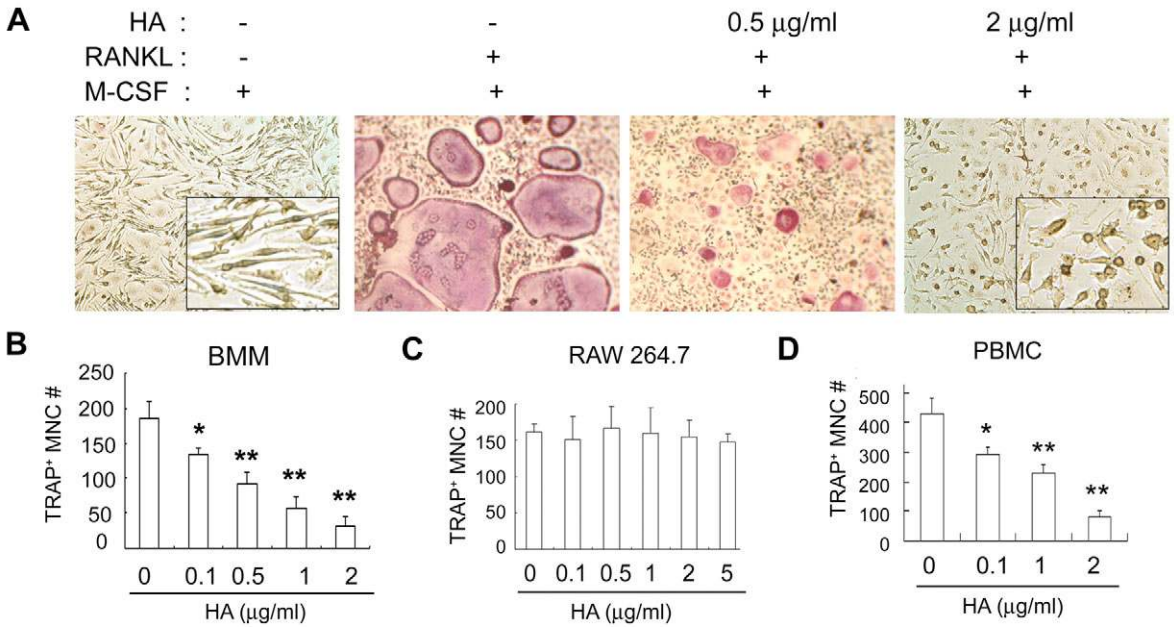

Fig. 1. Inhibition of osteoclast differentiation from BMMs and PBMCs by HA. (A,B) Effect of HA on osteoclastogenesis from BMMs. Mouse primary BMMs were cultured for 6 days with RANKL (100 ng/ml), M-CSF (30 ng/ml) and indicated concentrations of HA. After TRAP staining, $TRAP^+$ multinuclear cells ($TRAP^+$ MNCs) with more than three nuclei were scored as osteoclasts. $*P<0.05$ and $**P<0.01$ compared with vehicle-treated control. (C) No effects of HA on osteoclastic differentiation of RAW264.7 cells. Cells were cultured for 4 days with RANKL (100 ng/ml) and HA (0.1-5 μg/ml) and stained for TRAP, and $TRAP^+$ MNCs were counted. (D) Effect of HA on human PBMC differentiation to osteoclasts. Human PBMCs were cultured for 9 days with RANKL (200 ng/ml), M-CSF (30 ng/ml) and indicated concentrations of HA. Cells were stained for TRAP and $TRAP^+$ MNCs were counted. $*P<0.05$ and $**P<0.01$ compared with vehicle-treated control.

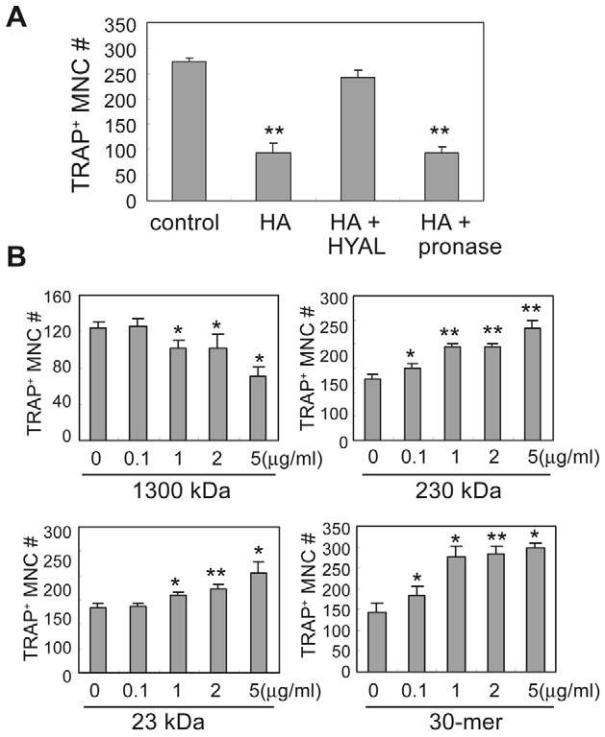

Fig. 2. Requirement of HMM HA for osteoclastogenesis suppression. (A) Hyaluronidase treatment abolishes the effect of HA. HA preparation from umbilical cord (1 μg/ml) was digested with 150 U/ml hyaluronidase (HYAL) or pronase for 30 minutes followed by boiling for 10 minutes. Digested and undigested HA was added to BMMs and osteoclastogenesis was induced by culturing the cells with RANKL (100 ng/ml) and M-CSF (30 ng/ml) for 6 days. After TRAP staining, $TRAP^+$ MNCs were scored. $^{**}P<0.01$ compared with vehicle-treated control. (B) Stimulatory effects of LMM-HA on BMM osteoclastogenesis. BMMs were cultured for 4.5 days with RANKL (100 ng/ml), M-CSF (30 ng/ml), and indicated concentrations of different sizes of HA. Cells were stained for TRAP and $TRAP^+$ MNCs were counted. $^{*}P<0.05$ and $^{**}P<0.01$ compared with vehicle-treated control.

in Fig. 1. By contrast, 230-kDa, 23-kDa and 30-mer HA had stimulatory effects (Fig. 2B). Collectively, these data suggest that HMM-HA has inhibitory and LMM-HA has stimulatory effects on osteoclast differentiation. To know the nature of the HA preparation from umbilical cord used in our study, its molecular mass was determined by gel-permeation chromatography. The HA preparation was found to be heterogeneous and the molecular mass ranged from 13,715 kDa to 25 kDa with the average mass of 846 kDa. Taken into account the data shown in Fig. 2A,B, the HA responsible for the anti-osteoclastogenic activity of the heterogeneous HA preparation must be the HMM component.

