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The effects of hyaluronan on matrix metalloproteinase-3 (MMP-3), interleukin-1β (IL-1β), and tissue inhibitor of metalloproteinase-1 (TIMP-1) gene expression during the development of osteoarthritis

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Summary

Objective: To assess the influence of intra-articular injection of hyaluronan (HA) on expression of matrix metalloproteinase-3 (MMP-3), interleukin-1β (IL-1β), and tissue inhibitor of metalloproteinase-1 (TIMP-1) in cartilage and synovium during the process of osteoarthritis (OA).

Design: Eighteen mature New Zealand white rabbits underwent unilateral anterior cruciate ligament transection (ACLT) and were divided into two groups. The first group (HA injection group) received 0.3 ml of intra-articular HA injections into the ACLT knees 4 weeks after transection, once a week for 5 weeks as per clinical treatment presently utilized. The animals in the second group (no injection group) were not injected after ACLT. At death, 9 weeks following surgery, synovium and cartilage were harvested and total RNA was extracted. Gene expressions of MMP-3, IL-1β and TIMP-1 were analyzed using reverse transcription-polymerase chain reaction (RT-PCR) for each subgroup created according to morphological grade of OA.

Results: The extent and grade of cartilage damage in the HA injection group was less severe than in the no injection group. In synovium, expression of MMP-3 and IL-1β mRNA was suppressed in the mild grades of OA in the HA injection group. HA treatment had either no effect on MMP-3 expression in cartilage at all grades of OA or on enhanced MMP-3 and IL-1β expression in synovium at a progressed grade. No effect of HA treatment on TIMP-1 expression was observed in either cartilage or synovium.

Conclusions: These results suggest that one of the mechanism of therapeutic effect of HA is down-regulation of MMP-3 and IL-1β in synovium during early development of OA.

Key words: Hyaluronan, Osteoarthritis, Cytokine, Matrix metalloproteinase.

Introduction

Osteoarthritis (OA) is a degenerative joint disease resulting in cartilage erosion, subchondral bone remodeling, osteophyte formation, and synovial inflammation. Although OA might have multiple origins, current evidence suggests that both mechanical and biochemical factors play an important role in its progression.

Matrix metalloproteinases (MMPs) synthesized and secreted from connective tissue cells have been thought to participate in the degradation of extracellular matrix since many of them have their optimum enzymic activity around physiological pH. In fact, increased MMP activities that degrade proteoglycans have been measured in OA cartilage in human [1] and experimental animal models [2]. Among MMPs, MMP-3 (stromelysin) degrades several extracellular matrix molecules, including cartilage proteoglycan and type II collagen [3, 4].

Tissue inhibitor of metalloproteinase (TIMP) is a glycoprotein and inhibits all MMPs on a 1:1 basis by forming high-affinity complexes [5]. TIMP was not elevated in OA cartilage and synovium as much as MMPs [1, 6]. An imbalance between the activities of MMPs and TIMP is thought to be important in the progression of OA.

Among the proinflammatory cytokines, interleukin-1β (IL-1β) is abundantly synthesized by OA synovium [7] and cartilage [8]. IL-1β induces the expression of MMP-3 [9] as well as prostaglandin [10] and inhibits the formation of extracellular

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matrix [11]. IL-1β is also an inhibitor of chondrocyte proliferation and induces in chondrocytes the production of high levels of nitric oxide (NO) and apoptosis [12, 13]. IL-1β appears to be primarily responsible for cartilage catabolism.

Intra-articular injection of hyaluronan (HA) is now widely used in the treatment of OA. Hyaluronan, one of the principal components of cartilage matrix, has been reported to have a positive effect on the maintenance of cartilage matrix integrity during the development of OA [14, 15]. Exogenous HA, previously used simply as a lubricative agent, has been shown to delay degradation of cartilage by inhibiting glycosaminoglycan (GAG) release from cartilage tissue [16] and has antiinflammatory effects [17, 18]. However, the mechanisms of such therapeutic effect of HA on OA remains to be elucidated. In this study, we are investigating the mechanisms of effects of HA on OA; expression of MMP-3, IL-1β, and TIMP-1 mRNA was assessed in the articular cartilage and synovium of experimentally induced OA rabbits with or without intra-articular injection of HA.

