Potential Role of Hyaluronic Acid on Bone in Osteoarthritis: Matrix Metalloproteinases, Aggrecanases, and RANKL Expression are Partially Prevented by Hyaluronic Acid in Interleukin 1-stimulated Osteoblasts

Zvezdana Mladenovic, Anne-Sophie Saurel, Francis Berenbaum, and Claire Jacques

ABSTRACT. ***Objective.*** To determine the effect of hyaluronic acid (HA) on proteolytic enzymes and bone remodeling mediators induced by interleukin 1β (IL-1β) and related to cartilage catabolism in murine osteoblasts.

Methods. Osteoblasts were obtained from Swiss mice and cultured for 3 weeks. HA-treated osteoblasts were incubated with 100 μg/ml HA during the last week of culture, then stimulated with IL-1β (10 ng/ml) for 24 h. The expression of matrix metalloproteinases 3 and 13 (MMP-3 and MMP-13), ADAMTS-4 and ADAMTS-5, tissue inhibitor of metalloproteinases (TIMP), osteoprotegerin, and receptor activator of nuclear factor-κB ligand (RANKL) was determined by real-time polymerase chain reaction. MMP-3 and MMP-13 release was assessed by Western blot analysis.

Results. IL-1β increased the mRNA levels of MMP-3 and MMP-13 and ADAMTS-4 and ADAMTS-5 and release of MMP-3 and MMP-13. Seven days of HA treatment significantly prevented the IL-1β-increased mRNA levels of MMP-3 (–61%, $p < 0.01$), MMP-13 (–56%, $p < 0.01$), ADAMTS-4 (–58%, $p < 0.05$), ADAMTS-5 (–52%, $p < 0.01$), and RANKL (–49%, $p < 0.05$), but not TIMP. As well, IL-1β-induced production of MMP-3 and MMP-13 was inhibited, by 27% ($p < 0.01$) and 40% ($p < 0.01$), respectively.

Conclusion. In an inflammatory context in murine osteoblasts, HA can inhibit the expression of MMP and ADAMTS. Because HA can counteract the production of these mediators in chondrocytes, its beneficial effect in osteoarthritis may be due to its action on cartilage and subchondral bone. (J Rheumatol First Release April 15 2014; doi:10.3899/jrheum.130378)

Key Indexing Terms:
HYALURONIC ACID OSTEOBLASTS METALLOPROTEINASES ADAMTS
TISSUE INHIBITOR OF METALLOPROTEINASES RANK LIGAND

Osteoarthritis (OA) is characterized by articular cartilage destruction and by synovial inflammation and abnormal bone remodeling as shown by subchondral bone sclerosis and varying degrees of osteophyte formation[1]. Cartilage and subchondral bone remodeling in OA are not independent events. Rather, cartilage and subchondral bone act together as a single functional unit[2], which underlines the strong functional interactions between the tissues. Indeed, preventing cartilage degradation and blocking bone remodeling in OA experimental models limited bone remodeling and cartilage breakdown, respectively[3,4,5]. Evidence suggests that subchondral bone remodeling may be a primary attribute of OA and may be responsible for cartilage damage[6]. Moreover, changes in OA subchondral bone are believed to consist of phases of bone resorption and abnormal bone sclerosis.

At the bone-cartilage interface, matrix metalloproteinases (MMP) play a role in resorbing both the bone and articular matrix[6]. We reported that murine osteoblasts produce MMP-3 and MMP-13[7]. Beside MMP, ADAMTS, also called aggrecanases, are involved in matrix degradation[8]. The production of ADAMTS-4 and ADAMTS-5 was increased in experimental models of OA and in articular cartilage from patients with OA[9,10]. All active MMP are inhibited by tissue inhibitors of metalloproteinases (TIMP)[11,12]. TIMP are secreted as endogenous inhibitors,

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and 4 mammalian TIMP have been cloned[13,14]. TIMP bind tightly to active MMP in a 1:1 ratio; therefore, connective tissue turnover is prevented if TIMP levels exceed those of the active enzyme. Thus, the regulation of MMP or TIMP at the bone-cartilage interface seems crucial for the remodeling process.

Moreover, subchondral bone plate vascularity is associated with severity of OA cartilage changes and clinical disease activity[15]. A higher number of vessels penetrate the articular cartilage in OA than in non-OA, and this number is positively correlated with the modified Mankin score of cartilage damage[16]. These channels contain vessels as well as osteoblasts and osteoclasts[16]. These subchondral bone resorption pits with vascular extension are considered part of the remodeling process of subchondral bone[17,18,19]. Interestingly, the expression of proteases, especially MMP, and matrix degradation have been observed around vascular channels[16,18].

Moreover, osteoblasts from human OA subchondral bone can produce an excess of many biochemical factors favoring the maturation/activation of osteoclasts and/or resorption of bone matrix. Abnormal levels of 2 major factors that play a major role in bone resorption, osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL), have been found in human OA subchondral bone osteoblasts[20]. Both factors are synthesized by osteoblasts. OPG, a 55-kDa protein, belongs to the tumor necrosis factor (TNF) receptor family[21] and is considered a decoy receptor that inhibits the binding of RANKL — also a member of the TNF family — to its membrane receptor RANK. The molecular triad OPG-RANKL-RANK is involved in orchestrating pathophysiological bone remodeling. RANKL is an essential cytokine for osteoclast differentiation and bone loss and acts as a survival factor for osteoclast precursors. However, OPG can inhibit the terminal stage of osteoclastic differentiation and suppress its activation as well as induce apoptosis of mature osteoclasts[22].