CD44 is not involved in the inhibition of osteoclastogenesis by HMM-HA

HA is known to bind several receptors. Among them, CD44 is the most extensively characterized receptor for HA. CD44 is expressed in osteoclasts and has been shown to regulate cell migration (Spessotto et al., 2002; Suzuki et al., 2002; Chellaiah et al., 2003). To explore whether CD44 mediates the anti-osteoclastogenic effects of HA on BMMs, we first examined the CD44 expression level by FACS analyses. HA had no effect on the cell surface levels of CD44 (Fig. 3A). Next, effect of a CD44-neutralizing antibody (Ab) (Cao et al., 2005) on osteoclast differentiation was assessed. The CD44 blockade did not affect $TRAP^+$ MNC formation either alone or in combination with HA (Fig. 3B). The lack of effect of the CD44 Ab was not due to its inefficacy because this Ab was active in suppressing BMM migration in the presence of osteopontin (another CD44 ligand) (Fig. 3C). HA had no effect on this osteopontin-CD44 mediated response (Fig. 3C). This CD44 Ab also reduced HA binding to CD44 in HEK-293 cells transfected to overexpress CD44 (see supplementary material, Fig. S1), demonstrating its capacity to neutralize CD44-HA interaction. A recent report showed that osteoclast formation was not defective in $CD44^{-/-}$ cells (de Vries et al., 2005). This report further supports the notion that CD44 involvement in HA-mediated osteoclastogenesis suppression is unlikely.

Anti-osteoclastogenic function of HMM-HA is mediated by TLR4

We next investigated whether TLR4 is the receptor that mediates the inhibitory function of HMM-HA on osteoclast differentiation. To this end, a neutralizing Ab against human TLR4 (Shimazu et al., 1999) was tested in PBMC cultures. The TLR4-neutralizing Ab blocked the HA-induced inhibition of PBMC differentiation to osteoclasts (Fig. 4A). Next, BMMs were derived from C3H/HeJ mice, which have a missense mutation in the *TLR4* gene (Poltorak et al., 1998) and effects of HA were tested. HMM-HA had no effect on the $TRAP^+$ MNC formation from the TLR4 mutant BMMs (Fig. 4B). These results indicate that the inhibitory effect of HA on osteoclastogenesis was mediated by TLR4.

BMMs express TLR4 on the cell surface (see supplementary material, Fig. S2). To elicit HA responses through TLR4, HA may associate directly or indirectly with the receptor. In order to know whether HA interacts with TLR4 and whether specificity is present in this interaction, we transfected HEK-293 cells with various plasmids encoding TLR members or with a control vector. The transfected cells were then incubated with FITC-conjugated HA for FACS analyses. The TLR4-transfected cells showed strong binding to FITC-HA, whereas TLR2-transfected cells displayed a very weak binding to FITC-HA (Fig. 4C). No HA-binding was detected in cells transfected with TLR5 and TLR9 (Fig. 4C). These data suggest that HA is likely to bind TLR4 to cause its anti-osteoclastogenic effects. However, we can not exclude the possibility that the increased HA binding ability to TLR4-transfected cells is the result of a secondary effect elicited only upon TLR4 overexpression, or that TLR4 is specifically required as a co-receptor for HA binding to an unidentified receptor present in HEK-293 and BMM cells.

TLR4 also mediates the suppression of osteoclast differentiation by lipopolysaccharides (LPS) if LPS is added from the beginning of BMM culture (Takami et al., 2002). The possibility of LPS contamination accounting for the anti-osteoclastogenic effect of the HA preparation was excluded because endotoxin was undetectable in the Limulus test, and because LPS augmented mature osteoclast formation when added to RANKL-primed prefusion cells whereas HA suppressed it (see supplementary material, Fig. S3).

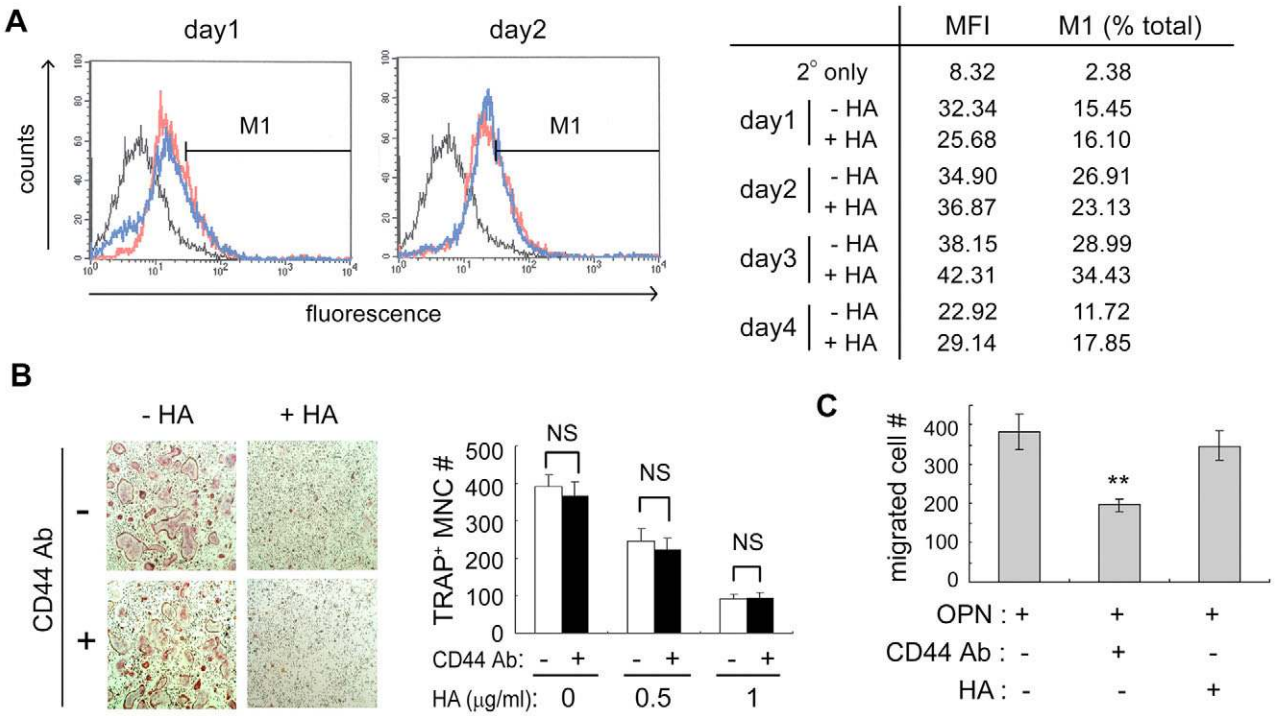

Fig. 3. Independence of HA inhibition of osteoclastogenesis on CD44. (A) HA does not suppress CD44 expression. BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 days in the presence or absence of HA (1 μg/ml). At each day, cells were collected and surface expression of CD44 was examined by flow cytometry. Red line, –HA; blue line, + HA; black line, secondary (2°) Ab control. MFI, mean fluorescence intensity. (B) CD44 independence of osteoclastogenesis inhibition by HA. BMMs were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 6 days. Anti-CD44 Ab (1 μg/ml) and HA (0.5-1 μg/ml) were included for the whole culture period where indicated. Cells were stained for TRAP, and $TRAP^+$ MNCs were counted. NS, no significant difference. (C) Effectiveness of the anti-CD44 Ab to neutralize the CD44-OPN-dependent response of BMMs. BMMs were placed on the top chamber of a transwell plate. Anti-CD44 Ab (1 μg/ml) and HA (1 μg/ml) were added to the top chamber and OPN (a CD44 ligand, 2 μg/ml) was added to the bottom chamber. The plate was incubated for 8 hours. The number of migrated cells was determined after hematoxylin staining. $^{**}P<0.01$ compared with OPN-treated control.