Materials and methods

ANIMALS

Eighteen mature New Zealand White rabbits underwent unilateral anterior cruciate ligament transection (ACLT). According to a protocol approved by the UCSD Institutional Review Board, ACLT was performed using a medial arthrotomy method [19]. Postoperatively the animals were permitted cage (60 cm × 60 cm × 40 cm) activity without immobilization. We divided the animals into two groups. The first group (HA injection group, $N=10$) received 0.3 ml of intra-articular HA (MW = 8×10^5, 10 mg/ml, Seikagaku Corp., Tokyo, Japan) injections into the operated knees 4 weeks after ACLT, once a week for 5 weeks as per clinical treatment presently utilized. The second group (no injection group, $N=8$) was not injected after ACLT. All animals were killed at 9 weeks following surgery. Four untreated rabbits were used as normal controls.

GROSS MORPHOLOGY

At death, all operated knees were evaluated for gross morphological changes in the cartilage. The femoral condyles were photographed using a 35-mm camera equiped with a close-up micro lens. Gross morphological changes of the femoral condyles were assessed following the application of India Ink and classified into four grades [Grade 1 (intact surface), Grade 2 (minimal fibrillation), Grade 3 (overt fibrillation) and Grade 4 (erosion)] as previously described [19].

EXTRACTION OF RNA AND SYNTHESIS OF FIRST-STRAND CDNA

Synovium (entire tissue) around the infrapatellar fat pads and cartilage tissue from the femoral condyle and the tibial plateau were harvested from the gross morphologically evaluated knees. Cartilage tissue or synovium from rabbit knees of the same Grade of OA were pooled and immediately frozen in liquid nitrogen.The frozen tissue was crushed and homogenized in 4 ml of 4 M guanidinium thiocyanate containing 25 mM Na-citrate, 0.5% Na-sarkosyl and 0.1 M 2-mercapto-ethanol. Total RNA was extracted by the acid-guanidinium-thiocyanate-phenol-chloroform method as previously described [20]. Chang *et al.* [21], using a video imaging technique to quantify India ink staining, demonstrated that the OA grade of the condyles and tibial plateau were correlated. Therefore, in order to obtain enough cartilage for RNA extraction, we determined the OA grade on the femoral condyles and pooled the cartilage from both the femoral condyles and the tibial plateau. Enough RNA could not be extracted to assess the expression of MMP-3, IL-1β and TIMP-1 in a small number of Grade 3 specimens. Therefore, we chose to use RNA from Grade 1, 2, or 4, respectively, in both HA and no injection groups. Total RNA extracted from the untreated rabbits was used as a normal control. To ensure that PCR amplification from contaminating genomic DNA would not affect the data, total extracted RNA was treated with 10 units/ml of RNase-free DNase (RQ1, Fisher Scientific, Pittsburgh, PA) at 42°C for 30 min. DNase was then removed by extraction and ethanol precipitation. Two micrograms of total RNA were incubated at 65°C for 5 min, chilled on ice, and reverse-transcribed in a final volume of 100 μl containing 10 μl 10 × PCR reaction buffer (Promega Co. Madison, WI), 5 mM $MgCl_2$, 1 mM each of dATP, dCTP, dGTP, and dTTT (Promega Co.), 2.5 μM oligo $(dT)_{16}$ primer, 100 units RNasin (Ribonuclease inhibitor, Promega Co.), and 2500 units M-MLV reverse transcriptase (Promega Co.). The mixture was incubated at 42°C for 1 h, heated to 94°C for 10 min, and stored at −20°C.

POLYMERASE CHAIN REACTION (PCR)

First strand cDNA for MMP-3, IL-1β and TIMP-1 was amplified by polymerase chain reaction (PCR).

