

EFFECTS OF SODIUM HYALURONATE ON EXPERIMENTAL OSTEOARTHRITIS IN RABBIT KNEE JOINTS

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ABSTRACT

The aim of this study was to examine the effects of intraarticular administration of hyaluronan (HA) on cartilage degradation. Using a partial meniscectomy model of osteoarthritis (OA) in the rabbit knee, the authors investigated the catabolic and anabolic changes induced by intraarticular injection of HA. To analyze anabolic changes, the authors assessed cell proliferation by measuring [³H] thymidine uptake, and proteoglycan biosynthesis by noting [³⁵S] sulfate incorporation. For catabolic changes, messenger ribonucleic acid (mRNA) expression of interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), and tissue inhibitor of metalloproteinase-1 (TIMP-1) in cartilage and synovium were detected with reverse transcriptase polymerase chain reaction (RT-PCR). Of significance for blocking the development of early OA in chondrocytes was the finding that total proteoglycan synthesis in the HA treatment group was significantly higher than in the controls. At the mRNA level in cartilage and synovium, HA inhibited MMP-3 and TIMP-1 production in the same way in the HA treatment group, while not affecting MMP-1 production. Thus it can be concluded that HA affects cartilage catabolism and anabolism to prevent the progress of OA.

Key Words: anterior cruciate ligament (ACL), osteoarthritis, matrix metalloproteinase (MMP), Tissue inhibitor of metalloproteinase (TIMP), proteoglycan

INTRODUCTION

Osteoarthritis (OA) is a destructive joint disease commonly observed among elderly people, characterized by degenerative changes in articular cartilage and reactive proliferation of bone and cartilage around the joint. Recent findings indicate that OA is not a simple destructive process, but rather a complicated cell mediated process that may result from an inappropriate balance of chondrocyte metabolism, with anabolic and catabolic changes in the articular cartilage matrix that reportedly occur even in early OA. Hyaluronan (HA), a high molecular weight polymer of glucosamine and glucuronic acid residues, is one of the key components of the articular cartilage matrix. The molecular weight is approximately 2 to 3 millions in normal synovial fluid. HA is also a major ingredient of synovial fluid which aids in the absorption of mechanical impact, joint lubrication, and preservation of articular cartilage. Moreover, recent studies have revealed that HA modulates the function of various types of cells, including articular chondrocytes and macrophages. Previous reports have revealed that HA concentration

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and molecular weight in the synovial fluid are significantly lower in arthritic joints.^{3,5)} Intraarticular HA injection therapy has been used widely in the treatment of OA, and good therapeutic results have been reported in many clinical investigations.^{16,20)} Moreover, the effects of intraarticular administration of HA have been studied extensively in many animal OA models.^{22,24,32)} A marked inhibitory effect of HA on cartilage degeneration has been described in these reports. These previous studies have focused on extracellular matrix changes rather than histologic examination. Recent research on the pathophysiology of OA has shown that the destruction of cartilage matrix in OA is caused by various proteinases.^{17,26)} The matrix metalloproteinases (MMPs), which are specifically controlled by tissue inhibitor of metalloproteinase (TIMPs), are the most likely mediators of this effect.^{10,19)}

To clarify the biologic effects of intraarticular administration of HA on cartilage degeneration, the authors investigated the anabolic and catabolic changes that were observed with and without HA therapy in a rabbit model of OA. For analysis of the anabolic changes, the authors measured the incorporation of [³⁵S] sulfate into newly synthesized proteoglycan and [³H] thymidine for chondrocyte proliferation, and for analysis of catabolic changes the authors examined messenger ribonucleic acid (mRNA) expression of interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), and tissue inhibitor of metalloproteinase-1 (TIMP-1) in cartilage and synovium.

MATERIALS AND METHODS

Experiment 1

Eighteen healthy New Zealand White male rabbits weighing 2.4 to 3 kg, aged 12 to 16 weeks (Tokyo Laboratory Animal Science, Tokyo, Japan) were used. Standard care methods were used with the rabbits during the experimental period.

Surgical procedures were performed on 12 of the 18 rabbits using sodium pentobarbital anesthesia under sterile conditions. The right knee articular capsule was incised longitudinally, the anterior cruciate ligament (ACL), posterior cruciate ligament (PCL), and medial collateral ligament were amputated, and 50% of the meniscus was resected. After washing with physiologic saline, the articular capsule and skin were sutured.