HA inhibits M-CSF signaling in BMMs and RANKL signaling in differentiating cells

To gain insights into the mechanisms by which HA inhibits osteoclast differentiation, we investigated signaling pathways triggered by M-CSF and RANKL. First, M-CSF signaling pathways were analyzed. In BMMs, M-CSF activated the MAPKs ERK, JNK and p38, and HA attenuated the M-CSF-induced activation of all three MAPK families (Fig. 5A). The JNK activation was most profoundly reduced by HA (Fig. 5Ab). Recently, it has been recognized that the generation of reactive oxygen species (ROS) by NADPH oxidase, which requires Rac activity, is important in the early stage of receptor tyrosine kinase signaling (Bokoch and Diebold, 2002; Choi et al., 2005). The M-CSF receptor, c-Fms, is a receptor tyrosine kinase. Therefore, we evaluated effects of HA on ROS production. M-CSF increased ROS production in BMMs, and HA pretreatment attenuated the response (Fig. 5B). The ROS increase by M-CSF was sensitive to DPI, a NADPH oxidase inhibitor (data not shown). The activation of Rac by M-CSF was also detected in BMMs and this effect was reduced by HA as well (Fig. 5C). These results demonstrate that HA hampers M-CSF signaling pathways to ROS and MAPKs in BMM osteoclast precursors.

We also investigated RANKL signaling pathways. In BMMs, RANKL activated ERK, JNK and p38 (Fig. 6A). Unlike in M-CSF signaling, the RANKL stimulation of MAPKs was not affected by HA (Fig. 6A). However, it is a possibility that RANKL signaling is affected after long exposure to HA if HA changes the expression levels of genes or proteins involved in RANKL signaling, which usually requires longer time to occur. To explore this possibility, we cultured BMMs with or without HA for 2 days in differentiation medium (containing M-CSF and RANKL). The cells were deprived of the cytokines and stimulated with RANKL. In the HA-unprimed cells, RANKL could still activate MAPKs (Fig. 6B). By contrast, the RANKL stimulation of MAPKs was substantially reduced in HA-primed cells (Fig. 6B). This striking difference between HA-unprimed and HA-primed cells in the activation of RANKL signaling pathways raised the question on the expression level of RANK (the RANKL receptor) in response to HA treatment.

HA reduces RANK expression via suppression of activation of AP-1 and MITF by M-CSF

As HA suppressed RANKL signaling in HA-primed but not in HA-unprimed cells (Fig. 6), we checked whether RANK expression was changed by HA treatment. The RANK mRNA level was reduced by HA, whereas the mRNA level of the M-

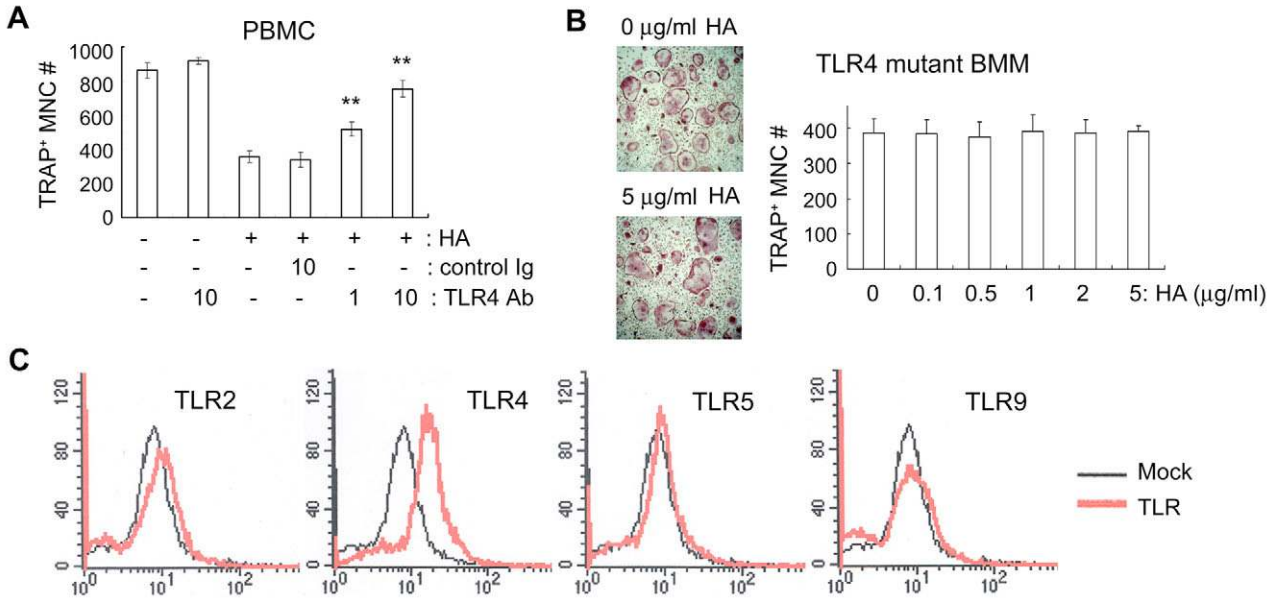

Fig. 4. Requirement of TLR4 for HMM-HA inhibition of osteoclast differentiation. (A) Blockade of HA effects by a TLR4-neutralizing Ab. Human PBMCs were cultured for 9 days with RANKL (200 ng/ml), M-CSF (30 ng/ml), HA (1 μg/ml) and a TLR4 Ab (1-10 μg/ml) or an isotype-matched control Ab (10 μg/ml). Cells were stained for TRAP, and TRAP⁺ MNCs were counted. $^{**}P<0.01$ compared with HA-treated control. (B) Lack of HA effects in TLR4-mutant cells. BMMs from TLR4-mutant mice were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) together with HA (0.1-5 μg/ml) for 6 days. TRAP⁺ MNCs were counted. (C) Binding of HA to TLR4. HEK-293 cells were transfected with TLR2, TLR4, TLR5, TLR9, or a control (mock) plasmid. At 24 hours after transfection, cells were incubated with anti-CD44 (1 μg/ml, 1 hour) to block potential HA binding to CD44. Cells were then incubated with FITC-HA for 30 minutes and analyzed in a flow cytometer. Red line, TLR-transfected cells; black line, mock-transfected cells.

CSF receptor (c-Fms) was not affected (Fig. 7A, left). By contrast, the RANK expression in BMMs from TLR4 mutant mice was not affected by HA (Fig. 7A, right). The surface level of RANK was also examined by FACS analyses with TRITC-RANKL. HA reduced the cell surface levels of RANK (Fig. 7B). Surface expression of c-Fms was not affected (see supplementary material, Fig. S4).