In order to ensure the quality of the RNA preparation and to normalize the RT-PCR protocol, β-actin RT-PCR products were also produced for all samples. cDNA was heated to 94°C for 10 min and cooled on ice for 5 min. cDNA (5 μl) was added to a 50 μl reaction mixture containing 5 μl 10 × PCR reaction buffer (Promega Co.), 2.5 mM $MgCl_2$, 20 μM of each dATP, dCTP, dGTP, and dTTT (Promega Co.), 200 nM of each priming oligomer, and 1.0 unit Taq DNA polymerase (Promega Co.). Primers used for rabbit MMP-3, IL-1β, TIMP-1 and β-actin were as follows [22–25]. MMP-3: sence, 5′-TTCCCTGGCACCCCAAAGTG-3′, position 361–380; antisence, 5′-AATCCTGAGG GACTT-GCGCC-3′, position 998–1017; IL-1β: sence, 5′-TGCTGTCCAGACGAGGGCAT-3′, position 184–203; antisence, 5′-ACTCTCCAGCTGCAGGGTAG-3′, position 638–657; TIMP-1: sence, 5′-ACCTTGTC ATCAGGGCCA-3′, position 131–148; antisence, 5′-ACAGGCAAACACTGTGCA-3′, position 463–480; β-actin: sence, 5′-ACGTTCAACACGCCGG CCAT-3′, position 376–395; antisence, 5′-GGATGT CCACGTCGCACTTC-3′, position 849–868. Amplification was performed using a DNA thermal cycler (GeneAmp PCR System 2400, Perkin Elmer, Norwalk, CT) for 35 cycles. A cycle profile consisted of 30 sec at 94°C for denaturation, 30 sec at 58°C for annealing, and 45 sec at 72°C for extention. Electrophoresis of 10 μl of the reaction mixture on a 1.5% agarose (Fisher Scientific) gel containing ethidium bromide was performed to evaluate amplification and size of generated fragments. 100 bp DNA Ladder (GIBCO BRL Co., Gaithersburg, MD) was used as a standard size marker. National Institute of Health analysis software (version 1.60; National Institute of Health, Bethesda, MD) was used to scan the RT-PCR agarose gels after photographic documentation. The software measures relative mean density over a fixed gray scale range after correction for background.

QUANTIFICATION OF TIMP-1 MRNA BY RT-PCR

Template cDNA of TIMP-1 was obtained in the following manner to assess the kinetics of PCR. The 349 bp RT-PCR product amplified from rabbit fibroblast mRNA by using the specific primer sets [24] was subcloned into pGEM-T (Promega Co.) and purified. Serially diluted TIMP-1 templates of 10^7–10^4 copies/μl were prepared. Six μl of each template were amplified in a 60 μl reaction mixture by PCR for 38 cycles by using a step-cycle program of 30 sec at 94°C for denaturation, 30 sec at 58°C for annealing, and 45 sec at 72°C for extention. Five μl of the reaction mixture was removed at every other cycle from 16 to 38 cycles and electrophoresed on 1.5% agarose gels. We analyzed all samples of cDNA from OA and control rabbits the same way.

Results

GROSS MORPHOLOGICAL ASSESSMENT

All specimens from the ACLT knees exhibited complete transection of the ACL at death. Twenty percent (two of 10) ACLT condyles from the HA injection group showed Grade 1 (intact articular cartilage surface) [Fig. 1(a)], while none of the no injection group showed Grade 1. Two of 10 condyles from the HA injection group and two of eight from the no injection group showed Grade 2 (minimal fibrillation on the articular cartilage surface) [Fig. 1(b)] and two of the HA injection group and one of the no injection group developed Grade 3 (overt fibrillation) [Fig. 1(c)]. Five of eight condyles from the no injection group developed Grade 4 damage [Fig. 1(d)]. Four of 10 condyles from the HA injection group also showed full-thickness ulceration of the articular cartilage. Averaged scores of the HA and no injection groups were 2.8 and 4.0, respectively. The extent and grade of cartilage damage in the HA injection group was less severe than in the no injection group (Fig. 2). All knees from untreated rabbits (normal controls) had intact cartilage (Grade 1).

ASSESSMENT OF THE EFFECT OF HYALURONAN ON MMP-3 AND IL-1β MRNA EXPRESSION BY RT-PCR

Triplicate experiments for each PCR yielded nearly identical results. Figures are those of the representative cases of the repeated experiments.