Hyaluronan (HA), a large glycosaminoglycan composed of repeating disaccharides of D-glucuronic acid and N-acetylglucosamine, is one of the major components of joint fluid and connective tissues. It is a structural component that helps maintain the extracellular matrix architecture by joint lubrication. Recent studies have shown that HA can be a biological inhibitor of joint degradation. HA could inhibit interleukin 6 (IL-6)-induced MMP production in human chondrocytes[23]. Moreover, intra-articular injection of HA is a widely used symptom-modifying approach for reducing pain with OA[24,25]. HA can penetrate osteoarthritic cartilage explants and bind to chondrocytes[26]. Recently, fluorescent HA was found penetrating into subchondral bone after its injection into OA joints of a rabbit OA model[27]. However, the mechanism(s) of HA remains unknown. HA may act on subchondral bone osteoblasts, and therapies that interfere with bone remodeling may block or at least attenuate the progression of cartilage alterations.

To further assess the effects of HA on OA subchondral bone, we investigated the effect of HA on proteolytic enzymes induced by IL-1β and related to cartilage catabolism in murine osteoblasts. Our findings suggest that HA inhibits the synthesis of proteolytic enzymes known to have deleterious effects at the bone-cartilage interface.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma-Aldrich, unless stated otherwise. Fetal calf serum was obtained from Invitrogen. Collagenase A, trypsin, hyaluronidase, collagenase D, and complete protease inhibitor mixture were from Roche Diagnostics. The enhanced chemiluminescence Western blot analysis system was from Amersham Pharmacia Biotech. Polyvinylidene difluoride membranes for Western blot and kaleidoscope prestained standards were from Bio-Rad. Recombinant human IL-1β was from PeproTech (Tebu Bio). HA (mean MW 3100 kDa) was from Pierre Fabre Laboratories.

Primary calvaria mouse osteoblast isolation and culture. As described[28], calvaria osteoblasts were obtained by enzymatic digestion of calvaria cortical bone of 5-day-old to 6-day-old Swiss mice. All enzymatic digestions were performed in DMEM/HAMF12 containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 4 mM glutamine, and 1 g/l glucose (final). This medium was called "basal medium." Calvarias from 12 to 14 newborn mice from the same mouse litter were pooled and successively incubated for 20 min at 37°C under agitation with trypsin (15 ml trypsin, 5 mg/l, with EDTA, 2 mg/l) and collagenase D (10 ml of 3 mg/ml of collagenase D; Roche) to eliminate surrounding soft tissues. After several washes, calvarias then underwent 2 successive collagenase digestions (25 ml of 3 mg/ml collagenase D in basal media for 45 min at 37°C under agitation). The isolated cells were collected by centrifugation, rinsed in basal medium, counted by trypan blue staining and seeded in 12-well multiplates (20,000 cells/cm^2) with 1 ml/well basal medium supplemented with 15% fetal bovine serum (FBS). After 14 h, plates were washed 3 times with phosphate buffered saline to remove most of the less-adherent cells such as adipocytes and osteoclasts. Osteoblasts were cultured for 21 days in basal medium supplemented with 10% FBS and 50 μg/ml ascorbic acid. During the last week, 2 mM β-glycerophosphate was added. On Day 15, osteoblasts were incubated with 10% fetal calf serum-containing medium with HA (100 μg/ml) with medium changes every 2 to 3 days, each set of wells having the same medium replacement protocol. On Day 20, IL-1β (1 or 10 ng/ml) was added for 24 h. Osteoblasts were then isolated from the extracellular matrix by enzymatic digestion with collagenase D (3 mg/ml, 30 min at 37°C under agitation). After centrifugation, osteoblasts were stored at –80°C. Cell viability was assessed by trypan blue exclusion. All experiments were performed in duplicate or triplicate, 3 or 4 times with different calvaria osteoblast populations.

RNA extraction, reverse transcription, and real-time quantitative RT-PCR. Total RNA was extracted from osteoblasts by use of the RNeasy kit (Qiagen). RNA concentration was measured by spectrophotometry. The migration in agarose gel was used for quality control. Total RNA (1 μg) was reverse transcribed by use of the Omniscript Kit (Qiagen) in a final volume of 20 μl containing 50 ng oligo(dT). The enzyme was then inactivated by heating, and the cDNA of interest [hypoxanthine phosphoribosyltransferase (HPRT); MMP-3, MMP-13; ADAMTS-4, ADAMTS-5; TIMP-1, TIMP-2, TIMP-3; OPG, and RANKL] were quantified by real-time quantitative PCR by use of LC480 LightCycler Real Time PCR (Roche) and Fast Start DNA master plus SYBR kits (Roche). Specific primers for cDNA were designed by use of the LightCycler Probe Design 2 program and were based on mouse sequence information. Primer sequences for MMP-3 were forward, 5′-TAC GGG TCT CCC CCAG TTT C -3′ and