The interference with M-CSF signaling by HA in BMMs (Fig. 5) prompted us to reason that the reduction of RANK expression in HA-treated cells would involve M-CSF signaling inhibition. An analysis of mouse RANK promoter sequences revealed AP-1-binding sites and MITF-binding E-box elements (Kwon et al., 2005). Because the effect of HA on M-CSF stimulation of JNK was prominent (Fig. 5Ab), we examined the activation of JUN, an AP-1 component. M-CSF increased JUN phosphorylation and this effect was attenuated by HA (Fig. 7C). M-CSF increases the transcription potential of MITF by phosphorylation (Weibaecher et al., 2001). HA suppressed the M-CSF-induced MITF phosphorylation in BMMs (Fig. 7C). Next, we examined the DNA-binding activity of AP-1 and MITF. In gel mobility shift assays with the AP-1 binding site or MITF binding (E-box) sequences, M-CSF induced DNA binding activities and HA blocked those activities (Fig. 7D, lanes 1-6). The DNA binding activities were not observed with mutant probes and also with inclusion of excessive unlabeled probes (Fig. 7D, lanes 7-9), demonstrating the specificity of the probes used. Taken together, these results suggest that HA interferes with M-CSF signaling pathways to transcription factors AP-1 and MITF and subsequently reduces RANK expression to cause a suppression in osteoclastogenesis.

HA attenuates RANKL-induced bone resorption in vivo

HA has been used clinically for more than 30 years for osteoarthritis and cartilage diseases (Goldberg and Buckwalter, 2005). Given the in vivo applicability and the in vitro anti-osteoclastogenic efficacy of HA, we set out to determine the in vivo relevance of HA effects using the calvarial bone resorption model. Mouse calvarias were implanted with collagen films absorbing RANKL and with or without HA. Five days later, whole calvarias were subjected to micro-computed tomography (μ-CT) analyses and TRAP-staining. In μ-CT analyses, a significant reduction in RANKL-induced bone erosion was detected in HA-treated calvarias (Fig. 8A). The number of TRAP-stained osteoclasts on the surface of calvarias was also reduced by HA (Fig. 8B). Compared with the control mouse calvarias, the relative bone volume of RANKL-treated calvarias was 71.96±2.82% and that of calvarias treated with HA and RANKL was 82.0±5.39% (Fig. 8C). In histological sections stained with hematoxylin and eosin, an increase in bone marrow space (resulting from stimulated bone resorption) was also detected in RANKL-treated calvarias, and HA significantly suppressed the RANKL-induced increase (Fig. 8D). The percentage of marrow area in RANKL-treated calvarias increased to 31.54±3.85% compared with 6.29±1.63% in control calvarias. whereas treatment with HA and RANKL together increased the marrow area only to 16.96±2.33% (Fig. 8E).

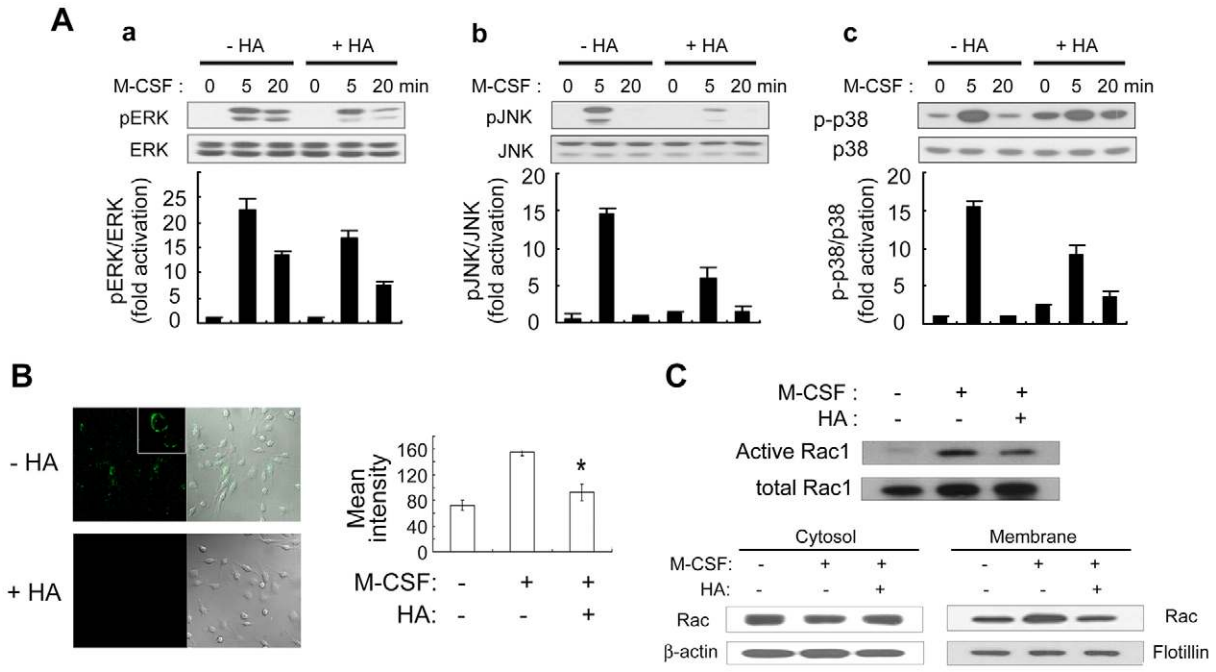

Fig. 5. Suppression of M-CSF signaling by HA. (A) BMMs were serum-starved, pretreated with HA (1 μg/ml) for 30 minutes and stimulated with M-CSF (300 ng/ml). Cell lysates were immunoblotted with phosphorylation-specific antibodies to detect the activation of ERK (a), JNK (b), and p38 (c). The same membranes were stripped and reprobed to detect total levels of each MAPK. The relative levels of phosphorylated forms were determined by densitometry. (B) BMMs were serum-starved and pretreated with HA (1 μg/ml) for 30 minutes. The cells were loaded with DCF-DA and stimulated with M-CSF (300 ng/ml) for 5 minutes. The DCF fluorescence was detected by confocal microscopy. The inbox in the upper left panel shows single-cell fluorescence. The average of the mean fluorescence intensity of several fields is presented as a histogram. **P*<0.05 compared with HA-untreated group. (C) BMMs were serum-starved, pretreated with HA (1 μg/ml) for 30 minutes and stimulated with M-CSF (300 ng/ml) for 5 minutes. Total cell lysates were prepared and subjected to the Rac activity assay (*top*). In the bottom panel, stimulated cells were lysed and cytosolic and membrane fractions were immunoblotted with anti-Rac antibody. The same membranes were reprobed with anti-β-actin and anti-flotillin antibodies to ensure comparable amounts of loading.

Discussion

As an important ECM component, HA functions in maintenance of matrix structure and regulation of matrix-associated cell responses. It has been suggested that HA relieves symptoms of arthritis and modifies the structure of diseased joints through complex biochemical responses that result from interactions with cartilage, subchondral bone and other matrix components (Goldberg and Buckwalter, 2005). Although there are ample documentations on the effects of HA on chondrocytes and synovial cells, reports on the relationship between HA and bone cells are scanty. Our data presented in this study demonstrate that HMM-HA potently inhibits osteoclastogenesis from primary BMMs and PBMCs. The mechanism responsible for the HA inhibition of osteoclast differentiation is the interference with M-CSF signaling that controls RANK expression. HA blocks M-CSF stimulation of Rac activity, ROS production and MAPKs activity. This leads to reduced DNA binding of transcription factors to AP-1 and E-box sites, resulting in a decrease in RANK transcription.