PCR products for β-actin were clearly detected in RNA preparations from all synovium [Fig. 3(c)] and cartilage [Fig. 3(d)], which confirmed uniformity of RNA preparation. mRNA for MMP-3 was slightly detected in normal synovium and Grade 2 of the HA injection group and was upregulated in Grade 4 of both HA and no injection groups, while MMP-3 expression was completely suppressed in Grades 1 and 2 of the HA injection group [Fig. 3(a)]. In normal cartilage, no MMP-3 mRNA was detected. An upregulation of MMP-3 expression was observed along with the progression of OA in the no injection group. The HA injection group showed MMP-3 expression in Grade 1 and upregulation in Grades 2 and 4 of articular cartilage [Fig. 3(b)]. IL-1β expression was detected in control synovium and was upregulated in the Grade 4 of both HA and no injection groups, while being suppressed in Grades 1 and 2 of the HA injection

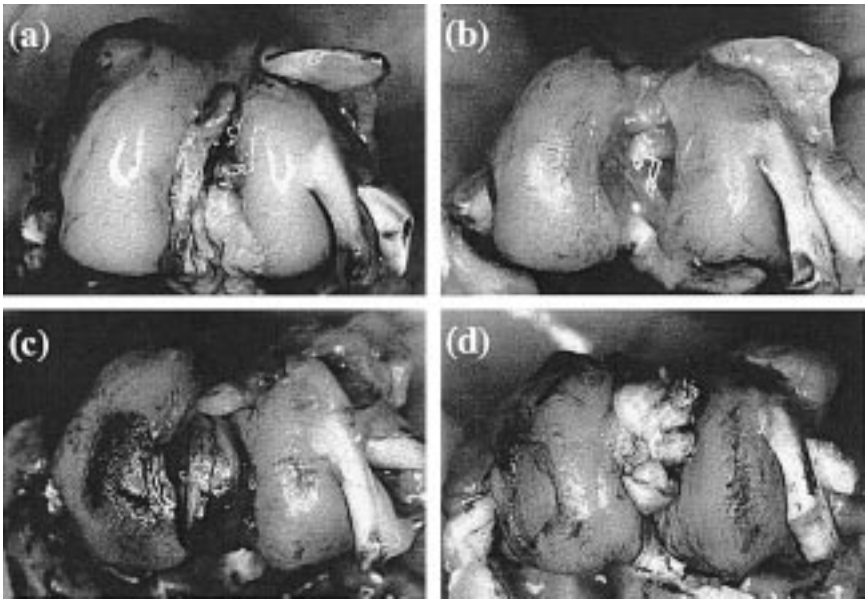

FIG. 1. Gross morphological change of the femoral condyles during developing OA. (a) Grade 1 specimen showing smooth cartilage surface and normal appearance. (b) Grade 2 specimen showing minimal fibrillation of articular cartilage. (c) Grade 3 specimen showing overt fibrillation on the medial condyle. (d) Grade 4 specimen. Full-thickness ulceration is marked on the medial condyle along with overt fibrillation on the lateral condyle.

group (Fig. 4). No IL-1β expression was observed in any cartilage. TIMP-1 mRNA expression was detected in all samples by RT-PCR, and the expression seemed to be similar.

ASSESSMENT OF THE EFFECT OF HYALURONAN ON TIMP-1 mRNA EXPRESSION BY RT-PCR

In order to further analyze TIMP-1 expression, quantification of mRNA by RT-PCR was performed. Serially diluted TIMP-1 templates were used for PCR to assess the kinetics of PCR of this fragments. As shown in Fig. 5, PCR was performed for 16–38 cycles at 2-cycle intervals. The amount of PCR products increased exponentially in the early cycles of the reaction and reached a plateau. The more cDNA exists, the earlier the ethidium-bromide luminescence of products showed up and reached a plateau level. The cycles in the middle of exponentially increasing period quantitatively reflected the amount of the initial template. Table I indicates the cycles in the middle of exponentially increasing period in PCR for serially diluted templates and for samples of cDNA from normal control or ACLT rabbits. TIMP-1 expression was upregulated in Grade 4 in both HA and no injection groups in synovium; however, no difference was observed between the HA and no injection groups. In cartilage, no differences were observed among samples.