HA (Seikagaku Kogyo, Tokyo, Japan) that was extracted from rooster combs and had an average molecular weight of 8×10^5 Da, was dissolved at 1% (weight/volume) in physiologic saline. After the surgery, the 12 animals were separated into two groups of six. One group received HA saline solution treatment and the other group received physiologic saline as a control treatment. The remaining six rabbits did not undergo surgery and did not receive any treatment. Both solutions were administered in a volume of 0.2 mL by injection into the right knee joint twice a week. All animals were euthanized 4 weeks after the operation.

Cartilage Culture

After death, articular cartilage was obtained from the tibialis plates for labeling. The slices from each joint were cultured in 3 mL of Dulbecco's modified Eagle's medium (Nipro, Tokyo, Japan) containing penicillin G (100 units/mL), and heat inactivated newborn calf serum (10% volume/volume). In addition, each right knee was washed with 2 mL saline twice, and collected. Cartilage was cultured for radiochemical specific activity and concentration of 1 mCi/mL [³⁵S] sulfate and 1 mCi/mL [³H] thymidine for 12 hours.

Analysis of Cartilage Proteoglycan Content and Synthesis

After labeling, samples were washed three times with phosphate buffered saline, and the tis-

sue explants were freeze dried and weighed. Proteoglycan synthesis and chondrocyte proliferation were determined by measuring the incorporation of [^{35}S] sulfate and thymidine after prior removal of unincorporated radioactive label, using barium sulfate precipitation.¹⁵⁾ The samples were digested with 0.25% proteinase overnight at 55°C. The incorporated radioactivity was measured by liquid scintillation photometry.

Each cartilage sample was digested with chondroitinase ABC (Seikagaku Kogyo) for 2 hours at 37°C. After centrifugation, samples were analyzed by high performance liquid chromatography, and fraction corresponding amounts of chondroitin 4 and 6 sulfate were collected. The amount of chondroitin 4 and 6 sulfate in each sample was calculated according to calibrated standards. Also the radioactivity of [^{35}S] sulfate was measured.

Experiment 2

Six male New Zealand White rabbits, weighing 2 to 3 kg, were subjected to dissection of the ACL and medial collateral ligament of both knee joints. Intraarticular HA injection was performed as described in Experiment 1. Right knees were treated with HA and left knees were treated with saline as controls. All animals were euthanized 2 weeks after surgery.

After death, one of the animals was used for a histopathologic study, in which synovium and cartilage samples were obtained from its knee joint. The remaining five animal's tissues were used for mRNA detection. In the mRNA detection study, samples were obtained from the knee joint, and were separated into synovia, patellofemoral joint cartilage, and femorotibial joint cartilage groups. The tissues were soaked gently in saline, wrapped in plastic foil, frozen in liquid nitrogen, and stored at -80°C until analysis. The tissues were fixed in a periodate-lysine-paraformaldehyde fixative at 4°C overnight, dehydrated in a graded ethanol series twice, and embedded in paraffin.

mRNA Preparation

The samples were ground to powder in liquid nitrogen by hand milling. Total RNA isolation was performed by the single step guanidinium-thiocyanate-phenol-chloroform extraction procedure as described by Chomczynski and Sacchi.⁴⁾ The RNA then was estimated on an aliquot by spectrophotometry at 260 nm. After denaturation of freshly prepared RNA for 10 minutes at 65°C, complementary deoxyribonucleic acid (cDNA) was produced by reverse transcription at 42°C for 45 minutes in a 50 μL reaction mixture containing: 1 μg of total cellular RNA, reverse transcriptase buffer (10 mmol/L Tris-HCl pH 8.3, 5 mmol/L MgCl_2 , 50 mmol/L KCl); 1 mmol/L of dATP, dCTP, dGTP, and dTTP, 1 U/ μL of RNAase inhibitor (Perkin-Elmer, Norwalk, CT); and 1 U/ μL of MuML reverse transcriptase (Perkin-Elmer). After heating at 99°C for 5 minutes for denaturing and cooling to 5°C for 5 minutes, the cDNA was used for amplification.