In a recent study, it was shown that HA fragments (a 4-mer and 12-mer, and a 8-kDa fragment) increased osteoclastic differentiation of RAW264.7 cells, whereas HMM-HA (2000 kDa) had no effect (Ariyoshi et al., 2005). Effects on primary cells were not examined in that study. In our study with primary culture BMMs, HMM-HA suppressed and LMM-HA enhanced osteoclastogenesis. HA is normally present in its HMM form but has been suggested to be degraded under inflammatory and injury conditions. However, direct evidence for the presence of oligomeric or small fragments of HA in pathologic or physiologic circumstances has not been clearly demonstrated. In synovial fluid from patients with rheumatoid arthritis and other joint diseases and temporomandibular disorders, the average molecular mass of HA was detected as 1570-5000 kDa, whereas that of normal individuals was 3000-7000 kDa (Dahl et al., 1985; Takahashi et al., 2004). Under even more radical in vitro digestion conditions, the size of end products was 10-40 kDa (Sampson et al., 1992; Saari et al., 1990). In addition, a recent attempt to detect small HA fragments in wound fluid was unsuccessful (Taylor et al., 2004). Although the possibility of low concentrations of small HA fragments in restricted local lesions can not be completely excluded – considering the HMM property of HA and insufficient evidence for the presence of small HA in patients with joint diseases – the significance of the very low molecular weight HA on osteoclast differentiation awaits further evaluation. Nonetheless, the lack of effects of HMM-HA on osteoclast formation from RAW264.7 cells was confirmed in our study. In contrast to the results with RAW264.7 cells, osteoclastogenesis from primary BMM and PBMCs was clearly inhibited by HMM-HA in our study. The difference in

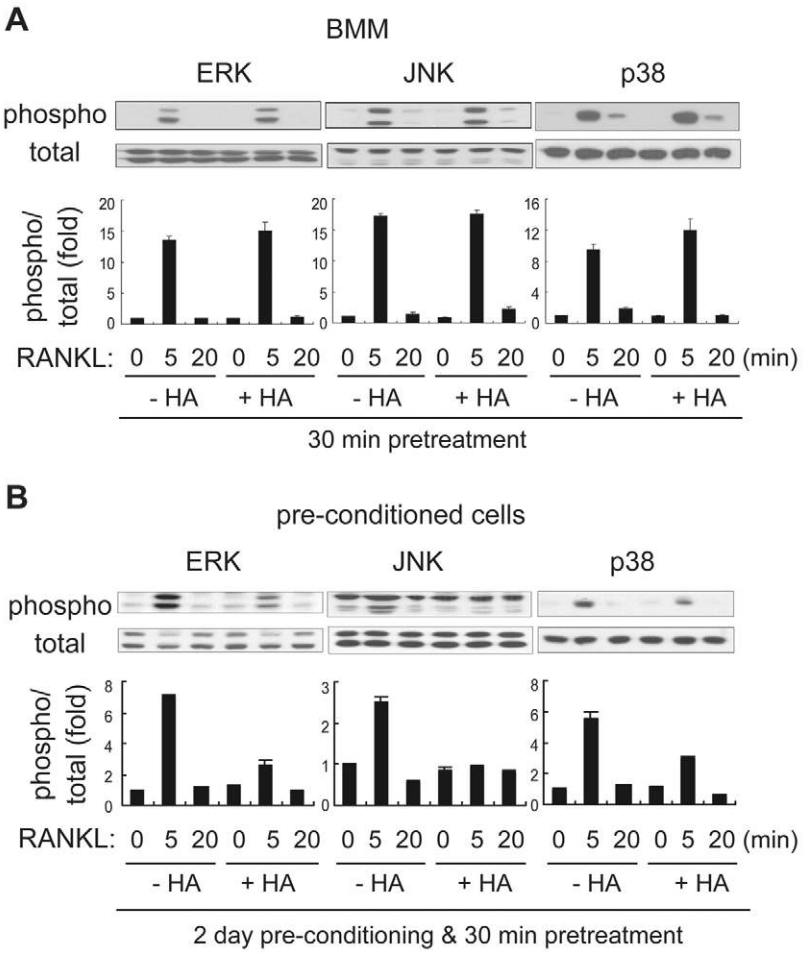

Fig. 6. Effects of HA on RANKL signaling. (A) BMMs were serum-starved, pretreated with HA (1 μg/ml) for 30 minutes, and stimulated with RANKL (500 ng/ml) for indicated times. Cell lysates were immunoblotted. (B) BMMs were cultured for 2 days with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in the presence or absence of HA (1 μg/ml). The pre-conditioned cells were serum-deprived, and cells pre-conditioned in the presence of HA were treated with HA (1 μg/ml) for 30 minutes, whereas cells pre-conditioned in the absence of HA were treated with the vehicle. Cells were then stimulated with RANKL (500 ng/ml) for indicated times. Cell lysates were subjected to western blot analyses.

effects of HMM-HA between RAW264.7 cells and primary osteoclast precursors may be ascribed to the independence of RAW264.7 cells on M-CSF for proliferation and osteoclastic differentiation. The M-CSF independence of this cell line was not due to endogenous generation of the cytokine (data not shown), suggesting the possibility of a transformation-derived attribute.

The function of HA as an extracellular signal to modulate intracellular M-CSF signaling may not be easily envisaged if this ECM molecule is viewed just as a scaffold to maintain tissue integrity. However, evidence for specific binding receptors for HA is growing in support of the role for HA in cell signaling (Laurent and Fraser, 1992; Lee and Spicer, 2000). CD44 has been indicated to mediate effects of HA in some cell types. The interaction between HA and CD44 was suggested to regulate the SDF-1-dependent trafficking of human hematopoietic stem cells (Avigdor et al., 2004). CD44 has also been implicated in the migration of differentiated osteoclasts (Spessotto et al., 2002). A study with the FLG 29.1 pre-osteoclastic cell line demonstrated inhibitory effects of HA on osteoclast migration, which was restored by blocking CD44 (Spessotto et al., 2002). However, studies showing a link between HA synthesis and osteoclastic resorption (Prince, 2004) and those showing an attenuated motility and resorption activity of CD44-null osteoclasts (Suzuki et al., 2002) led to a suggestion that HA-CD44 interaction may play an important role in bone resorption (Prince, 2004). However, no direct evidence for involvement of HA in the CD44-mediated regulation of osteoclast activity or differentiation has been documented. Rather, osteopontin, another CD44 ligand, has been implicated in the role for CD44 in osteoclastic resorption (Suzuki et al., 2002; Ishijima et al., 2001). Moreover, a recent study showed no defect in osteoclast differentiation from CD44-deficient cells (de Vries et al., 2005). In our study, the inhibition of osteoclast differentiation by HWM-HA did clearly not depend on CD44 but, rather, TLR4 was involved. However, it cannot be ruled out that HA interacts with both CD44 and TLR4 during osteoclastogenesis. TLR4 is expressed in both BMM osteoclast precursor cells and differentiated osteoclasts (Takami et al., 2002). HA binds TLR4 in BMMs to inhibit the commitment to osteoclastic differentiation, whereas its interaction with CD44 in differentiated osteoclasts may inhibit resorption activity. The combined effects of HA may describe this ECM component as an important negative regulator of bone catabolism.