reverse, 5′-GGT TCG GGA GGC ACA GAT T-3′; MMP-13, forward, 5′-TTC TTC TGG CGG CTG CAT-3′ and reverse, 5′-GGA AGT TCT GGC CCA AAC G-3′; TIMP-1, forward, 5′-GCA AAG AGC TTT CTC AAA GAC C-3′, and reverse, 5′-AGG GAT AGA TAA ACA GGG AAA CAC T-3′; TIMP-2, forward, 5′-CGT TTT GCA ATG CAG ACG TA-3′, and reverse, 5′-GGA ATC CAC CTC CTT CTC G-3′; TIMP-3′, forward, 5′-CAC GGA AGC CTC TGA AAG TC-3′, and reverse, 5′-TCC CAC CTC TCC ACA AAG TT-3′; ADAMTS-4, forward, 5′-GGC AAG GAC TAT GAC GC-3′, and reverse 5′-TCA GCC CAA GGT GAG TG-3 (60°C, 155 bp); ADAMTS-5, forward 5′-TCA GCC ACC ATC ACA GAA-3′, and reverse 5′-CCA GGG CAC ACC GAG TA-3′ (60°C, 161 bp); OPG, forward, 5′-ATC AGA GCC TCA TCA CCT T-3′ and reverse, 5′-CTT AGG TCC AAC TAC AGA GGA AC-3′; RANKL, forward, 5′-TTT CGT GCT CCC TCC TTT-3′, and reverse, 5′-GCT TCT ATT ACC TGT ACG CCA-3′; CD44, forward, 5′-TTT CGT GCT CCC TCC TTT-3′, and reverse, 5′-GCT TCT ATT ACC TGT ACG CCA-3′; intercellular adhesion molecular 1 (ICAM-1), forward, 5′-TTT CGT GCT CCC TCC TTT-3′, and reverse, 5′-GCT TCT ATT ACC TGT ACG CCA-3′; and HPRT, forward, 5′-AGG ACC TCT CGA AGT GT-3′ and reverse, 5′-ATT CAA ATC CCT GAA GTA CTC AT-3′.

The PCR reactions were performed in a 12-µl final volume with 0.2 µl cDNA or 600 ng specific primers and 1× Fast Start DNA master plus SYBR mixture. PCR amplification conditions were denaturation for 5 min at 95°C, then 40 cycles of 10 s at 95°C, 15 s at 60°C and 10 s at 72°C. The generation of specific PCR products was confirmed by melting-curve analysis. For each real-time RT-PCR run, cDNA were run in triplicate. HPRT was used as an internal standard, and the ratio of each investigated PCR primer to HPRT was calculated.

Protein extraction and Western blot analysis. Protein obtained from supernatant and immunoblotting procedures were as described[29] with anti-mouse MMP-3 and MMP-13 polyclonal antibodies (Santa Cruz Biotechnology, Tebu-Bio). Signals were detected by enhanced chemiluminescence and were exposed to Fujifilm LAS-300 (Fujifilm Medical Systems). For densitometry analysis, we used Image-Gauge software (Science Lab 2004; Fujifilm).

Statistical analysis. Data are expressed as mean ± SD fold induction compared with the control (set to 1). Data are reported as mean ± SD. One-way ANOVA with Tukey Kramer multiple comparisons test was used to compare mean values among control, Il-1β or IL-1β+HA with GraphPad Prism software. $P < 0.05$ was considered statistically significant. We reported Bonferroni corrections for multiple comparisons. Significance was set at $p \leq 0.05$.

RESULTS

HA lowered IL-1β-stimulated MMP-3 and MMP-13 gene expression and protein secretion. Because the matrix breakdown that occurs in OA is mainly due to MMP (especially MMP-3 and MMP-13), we assessed the effect of HA on the synthesis of these enzymes by murine osteoblasts. Osteoblasts were treated for 7 days with HA (100 µg/ml), with IL-1β (10 ng/ml) for the last 24 h. HA did not significantly alter the basal mRNA level of MMP-3; stimulation with IL-1β for 24 h elevated the basal level 20-fold ($p < 0.001$; Figure 1A) and IL-1β-induced MMP-3 mRNA level was decreased by 61% with HA pre-incubation ($p < 0.01$). IL-1β stimulation for 24 h increased MMP-3 protein release 90-fold ($p < 0.001$; Figure 1B) and IL-1β-stimulated MMP-3 production was lowered by 27% with HA pre-incubation ($p < 0.01$).

In addition, HA did not significantly alter the basal levels of MMP-13. Stimulation with IL-1β elevated the basal level 15-fold ($p < 0.001$; Figure 2A) and IL-1β-induced MMP-13 mRNA level was decreased by 56% with HA pre-incubation. IL-1β stimulation for 24 h increased MMP-13 protein release 39-fold ($p < 0.001$; Figure 2B) and IL-1β-stimulated MMP-13 production was decreased 40% with HA pre-incubation ($p < 0.001$).

HA decreased IL-1β-stimulated ADAMTS-4 and ADAMTS-5 gene expression in mouse osteoblasts. Because the matrix breakdown that occurs in OA is also due to ADAMTS-4 and ADAMTS-5, we assessed the effect of HA on the synthesis of these enzymes by osteoblasts. Osteoblasts were treated as described. HA did not significantly alter the basal mRNA levels of ADAMTS-4 and ADAMTS-5. Stimulation with IL-1β for 24 h elevated the basal level 18-fold ($p < 0.001$; Figure 3A) and 7-fold ($p < 0.001$; Figure 3B), respectively. However, IL-1β-induced ADAMTS-4 and ADAMTS-5 mRNA level was decreased by 58% ($p < 0.05$; Figure 3A) and 52% ($p < 0.01$; Figure 3B), respectively, with HA pre-incubation.

HA did not modify IL-1β-stimulated TIMP gene expression in mouse osteoblasts. Because TIMP are the major natural MMP and aggrecanase inhibitors in cartilage, we analyzed their expression in mouse osteoblasts. Osteoblasts were treated as described. The expression of TIMP-4 was not detected in the presence or absence of IL-1β. Stimulation with IL-1β elevated the basal mRNA level of TIMP-1 by 27-fold ($p < 0.01$; Figure 4A) but did not modify TIMP-2 and TIMP-3 gene expression (Figure 4B and C). With HA (100 µg/ml) pre-incubation before IL-1β treatment, IL-1β-induced TIMP-1 to TIMP-3 gene expression was not modified (Figure 4A, B, and C).