Polymerase Chain Reaction (PCR) Amplification and Detection of Products

Enzymatic amplification of specific cDNA sequences by PCR was performed according to the recommended procedure²⁹⁾ with a DNA Thermal Cycler 480 (Perkin-Elmer) in a final volume of 50 μL Tris HCl/ MgCl_2 buffer containing 1.25 U of Amplitaq DNA polymerase (Perkin-Elmer), and 75 pmol each of sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MMP-1, MMP-3 and TIMP-1. Thirty cycles of amplification were performed for GAPDH following the recommended procedure by Perkin-Elmer (120 seconds at 95°C, 30 seconds at 60°C, 90 seconds at 72°C). Thirty-five cycles of amplification were performed for MMP-1, MMP-3 and TIMP-1. The oligonucleotide primer pairs used for this study are listed in Table 1.

Table 1. A list of primers used in this study

cDNA	Primer	Primer Sequence (5' to 3')	DAP	Reference
GAPDH	Sense	TGAACGGATTGGCCGCATT	387bp	2
	Antisense	ATTCACGCCCATCACAAACA		
MMP-1 Interstitial collagenase	Sense	AGAGCAAGATGTGGAGATGG	302bp	6
	Antisense	CTTGACAGGTCTGGTGTGTA		
MMP-3 Stromelysin-1	Sense	AAGTTCCTGGCTTGGAGGT	308bp	7
	Antisense	ATCTCCATGTTCTCGGACTC		
TIMP-1 Tissue inhibitor of metalloproteinase-1	Sense	CATACTACCTGTACCAGCG	368bp	8
	Antisense	TAGAGAGCTGTCTGTCCACA		

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DAP: deduced amplification product; bp: base pairs.

After adding 8 μ L of the solution containing PCR products to 2 μ L of loading buffer, the mixed solutions then were electrophoresed in a 1.5% agarose gel with 0.5 μ g/mL of ethidium bromide. Specific amplification for each primer pair was determined by the measurement standards of the product. Each reverse transcriptase polymerase chain reaction (RT-PCR) experiment comprised several negative controls: controls for reverse transcription (mixtures without MuML reverse transcriptase and without total cellular RNA) and controls for the PCR (mixtures without primers, without amplitaq polymerase, and without cDNA). The serial concentrations of reverse transcriptase products were tested to conform with the linearity of the amplification for performed MMP-1, MMP-3 and TIMP-1.

Image Analysis

Video images of agarose gels were obtained with a charge coupled device camera (Fotodyne, Hartland, WI).

Light Microscopic Analysis

After euthanasia, the femoral condyle and the tibialis plateau were resected and immediately fixed in a 10% formalin buffer (pH 7.4) containing 0.5% of cetylpyridinium chloride. The samples were decalcified in 0.5% ethylenediaminetetracetic acid (pH 7.4) and embedded in paraffin. The femoral condyle and the tibialis plateau were cut into coronal sections. Sections were stained with hematoxylin and eosin (H&E) for general morphologic analysis.

Electron Microscopic Analysis

Cartilage was collected from high stress areas of the tibia. Specimens were fixed with a 2% glutaraldehyde solution containing 0.1 Na-cacodylate (pH 7.4), followed by postfixing in osmium tetroxide and embedding in Spurr resin (Taab, Berkshire, England). Sections were cut on a Reichert Nissei Ultracut S (Leica, Leica AG Reichert Division, Vienna, Austria) ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a JEM1200 EX electron microscope (JEM, Tokyo, Japan) operating at 100 kV.

RESULTS

Chondrocyte Proliferation

We measured [^3H] thymidine uptake as a marker of chondrocyte proliferation. In this study, uptake of [^3H] thymidine in the hyaluronan group and saline group was significantly higher than the normal group (Fig. 1). An increase in chondrocyte proliferation was observed in both surgical groups. Moreover, there was no significant difference between the HA and saline groups with regard to cell proliferation.

Biosynthesis of Proteoglycan

The amount of [^{35}S] sulfate incorporation was used as a marker of proteoglycan biosynthesis. [^{35}S] sulfate uptake into cartilage in the HA group was higher than in the saline and normal groups (Fig. 2). In addition, total proteoglycan biosynthesis was measured as [^{35}S] sulfate incorporation into sulfate glycosaminoglycan, chondroitin 4 and 6 sulfate. Uptake in the HA group was significantly higher than that in the saline and normal groups, and the saline group was significantly higher than the normal group (Fig. 3).