Other cases of TLR4 involvement in mediating cellular responses to HA have been documented. In a manner dependent on TLR4, HA stimulates maturation of dendritic cells (Termeer et al., 2002) and IL-8 production by endothelial cells (Taylor et al., 2004). However, the mechanism of action by which TLR4 mediates effects of HA on cellular responses is not well understood. Our results, showing decreases in the

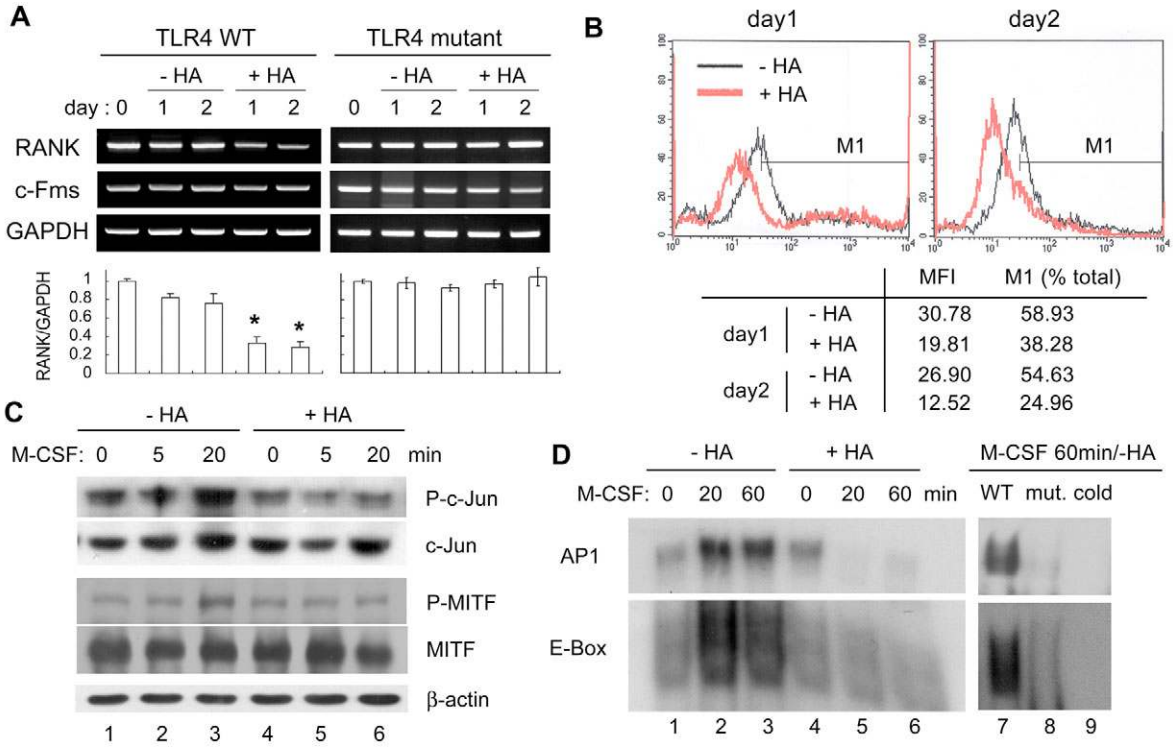

Fig. 7. Reduction of RANK expression by HA. (A) BMMs with wild-type (WT) or mutant TLR4 were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in the absence or presence of HA (1 μg/ml) for 1 or 2 days. Expression levels of RANK mRNA were measured by RT-PCR. (B) BMMs from wild-type mice were cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 1 or 2 days. The surface levels of RANK were determined by FACS using TRITC-conjugated RANKL. Black line, –HA; red line, + HA; MFI, mean fluorescence intensity. (C) Effects of HA on the phosphorylation of JUN and MITF by M-CSF. BMMs were serum-starved, pretreated with HA (1 μg/ml) for 30 minutes, and stimulated with M-CSF (300 ng/ml) for indicated times. Cell lysates were immunoprecipitated with an Ab against phosphorylated MITF (P-MITF) and western blotted with a regular MITF Ab (third panel). All other protein levels [phosphorylated JUN (P-c-Jun), unphosphorylated JUN (c-Jun) and β-actin] were determined with cell lysates. (D) Nuclear extracts were prepared from cells stimulated as in C and subjected to EMSA analyses with an AP-1-binding site oligonuleotide or an E-box sequence from RANK promoter (left). The DNA binding activity of M-CSF-stimulated nuclear extract to mutant probes (mut.) and to wild-type probes in the presence of 50-fold excess unlabeled probes (cold) was compared to the one with wild-type probes (WT).

M-CSF-induced MAPK activation, ROS production and Rac stimulation, suggest that TLR4 signaling pathways cross-talk with M-CSF receptor signaling pathways. c-Fms, the M-CSF receptor, contains multiple tyrosine phosphorylation sites to which a variety of signaling molecules, including Src and PI3K, are recruited (Weilbaecher et al., 2005). Src and PI3K pathways can activate the Rho-family-GTPase Rac, which plays a crucial role in ROS generation upon stimulation of receptor tyrosine kinases (Bokoch and Diebold, 2002; Choi et al., 2005). Therefore, it is plausible that Src and/or PI3K lie in the pathways to which HA-induced signals cross-talk. In line with this notion, we observed that HA inhibits the stimulation of Src tyrosine phosphorylation by M-CSF in BMMs (data not shown), but how this inhibition is achieved by HA-engaged TLR4 is unclear. However, a study showing involvement of Src family kinases in TLR4 signaling (Aki et al., 2005) suggests that TLR4 outcompetes c-Fms for limited amounts of Src family kinases. A recent study reported that the phosphatase SHIP1 is tyrosine phosphorylated and induced to negatively regulate TLR4-mediated signal transduction (An et al., 2005). Engagement of TLR4 by HA may facilitate SHIP1 recruitment to c-Fms signaling complexes and subsequently turn off c-Fms signaling pathways. Investigation on these possibilities will clarify the molecular nature of cross-talk between TLR4 and c-Fms. Also, analyses of TLR4-associated complexes with the help of proteomics strategies may be useful in identifying molecules targeted by TLR4 in response to HA.