HA increased OPG and inhibited RANKL gene expression with IL-1β stimulation in mouse osteoblasts. Osteoblasts were treated as described. HA did not significantly alter the basal levels of OPG. Stimulation with IL-1β elevated the basal level by 2.5-fold ($p < 0.001$; Figure 5A). However, IL-1β-induced OPG mRNA level was increased by 19% with HA pre-incubation ($p < 0.05$). Stimulation with IL-1β for 24 h significantly increased the basal mRNA level of RANKL 13-fold ($p < 0.001$; Figure 5B). Interestingly, IL-1β-induced RANKL mRNA level was decreased by 49% with HA pre-incubation ($p < 0.05$). Stimulation with IL-1β for 24 h decreased the basal ratio of OPG to RANKL mRNA level by 80% ($p > 0.05$) and IL-1β-induced OPG to RANKL mRNA level was induced, but not significantly, by 4-fold with HA pre-incubation ($p > 0.05$; Figure 5C).

HA decreased IL-1β-stimulated CD44 and ICAM-1 gene expression in mouse osteoblasts. Because HA is known to associate with several cell-surface molecules such as CD44 and ICAM-1 to transduce cell signaling, we assessed the effect of HA on the expression of these receptors by osteoblasts. Osteoblasts were treated as described. HA did not significantly alter the basal mRNA levels of CD44 and

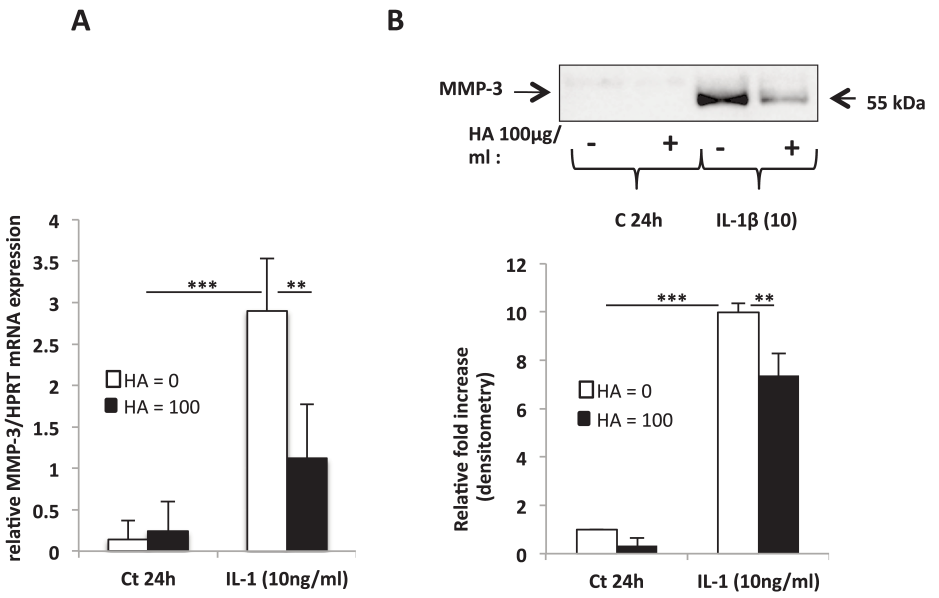

Figure 1. Effect of hyaluronan (HA) on interleukin 1β (IL-1β)-induced matrix metalloproteinase 3 (MMP-3) mRNA expression and protein release. Osteoblasts were treated for 7 days with or without HA (100 µg/ml). IL-1β (10 ng/ml) was added for the last 24 h. A. Quantitative PCR analysis of mRNA level of MMP-3. B. Western blot analysis of MMP-3 release into medium. Data are mean of 4 independent experiments analyzed in duplicate (corrected p values: ** < 0.01; *** < 0.001). HPRT: hypoxanthine phosphoribosyltransferase.