Discussion

Since OA in humans is most commonly a slowly progressive disease and its onset is not ordinarily definable, the development of the disease is difficult to study. We previously characterized the ACLT rabbit joints model and demonstrated its validity to study the development of OA. This OA model showed mild OA changes in all joints as early as 4 weeks after ACLT, whereas OA progressed in 40% of joints to develop full thickness of ulceration (Grade 4) by 8 weeks. By 12 weeks 60% of joints developed Grade 4 OA [19]. We also demonstrated a therapeutic effect of HA during development of OA by administering HA using a similar model [14, 15]. In the present study, ACLT rabbits in the no injection group developed Grade 4 OA in 63% of operated knees 9 weeks after ACLT while this grade was decreased to 40% of the condyles in the HA injection group. These results confirm the consistency and reproducibility of the OA model as well as the effect of HA during the development of OA. While the optimal protocol of HA treatment for rabbits is uncertain, we kept the number of injections similar to that used clinically for humans as well as maintaining consistency with our previous studies [14, 15]. The volume of injected solution (0.3 ml) was determined to be the maximum amount injectible without increasing joint fluid pressure.

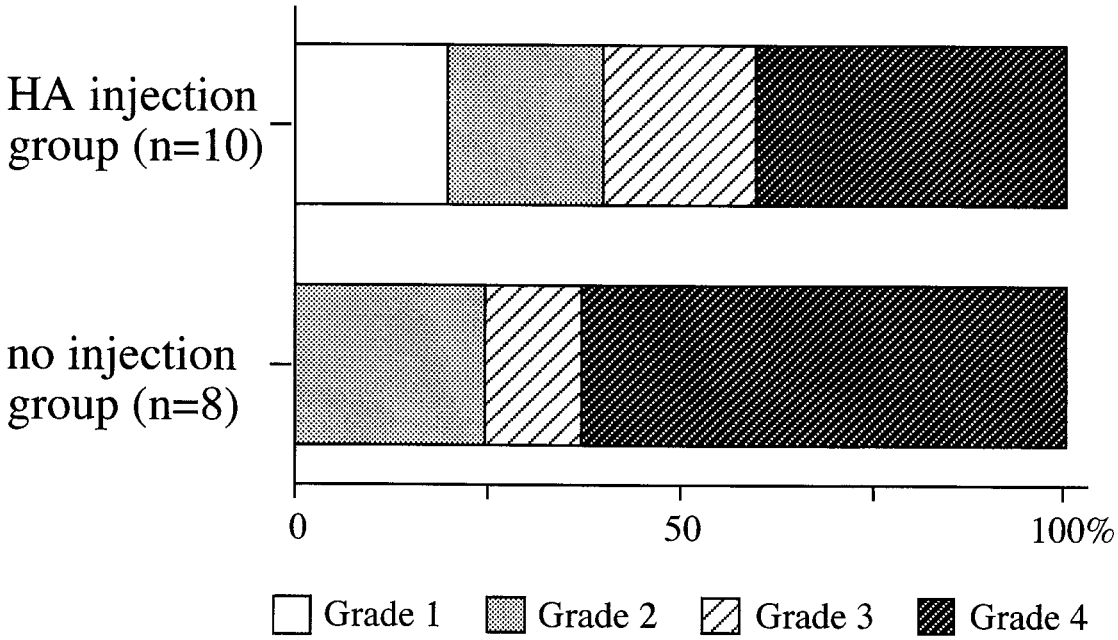

FIG. 2. Gross morphological assessment of the femoral condyles from the HA injection group (N=10) and the no injection group (N=8). Note that the grade of cartilage damage in the HA injection group was less severe than in the no injection group.