These data suggest that cartilage proteoglycan synthesis and chondroitin sulfate synthesis increased in the two surgical groups, and that HA has more of an enhancing effect than saline.

mRNA Expression

Expression of performed MMP-1, MMP-3 and TIMP-1 genes were examined on the basis of steady state levels of the corresponding mRNA (Fig. 4). In the HA treatment group, MMP-3 mRNA was detected in two of five samples from the femorotibial joint cartilage, three of five samples from the patellofemoral joint cartilage, and four of five samples from the synovia, whereas in the saline treatment group MMP-3 mRNA was detected from all of the samples collected from the femorotibial joint, patellofemoral joint, and synovia (Table 2). HA treatment

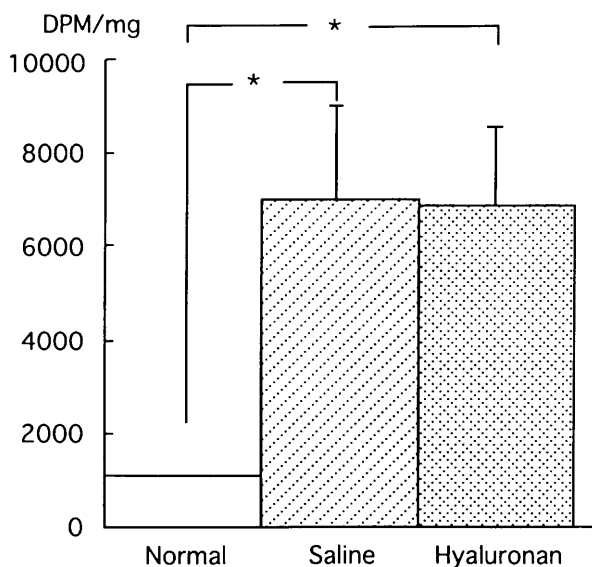


Fig. 1. [^3H] thymidine uptake of thymidine into cartilage. Hyaluronan: HA treatment group; Saline: saline treatment group; Normal: no surgery or treatment group. Each value represents the mean \pm standard deviation from six animals. * $p < 0.05$

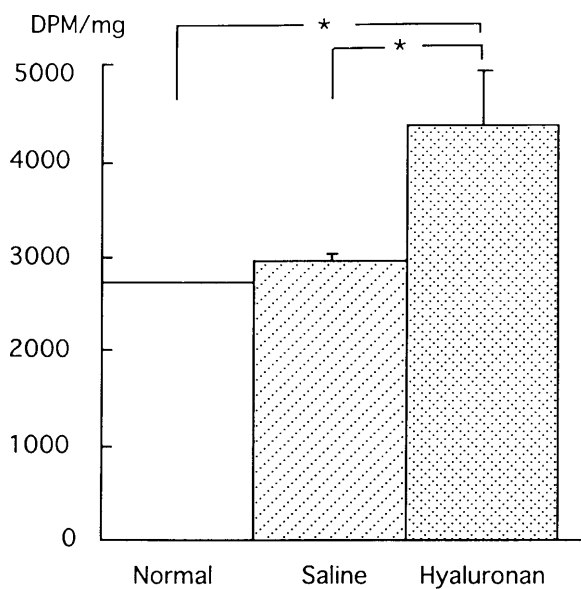


Fig. 2. ^{35}S sulfate uptake of cartilage. Hyaluronan: HA treatment group; Saline: saline treatment group; Normal: no surgery or treatment group. Each value represents the mean \pm standard deviation from six animals. * $p < 0.05$