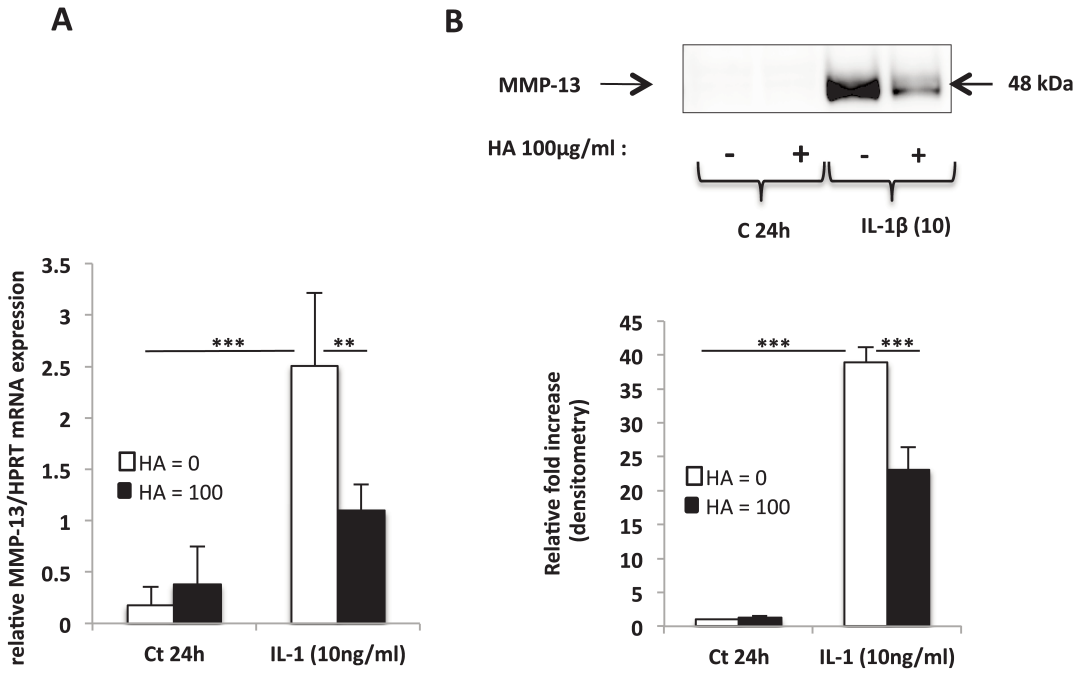

Figure 2. Effect of hyaluronan (HA) on interleukin 1β (IL-1β)-induced matrix metalloproteinase 13 (MMP-13) mRNA expression and protein release. Osteoblasts were treated as in Figure 1. A. Quantitative PCR analysis of mRNA level of MMP-13. B. Western blot analysis of MMP-13 release into medium. Data are mean of 4 independent experiments analyzed in duplicate (corrected p values: ** < 0.01; *** < 0.001). HPRT: hypoxanthine phosphoribosyltransferase.

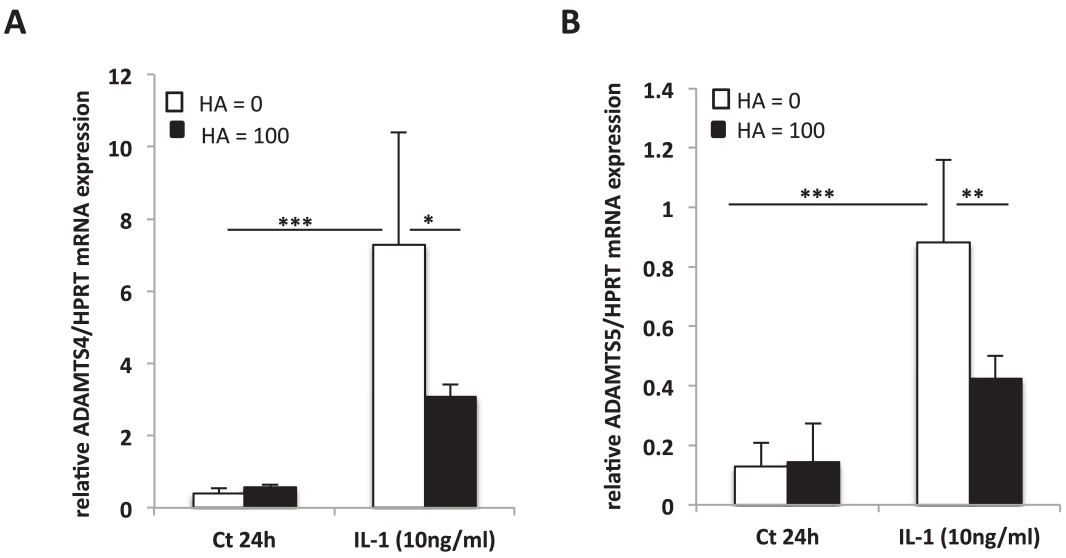

Figure 3. Effect of hyaluronan (HA) on interleukin 1β (IL-1β)-induced ADAMTS-4 and ADAMTS-5 mRNA expression. Osteoblasts were treated as in Figure 1. Quantitative PCR analysis of mRNA levels of ADAMTS-4 (A) and ADAMTS-5 (B). Data are mean of 4 independent experiments analyzed in duplicate (corrected p-values: * < 0.05; ** < 0.01; *** < 0.001). HPRT: hypoxanthine phosphoribosyltransferase.