The importance of TLRs in recognizing exogenous microbial pathogens by the innate defense system is well established (Takeda and Akira, 2005; Rifkin et al., 2005). However, growing evidence indicates the presence of endogenous ligands for TLRs, such as HA and mammalian chromatin (Rifkin et al., 2005). Although further evidence needs to be accumulated, the interaction of endogenous ligands with TLRs has been suggested to contribute to autoimmune diseases. Our result of the TLR4 involvement in HMM-HA-induced suppression of osteoclast differentiation may envision a potential relationship between innate immunity and osteoclastogenesis. The innate immune receptor TLR4 may play a role as a surveillant for osteoclast generation when bound to the ECM component HA while it alerts the immune system when bound to the microbial component LPS. It is intriguing to know whether other ECM components have any effect on osteoclastogenesis and whether other innate immune components are involved in the regulation of osteoclast differentiation by ECM constituents.

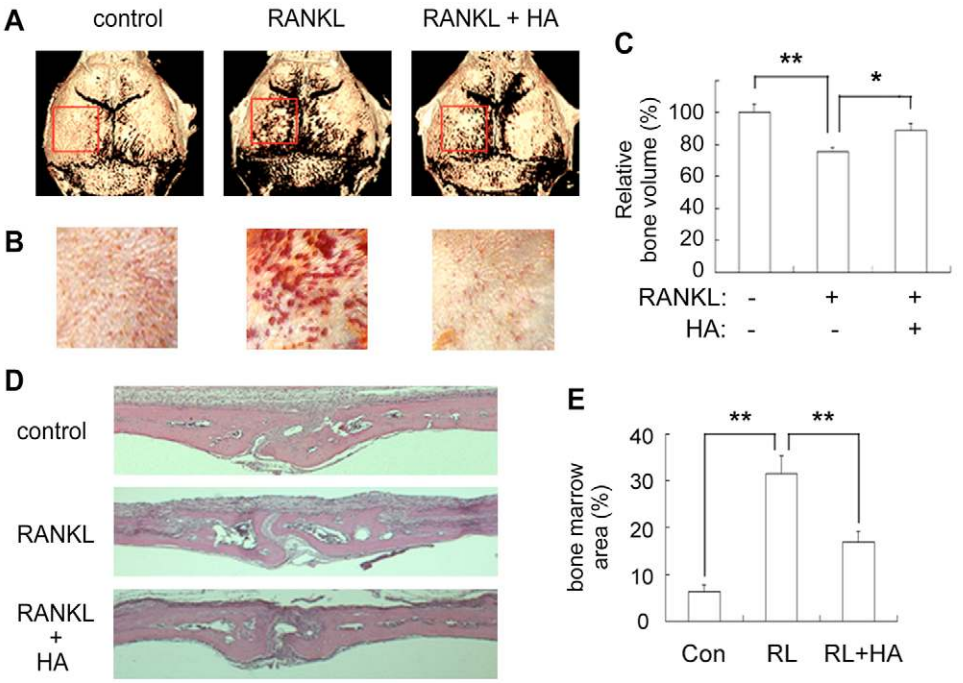

Fig. 8. In vivo effects of HA on bone resorption. (A-C) Mouse calvarias were implanted with collagen sheets soaked with RANKL alone or with RANKL and HA. Five days later, calvarias were collected and analysed using micro-computed tomography (μ-CT). (A,B) 3D configurations of whole TRAP-stained calvarias (A); boxed areas of A are shown in B. (C) Relative percentages of calvarial bone volume. **P*<0.05 and ***P*<0.01 between indicated groups. (D,E) Mouse calvarias treated as above were decalcified and embedded in paraffin. Coronal sections were stained with hematoxylin and eosine. Representative sections (D) and their respective percentage of marrow area (E) are shown. ***P*<0.01 between indicated groups.

The role of HA in the control of osteoclastogenesis will be physiologically important in the bone marrow environment where bone tissue and blood vessels are present. The progenitor cells of osteoclast lineage are continuously supplied near the bone surface by blood vessels. HA is present in the sinusoidal endothelium, endosteum and stromal ECM in bone marrow (Avigdor et al., 2004). The osteoclastic differentiation of progenitor cells may be in a negative control by HA in ECM and a positive control by M-CSF/RANKL expressed on marrow stromal cells and osteoblasts under unstimulated situations. Upon stimulation of osteoblasts by hormones and inflammatory mediators, the levels of M-CSF and RANKL will increase to trigger efficient osteoclastogenesis, overcoming the negative influence by HA through TLR4.

In conclusion, our study clearly shows that, HMM-HA inhibits osteoclastogenesis in vitro and in vivo, and TLR4 is involved in this response. The anti-osteoclastogenic property of HMM-HA may be a beneficial feature of its clinical use for bone erosion-associated joint diseases. Results of this study also open the question whether the regulation of osteoclastogenesis by innate immune receptors, via the interaction with endogenous ECM components, can be envisioned as a general concept in bone metabolism physiology.

Materials and Methods

Reagents and antibodies

Hyaluronan (H1751, from human umbilical cord) was purchased from Sigma (St Louis, MO). 1300-kDa HA was provided by Amorepacific R&D center (Seoul, Korea). 230-kDa HA, 23-kDa HA and 30-mer HA were generous gifts from the Seikagaku Corporation (Tokyo, Japan). The HA from Sigma was used throughout the study except for experiments shown in Fig. 2B.

Hyaluronidase (H1136, from *Steptomyces hyalurolyticus*) and the Leukocyte Acid Phosphatase Assay Kit were purchased from Sigma. RANKL and IL-1α were obtained from PeproTech (Rocky Hill, NJ). M-CSF was from R&D Systems (Minneapolis, MN). The antibody (Ab) against phosphorylated MITF (P-MITF) for immunoprecipitation has been previously described (Weilbaecher et al., 2001). Pronase (5370887, from *Steptomyces griseua*), the MITF Ab for western blot analyses and the CD44-neutralizing Ab (217594) were from Calbiochem (San Diego, CA). The TLR4-neutralizing Ab (MCA2061XZ) and the isotype (IgG2a)-matched control Ab (MCA929) were from Serotec (Raleigh, NC). All other antibodies were from Cell Signaling Technology (Beverly, MA). The EZ-Detect Rac1 Activation Kit (89856) was from Pierce (Rockford, IL).

Osteoclast culture

To generate mouse bone marrow derived osteoclasts, monocytes were isolated from tibiae of 6-week-old ICR or C3H/HeJ mice as previously described (Ha et al., 2003). All animal experiments were performed according to the guidelines by the Institutional Animal Care Committee of Seoul National University. Cells were seeded in 96-well plates (4×10^4 cells per well) and cultured with 30 ng/ml M-CSF for 72 hours. Cells at this stage were considered to be M-CSF-dependant bone marrow macrophages (BMMs) and used as osteoclast precursors (Kobayashi et al., 2000). Induction of differentiation to osteoclasts was achieved by culturing the cells with 100 ng/ml RANKL and 30 ng/ml M-CSF for 5-6 days. Differentiation of RAW264.7 cells to osteoclasts was conducted by culturing the cells in α-minimal esssential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) in the presence of 100 ng/ml RANKL without M-CSF for 4 days. Osteoclasts from human PBMCs were generated by seeding cells onto 96-well plates (2×10^5 cells per well), and culturing with 30 ng/ml M-CSF and 200 ng/ml RANKL for 7-9 days. Peripheral blood was obtained from healthy volunteers after obtaining informed consent and human subject protocols were approved by the Institutional Review Board. Osteoclasts were identified by staining for tartrate-resistant acid phosphatase (TRAP) activity using the Leukocyte Acid Phosphatase Assay Kit. Typically, TRAP-positive (pink- to purple-colored) mononuclear cells appeared around day 3. TRAP-positive cells with more than three nuclei ($TRAP^+$ MNCs) were counted as osteoclasts. $TRAP^+$ MNCs were observed approximately 5-6 days after the addition of RANKL to BMM cultures. Formation $TRAP^+$ MNC from PBMCs needed 7-9 days.