Increased attention has been focused on the involvement of enzymatic pathways associated with the degradation of extracellular matrix and its regulation by several cytokines. This study yielded data relating to this topic. This study showed that mRNA expressions of MMP-3 in cartilage and synovium and IL-1β and TIMP-1 in synovium were upregulated in experimental OA. MMP-3 mRNA expression in human OA synovium was reported to be enhanced [26] as well as the MMP-3 protein levels in human OA cartilage and synovium [9]. Previous studies also showed upregulation of TIMP-1 mRNA expression in human OA synovium and cartilage [26]. The level of IL-1β is reported to be increased in human and experimental OA in cartilage and synovium [7, 8]. Our results are consistent with these studies.

This study also showed that HA downregulated MMP-3 and IL-1β expression in synovium from 4 animals showing mild OA [Grade 1 (N=2) and Grade 2 (N=2)]. A previous study demonstrated the suppression of synovial hyperplasia by HA following induction of OA in a similar model [14]. IL-1β is one of the most potent proinflammatory cytokines and has been shown to induce the production of MMP-3 [9], as well as prostaglandin (PG) [10]. The results of this study provides additional evidence of an antiinflammatory effect of HA on synovium during early stages of OA.

Several explanations can be proposed to account for this suppression following HA administration. The protective effect of HA on cartilage might affect the expression of catabolic mediators in the synovium. A variable degree of synovial inflammation frequently observed in OA is believed to be secondary to the cartilage breakdown while inflamed synovium might result in increased synthesis of proteases leading to cartilage matrix breakdown. In an in-vitro study, it was shown that HA could inhibit GAG release from the cartilage tissue [16]. However, we compared expression of mediators between the samples with the same grade of cartilage degeneration. Alternatively, it could be argued that HA has a direct effect on synovial tissue by decreasing MMP-3 and IL-1β expression. Some antiinflammatory effects of HA on synoviocytes have been described *in vitro*. The levels of PGE_2 released by synovial cells in response to IL-1α activation was suppressed by HA [17]. In one study, HA was shown to inhibit arachidonic acid released from the synovial cells in a concentration and MW-dependent manner [18]. These direct antiinflammatory effects of HA on synovial cells might suppress MMP-3 expression secondary to IL-1β. There is also a possibility that HA may suppress the basal expression level of MMP-3 and IL-1β in synovium. While HA downregulates MMP-3 and IL-1β in synovium during the early stages of OA, it seemed to have no effect on MMP-3, IL-1β and TIMP-1 expression in synovium from joints having more advanced OA.