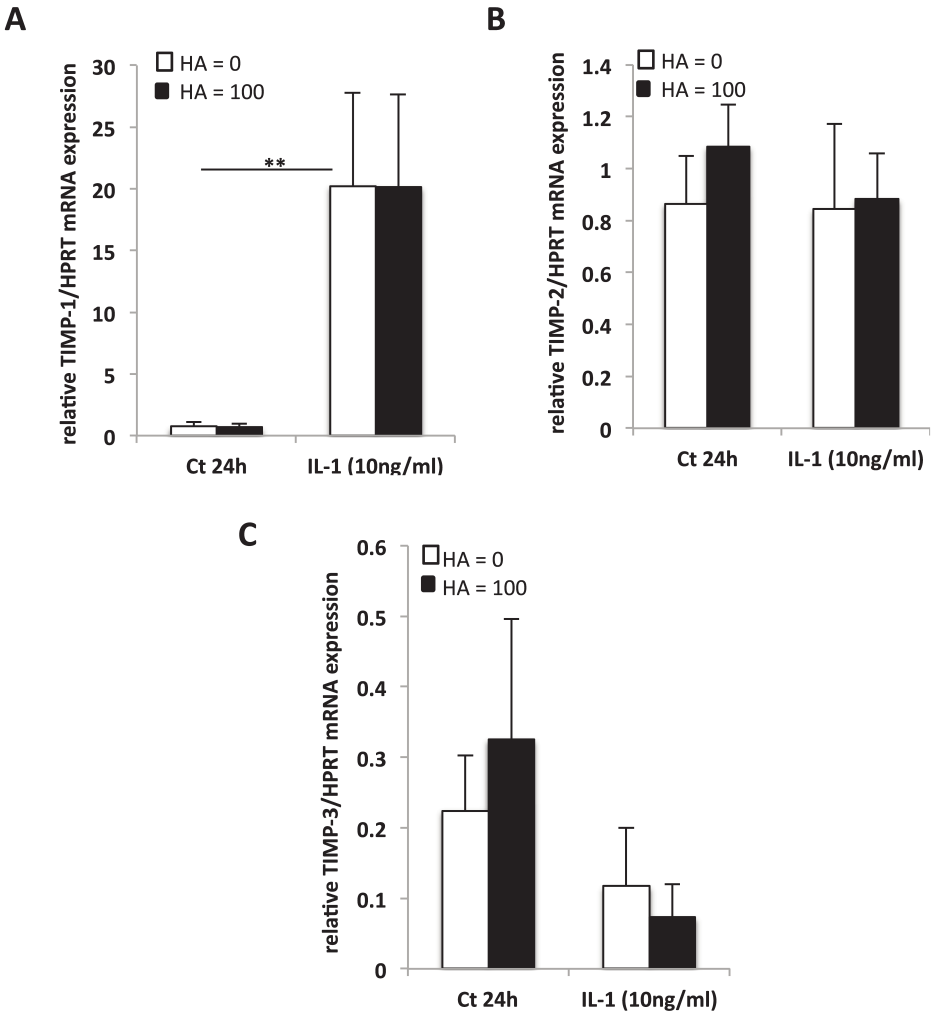

Figure 4. Effect of hyaluronan (HA) on interleukin 1β (IL-1β)-induced tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, and TIMP-3 mRNA expression. Osteoblasts were treated as in Figure 1. Quantitative PCR analysis of mRNA levels of TIMP-1 (A), TIMP-2 (B), and TIMP-3 (C). Data are mean of 4 independent experiments analyzed in duplicate (corrected p-value: ** < 0.01). HPRT: hypoxanthine phosphoribosyltransferase.

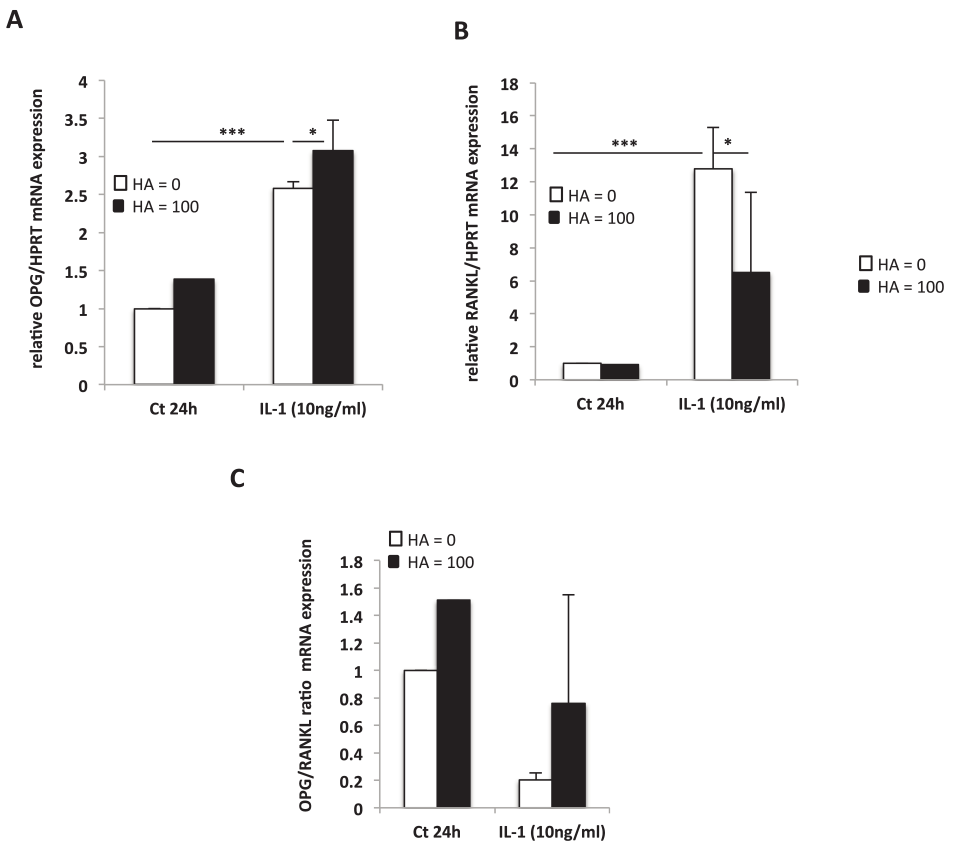

Figure 5. Effect of hyaluronan (HA) on interleukin 1β (IL-1β)-induced osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL) gene expression and ratio of OPG to RANKL. Osteoblasts were treated as in Figure 1. Quantitative PCR analysis of mRNA levels of OPG (A), RANKL (B), and ratio of OPG to RANKL (C). The mRNA levels of OPG and RANKL were normalized to that of hypoxanthine phosphoribosyltransferase (HPRT). Data are mean of 4 independent experiments analyzed in duplicate (corrected p values: * < 0.05; *** < 0.001).

ICAM-1. Moreover, stimulation with IL-1β for 24 h elevated the basal level 4-fold ($p < 0.001$; Figure 6A and 6B) and IL-1β-induced CD44 and ICAM-1 mRNA levels were decreased by 57% (Figure 6A) and 58% (both $p < 0.001$; Figure 6B), respectively, with HA pre-incubation.