Reverse transcription (RT)-PCR analysis

RT-PCR analyses were performed as previously described (Lee et al., 2003). The sequences of oligonucleotide primers used were 5′-AGTTTAAG-CCAGTGCTTCACG-3′ (RANK forward); 5′-ACGTAGACCACGATGATGTCG-3′ (RANK reverse); 5′-GATTGGGGACTTTGGACTGGC-3′ (c-Fms forward); 5′-TGCTGCCACCACCACTGTCA-3′ (c-Fms reverse); 5′-ACCGTCCTTTCTTGT TGG-3′ (MMP9 forward); 5′-GGATAGCTCGGTGGTGTT-3′ (MMP9 reverse); 5′-CAAGGCTGTGGGCAAGGTCA-3′ (GAPDH forward); and 5′-AGGTGGAA-GAGTGGGAGTTGCTG-3′ (GAPDH reverse). PCR reaction was as follows: denaturation, 1 minute at 94°C; annealing, 1 minute at ~56-60°C; extension, 30 seconds at 72°C. The number of cycles was determined to be in a linear range of amplification. RANK, c-Fms and MMP9 cDNAs were amplified in ~26-30 cycles and GAPDH cDNA was amplified in 22 cycles.

Western blot analysis

Cells were washed with ice-cold PBS and lysed in RIPA buffer (10 mM Tris-Cl pH 7.2, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 1% aprotinin,

2 mM PMSF). The whole-cell lysates were centrifuged at 10,000 ***g*** for 20 minutes and the supernatants were collected. The protein concentration was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). 30-50 μg of proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were probed with primary antibodies, followed by horseradish-peroxidase-conjugated secondary antibodies. All immunoblots were detected using an enhanced chemileuminesence system.

Electrophoretic gel mobility shift assay (EMSA)

BMM osteoclast precursor cells were serum-deprived for 3 hours, pretreated with 1 μg/ml HA for 30 minutes and stimulated with 300 ng/ml M-CSF. Nuclear extracts were prepared and gel mobility shift assays were performed as previously described (Lee et al., 2001). The sequence of AP-1-binding DNA was 5′-CGCTTGATGACT-CAGCCGGAA-3′ (WT) and 5′-CGCTTGATGACTTGGCCGGAA-3′ (mutant). The probe sequence for E-box EMSA was 5′-CTTTTTCAAGTGCACATGGAATT-3′ (WT) and 5′-CTTTTTGAAGTGGAGATGGAATT-3′ (mutant); underlined nucleotides represent sequence differences.

ROS detection

Cells were placed on coverslips and serum-starved for 2 hours, pretreated with HA for 30 minutes and loaded with 10 μM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Molecular Probes) for 1 hour at 37°C. Cells were then stimulated with 300 ng/ml M-CSF for 5 minutes. After fixing the cells, fluorescence images were captured under the Axiovert 200MBP microscope (Carl Zeiss, Oberkochen, Germany) equipped with 10× Plan-Neofluor and 40× C-apochromat objective lenses and the LSM5 Pascal confocal system (Carl Zeiss).

Flow cytometry (FACS)

Cells were gently scraped and washed with PBS twice. 1×10^6 cells were resuspended in PBS, incubated in 4% goat serum for 30 minutes on ice, and washed with cold PBS containing 0.5% bovine serum albumin (BSA). Cells were incubated with anti-CD44 (AHS4401, Biosource International, Camarillo, CA) for 1 hour on ice, washed and incubated with FITC-conjugated secondary antibody for 1 hour on ice. Cells stained with fluorescein (FITC)-conjugated secondary antibody only were used as negative control. For detection of surface RANK expression, tetramethylrhodamine isothiocyanate-conjugated RANKL was used. For HA-binding experiments, FITC-conjugated HA was used. The fluorescence intensity was detected using the FACScan flow cytometer (Becton Dickenson, Franklin Lakes, NJ).

Rac activity assay

BMMs seeded on 60-mm dishes were cultured in medium containing 30 ng/ml M-CSF for 1 day. Cells were serum-deprived for 3 hours, pretreated with 1 μg/ml HA for 30 minutes and stimulated with 300 ng/ml M-CSF for 5 minutes. Cells were washed with ice-cold PBS, and Rac activity assays were performed with 500 μg of total cell lysates using the Rac activity assay kit (Pierce) following the manufacturer's instruction. In the Rac translocation experiments, cells were lysed in a buffer containing 100 mM Hepes (pH 7.4), 0.2 M sucrose, 50 mM KCl, 1 mM EGTA, and 2 mM DTT. The lysates were centrifuged at 6000× ***g*** for 20 minutes. The supernatants were collected and centrifuged at 100,000 ***g*** for 60 minutes. The supernatants were collected and used as cytosolic fraction; the pellets were taken as membrane fractions. Cytosolic and membrane proteins were resolved by SDS-PAGE and immnoblotted with anti-Rac antibody.

Migration assay

Migration assay was performed using a 24-well Transwell plate (Corning) possessing 8 μm pores. BMMs were serum-starved for 3 h and 5 x 10^5 cells applied to the upper well. Osteopontin (2 μg/ml) was added to the lower well. Neutralizing CD44 Ab (1 μg/ml) or HA (1 μg/ml) were added to the upper well. Cells were incubated for 8 hours. The side of membranes facing the upper well were cleared of cells and the bottom side was stained with hematoxylin. The number of migrated cells was counted.

Calvarial bone resorption assay

A collagen sheet (100 mm^2) was soaked with PBS and RANKL (5 μg) or PBS, RANKL (5 μg) and HA (1 mg) in 30 μl volume and implanted into the center of calvarias. Five mice were used for each group. Five days later, mice calvarias were collected and micro-computed tomography (μ-CT) was performed with 1072 Microtomograph (SkyScan, Belgium). A total of 320 tomographic slices were acquired at a 4-μm resolution. 3D analyses were performed with the V-Works program (Cybermed, Korea). From a separate set of treated mice, calvarias were cut coronally in the middle of the sagittal suture into two pieces. Calvaria pieces were fixed for 2 days in 4% paraformaldehyde, decalcified in 13% EDTA for 4 weeks and embedded in paraffin. Paraffin blocks were cut into 4-μm thick sections using a microtome. Tissue sections were deparaffinized and stained with hematoxylin and eosin.

Statistical analysis

Each experiment was performed three to five times, except the calvarial resorption study. All quantitative experiments were performed at least in triplicate and the data are shown as the mean ± s.d. of one representative experiment. Statistical significance was analyzed by Student's *t* test.

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