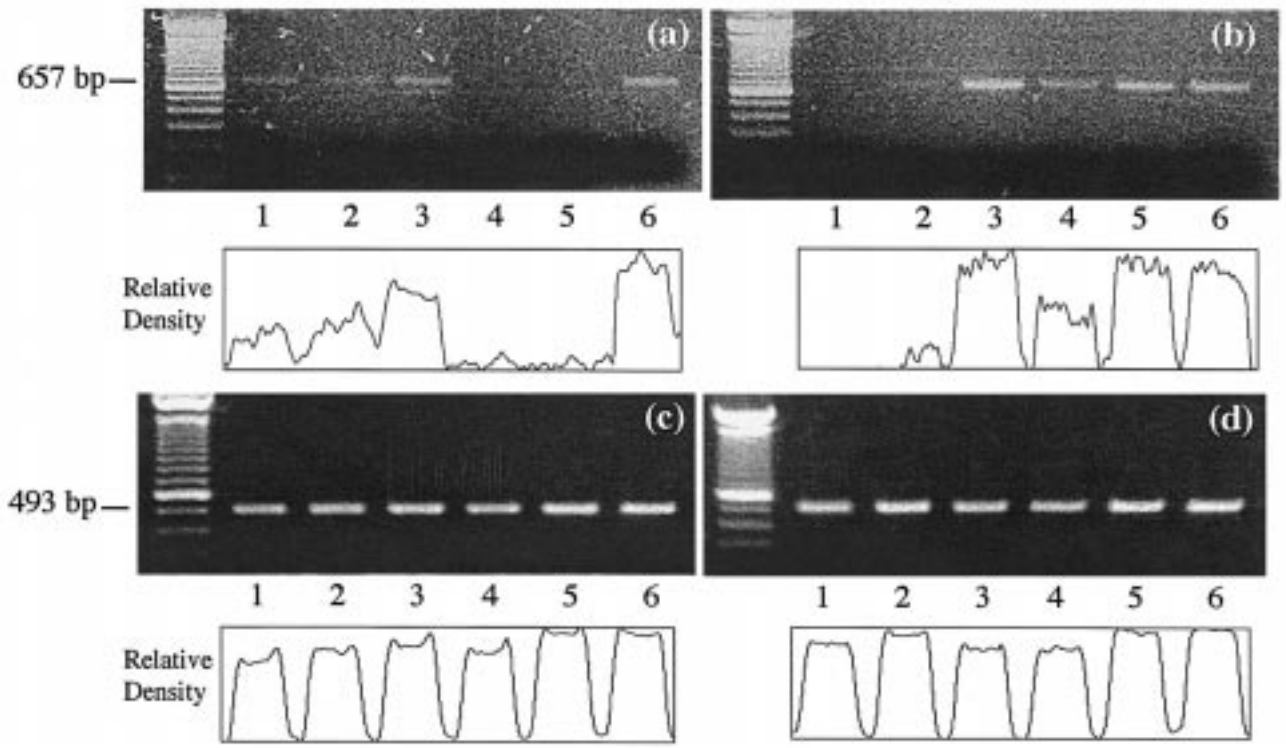

FIG. 3. RT-PCR of MMP-3 (a and b) and β-actin (c and d). One hundred nanogram of total RNA from synovium (a and c) and cartilage (b and d) from each grade of OA were used for all amplifications. lane1: Normal control. Lane 2: Grade 2 of no injection group. Lane 3: Grade 4 of no injection group. Lane 4: Grade 1 of HA injection group. Lane 5: Grade 2 of HA injection group. Lane 6: Grade 4 of HA injection group. National Institute of Health image analysis scans of meassuring the relative mean densities of the products are shown at the bottom.

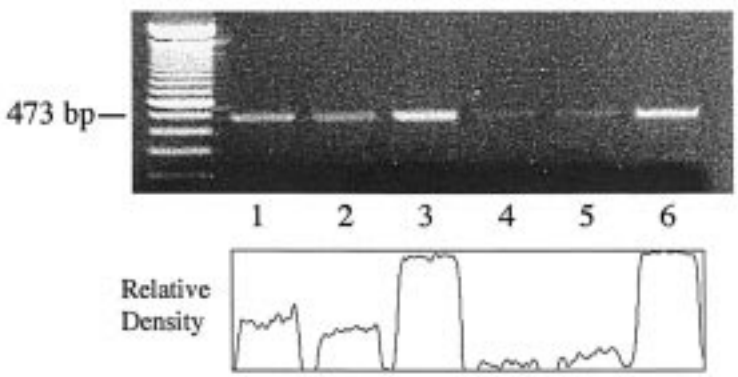

FIG. 4. IL-1β mRNA expression in synovium from each grade of OA. One hundred nanograms of total RNA were used for all RT-PCR. Lane 1: Normal control. Lane 2: Grade 2 of no injection group. Lane 3: Grade 4 of no injection group. Lane 4: Grade 1 of HA injection group. Lane 5: Grade 2 of HA injection group. Lane 6: Grade 4 of HA injection group. National Institute of Health image analysis scan measuring the relative mean densities of the products is shown at the bottom. No IL-1β expression was observed in any cartilage (data not shown).

In cartilage, HA had no effect on MMP-3 expression in the progressed grade of OA. However, MMP-3 expression seemed to be enhanced by HA during the early stages of OA. One related study has shown that HA enhanced MMP-3 activity in isolated bovine articular chondrocyte culture [27]. However, it was reported that HA did not penetrate into normal cartilage tissue but coated cartilage surface while deep intrusion was observed in severely damaged OA cartilage tissue [28]. Therefore, it seems unlikely that injected HA would dramatically influence gene expression in chondrocytes in intact or nearly intact cartilage. It may be possible that HA inhibited cartilage degeneration and improved OA grade in spite of its lack of effect on MMP-3 expression in