DISCUSSION

OA is now considered a global organ failure involving all tissues of the joint: the cartilage, synovial membrane, and subchondral bone[1]. In recent years, many studies have focused on the role of subchondral bone in the pathophysiologic features of the disease. Subchondral bone is a site of several dynamic morphological changes involving a remodeling process[1]. In OA, subchondral bone shows accelerated phases of bone resorption and bone formation[30,31,32]. These changes are associated with altered metabolism of osteoblasts, which leads to abnormal production of soluble mediators. Such mediators produced by bone cells can affect deep-zone chondrocytes through the bone-cartilage interface[2]. The hypothesis of a central role for subchondral bone has been recently strengthened by the demonstration of bone-marrow lesions seen more frequently on MRI in the subchondral bone compartment of symptomatic than nonsymptomatic patients with OA. Thus, subchondral bone cells could be targeted to treat OA and decrease cartilage degradation. To assess the role of subchondral bone osteoblasts in the OA process, we developed a model of mouse osteoblasts in culture[28].

Here, we investigated whether HA could counteract the expression of proteases (known to be deleterious for cartilage) and bone remodeling (ratio of OPG to RANKL) by murine osteoblasts in an inflammatory context. Osteoblasts were isolated from calvaria because of technical limitations in using subchondral bone cells from mice. Although calvaria-derived and long-bone-derived cells have distinct embryonic origins and show differences within tissues in architectural organization and protein synthesis pattern[33,34] when isolated and cultured, osteoblasts from calvaria or long bones have a similar response to mechanical strain or other stimuli[35,36]. Another possibility is to use human osteoblasts from subchondral bone. Four million cells are needed after cell culture for experiments.

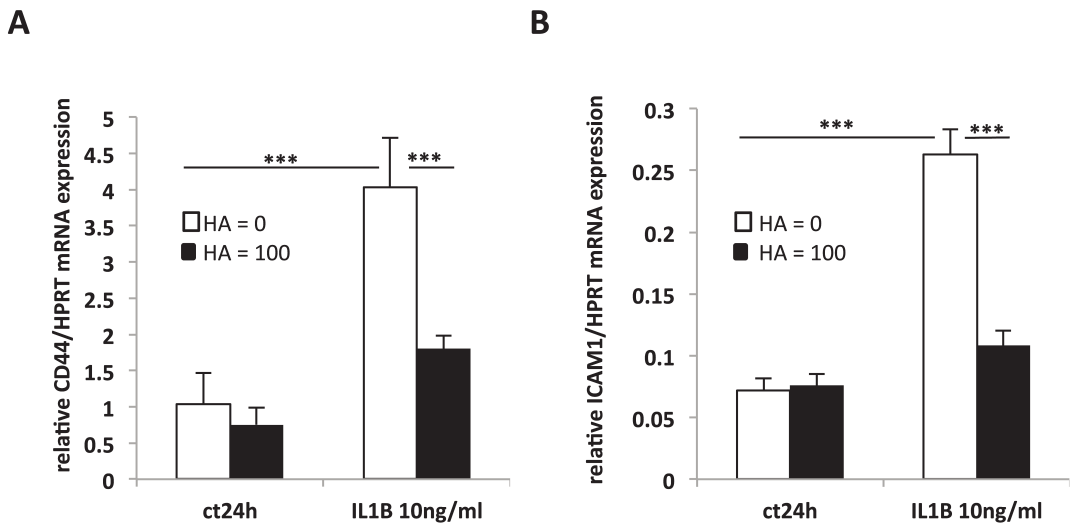

Figure 6. Effect of hyaluronan (HA) on interleukin 1β (IL-1β)-induced CD44 and intercellular adhesion molecule 1 (ICAM-1) mRNA expression. Osteoblasts were treated as in Figure 1. Quantitative PCR analysis of mRNA levels of CD44 (A) and ICAM-1 (B). Data are mean of 4 independent experiments analyzed in duplicate (corrected p value: *** < 0.001). HPRT: hypoxanthine phosphoribosyltransferase.

Unfortunately, one cannot obtain a sufficient number of cells from 1 patient with OA[37]. Moreover, we did not want to mix cells from different patients in the same experiment because of the well-known heterogeneity of the disease. Therefore, we used mouse calvaria osteoblast-lineage cells. Osteoblasts were incubated with 100 ∝g/ml HA during the last week of culture, then stimulated with IL-1β for 24 h. The physiological relevance of this choice of protocol was based on data demonstrating reduced concentration of HA in synovial fluid from patients with OA[38,39]. HA could facilitate solute exchange between the synovial capillaries and cartilage and other joint tissues. In addition, HA could form a pericellular coat around cells, interacting with proinflammatory mediators, where it modulates gene expression. So, the addition of HA to osteoblasts before stimulation with IL-1β could counteract the low concentration of HA observed in the synovial fluid of patients with OA.

We tried 4 different concentrations of HA (1, 10, 100, and 1000 μg/ml). The protein expression of MMP-13 decreased dose-dependently in the presence of HA (data not shown). The maximal decrease was seen at 1000 μg/ml, but at this concentration, the phenotype of the cells was modified. Indeed, we tested the effect of HA on osteoblast differentiation (alkaline phosphatase) and mineralization (alizarin red). HA with or without IL-1β did not modify these 2 variables in our culture, except at 1000 μg/ml. At 1000 μg/ml, osteoblast differentiation and mineralization decreased. So, we chose 100 μg/ml for our study.

IL-1β induced MMP-3 and MMP-13 gene expression and synthesis in murine osteoblasts, and HA could significantly counteract this effect. However, the specific antibodies used against MMP-3 and MMP-13 were not able to distinguish between pro-enzyme or activated enzyme forms. So, whether the effect of HA is primarily on mRNA expression or on translation of the MMP in this system requires additional experiments.

One possible role for MMP is to prepare recruitment sites for osteoclasts and progenitors by degrading the extracellular matrix covering the mineralized bone surface and then exposing RGD (Arg-Gly-Asp) sequences, which allow for osteoclast adhesion through the α5/β3 integrin receptor[40]. Previous studies suggested that osteoclasts adhere to denatured collagen through α5/β3 integrin, which leads to osteoclast activation. Further, degradative products of collagens may activate osteoclasts[41]. Thus, degradation of collagen on the bone surface allows for osteoclast attachment but may also stimulate osteoclasts to proceed to activation and resorption phases. MMP-3 contributes to the resorption of osteoid matrix by activating collagenases. Our results in osteoblasts agree with our hypothesis that HA may modulate osteoclast activation by inhibiting MMP-3 expression. Moreover, HA decreasing the production of MMP-3 and MMP-13 at the bone-cartilage interface by subchondral osteoblasts may have an antidegradative effect in the deep-zone cartilage in OA[16].

We demonstrated that IL-1β induces ADAMTS-4 and ADAMTS-5 gene expression, and that HA could significantly counteract this effect. The loss of aggrecan, through the actions of aggrecanase enzymes, is a key event in early OA, and ADAMTS-4 and ADAMTS-5 are the major cartilage aggrecanases in humans[42]. Studies in mice show that deletion of ADAMTS-5 protects against the devel-

opment of OA and inflammatory arthritis, so ADAMTS-5 may play a key role during OA development[3].

The posttranslational regulation of MMP activity depends on the counteracting interactions between MMP and inhibitory TIMP[43]. TIMP-1 is secreted as a 28-kDa glycoprotein that binds tightly to the active form of multiple MMP. TIMP-2 is a nonglycosylated 21-kDa protein with biochemical properties similar to those of TIMP-1 in inhibiting various MMP; it can preferentially inhibit MMP-2[44]. TIMP-3, unlike TIMP-1 and TIMP-2, binds to the extracellular matrix[45], and TIMP-4, the newest member of the TIMP family, blocks the activities of several MMP implicated in arthritic cartilage erosion[46]. Therefore, when TIMP levels exceed those of the active enzyme, connective tissue turnover is prevented. We did not find TIMP-4 expression in murine osteoblasts. Moreover, IL-1β could upregulate TIMP-1 expression but had no effect on TIMP-2 or TIMP-3 in mouse osteoblasts. As well, HA was not able to modify this expression.

Evidence has shown that subchondral bone alterations in OA are intimately involved in cartilage degradation, and the ratio of OPG to RANKL may be implicated[47]. We found that HA could modulate osteoclast activation by modulating the expression of these molecules. With HA pre-incubated for 7 days before IL-1β, OPG mRNA expression was increased by 19% and that of RANKL was decreased by 49% in mouse osteoblasts, so HA could increase the mRNA ratio of OPG to RANKL. Extracellular OPG was shown to modulate the half-life of membranous RANKL by enhancing its degradation through an internalization process[47]. Nonetheless, HA may also act indirectly through the production of other factors that in turn modulate the ratio of OPG to RANKL and/or resorption activity. Hence, HA, through RANKL-dependent and RANKL-independent mechanisms, may explain the additive-reduced resorption.

Thus, we demonstrate that, in an inflammatory context, HA inhibits the production of MMP and the expression of ADAMTS-4 and ADAMTS-5 and RANKL. The mechanism of action of HA on these proteins remains to be elucidated. HA is known to associate with several cell-surface molecules such as CD44 and ICAM-1. In our present study, we demonstrated that CD44 and ICAM-1 mRNA are expressed in mouse osteoblasts. Moreover, IL-1β induced CD44 and ICAM-1 gene expression and HA could significantly prevent this effect. Whether CD44/ICAM-1 pathways are involved in HA modulation of the IL-1β effects requires additional experiments. Previous studies have shown that the anticatabolic effects mediated by HA require interactions with CD44 or ICAM-1 in a cell-type-dependent and target-gene-dependent manner[48,49]. However, the downstream signaling components involved in this inhibitory effect remain unclear. Therefore, further studies are needed to fully elucidate these signaling findings.

In a rabbit OA model, intraarticularly injected (IAI) high-molecular-weight HA reached the subchondral bone and decreased the OA-enhanced MMP-13 expression in subchondral bone, likely through interaction with CD44[27]. In OA joints, vascularization and microcracks arise between the cartilage and subchondral bone[16], allowing substances to move directly between the cartilage and subchondral bone beyond the tidemark. Human OA joint fluid has a lower concentration of HA (normally 0.3–0.4%; in OA, 0.1–0.2%)[50]. IAI-HA in the clinic increases the concentration of hyaluronan and can promote the penetration of HA into the subchondral bone. IAI-HA may inhibit cartilage degeneration by inhibiting the resorption of subchondral bone and protecting subchondral bone microarchitecture by suppressing MMP-13 expression. It may act by regulating MMP-13 expression in subchondral bone, so inhibition of MMP-13 in subchondral bone may be a new approach for treating OA. Further investigation of bone metabolism in subchondral bone is necessary to improve treatment.

Our data demonstrate that, in an inflammatory context, HA inhibits the production of MMP and the expression of ADAMTS-4 and ADAMTS-5 and RANKL. The beneficial effect of HA in OA may be due to its action on cartilage and subchondral bone.

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