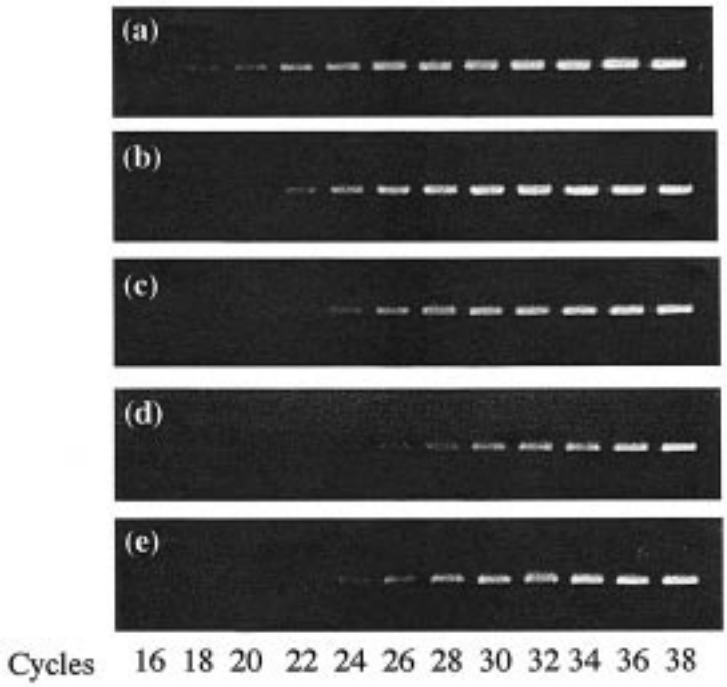

FIG. 5. Kinetics of PCR for TIMP-1 cDNA fragments. Six μl of serially diluted TIMP-1 templates of 10^7 (a), 10^6 (b), 10^5 (c), and 10^4 (d) copies/μl were amplified in a 60 μl reaction mixture. Five μl of the reaction mixture was removed for the electrophoresis at every other cycle from 16 to 30 cycles. Note that the cycles in the middle of the exponentially increased period quantatively reflected the amount of the initial template. All samples of cDNA from OA and control rabbits were assessed the same way. A representative electrophoresis of samples was shown (e).

Table I
Quantification of TIMP-1 mRNA expression

Templates	10^7	10^6	10^5	10^4		
	18	20	24	26		
Group	Control	No injection		HA injection		
OA Grade	1	2	4	1	2	4
Synovium	24	24	22	24	24	22
Cartilage	22	22	22	22	22	22

The cycles in the middle of the exponentially increased period in PCR for control plasmid templates and for samples from rabbits were shown. The numbers of cycles were averaged by the results from triplicated experiments.

cartilage. It should be noted that MMP-3 could be secreted as a proenzymatic form and activated in tissue [29].

In the present study, we could clearly detect an influence of HA on MMP-3 and IL-1β mRNA expression during development of OA while TIMP-1 expression was not different between the groups. Since it was difficult to detect small differences in TIMP-1 expression using ordinary RT-PCR techniques, we performed a more refined quantification in order to further analyze TIMP-1 mRNA. Even by this method, however, we could not establish an effect of HA on TIMP-1 expression in either cartilage or synovium. Specific cell membrane receptors for HA which belong to the CD44 adhesion glycoprotein family have now been identified and allow HA to modulate cell function directly [30, 31]. The binding of HA to these receptors appears to be molecular weight-dependent. In addition to the difficulty of intrusion of HA into intact cartilage, depolymerization or degradation of injected HA might also arise and explain the different TIMP-1 results between our in-vivo study and the in-vitro study which showed an enhancement of TIMP-1 production by HA in bovine articular chondrocytes cultured in the presence of IL-1α [27].

In conclusion, this study has demonstrated that intra-articular injection of HA suppressed MMP-3 and IL-1β expression in synovium during the early

stages of OA in an experimental rabbit model. HA treatment had no effect on either MMP-3 or IL-1β expression in cartilage at all grades of OA or on MMP-3 expression in synovium at a progressed grade despite the delay in morphological degeneration of cartilage. TIMP-1 expression was not influenced by HA. These results suggest that one of the mechanisms of the therapeutic effect of HA is downregulation of MMP-3 and IL-1β in synovium during early development of OA.

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