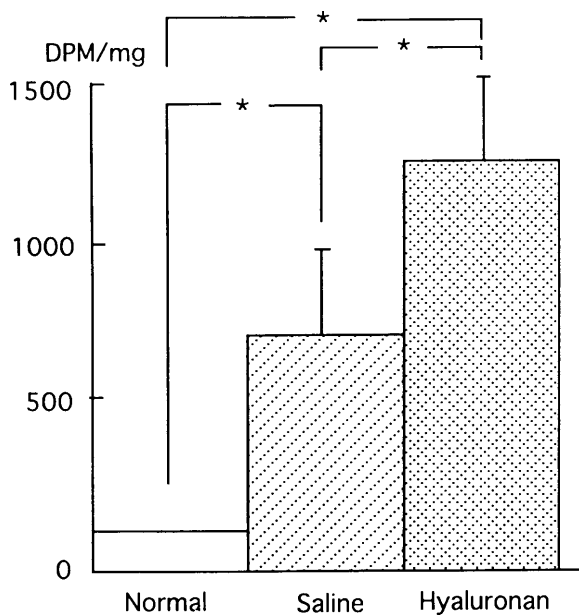


Fig. 3. ^{35}S sulfate incorporation into sulfate glycosaminoglycan, chondroitin 4 and 6 sulfate in cartilage. Hyaluronan: HA treatment group; Saline: saline treatment group; Normal: no surgery or treatment group. Each value represents the mean \pm standard deviation from six animals. * $p < 0.05$

HYALURONAN IN OSTEOARTHRITIS

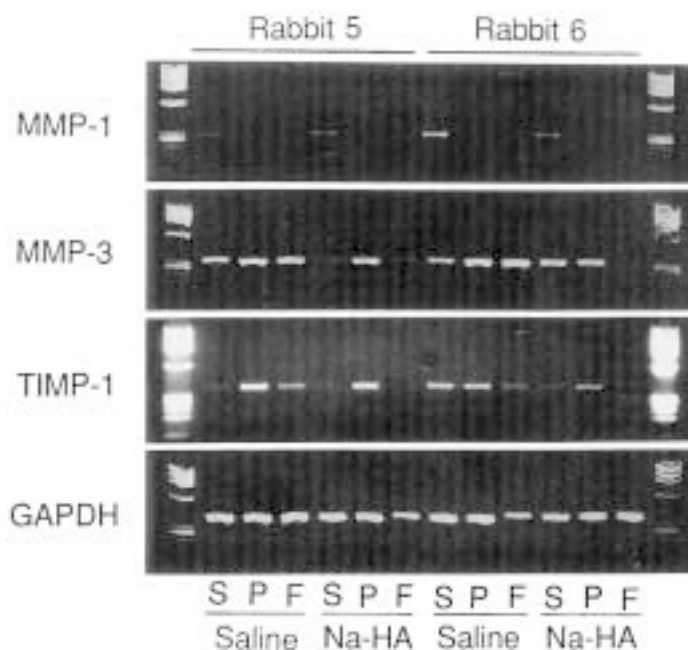


Fig. 4. MMP-1, MMP-3 and TIMP-1 mRNAs in rabbits 5 and 6. MMP-1: interstitial collagenase; MMP-3: stromelysin 1; TIMP-1: tissue inhibitor of metalloproteinase 1; GAPDH: glyceraldehyde-3-phosphate; S: Synovia; P: Patellofemoral joint; F: Femorotibial joint; Na-HA: HA treatment group; Saline: Saline treatment group.

Table 2. Frequency of MMP-1, MMP-3 and TIMP-1 mRNAs detection

Treatment group	MMP-1 mRNA			MMP-3 mRNA			TIMP-1 mRNA		
	FT	PT	S	FT	PT	S	FT	PT	S
HA	0/5	1/5	5/5	2/5	3/5	4/5	2/5	3/5	4/5
Saline	0/5	1/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5

MMP-1: interstitial collagenase; MMP-3: stromelysin 1; TIMP-1: tissue inhibitor of metalloproteinase 1; FT: Femorotibial joint; PT: Patellofemoral joint; S: Synovia; HA: HA treatment group; Saline: Saline treatment group.

thus inhibited MMP-3 mRNA production compared with saline.

A similar effect on TIMP-1 mRNA levels was observed. TIMP-1 mRNA was detected in all of the samples from the saline treatment group. In contrast, in the HA treatment group TIMP-1 mRNA was detected in only two of five samples from the femorotibial joint cartilage, three of five samples from the patellofemoral joint cartilage, and four of five samples from the synovia. As seen in Table 2, TIMP-1 mRNA was detected with the same frequency as MMP-3 mRNA.

Table 2 also shows that MMP-1 mRNA expressions levels were identical in the HA and saline groups. These results suggest that HA treatment inhibited both MMP-3 and TIMP-1 mRNA production similarly. HA treatment had no significant effect on MMP-1 at the mRNA levels in the cartilage and synovium.

Light Microscopic Findings

Although cartilage fibrillation, chondrocyte clustering, and abnormal disposition of chondrocytes were seen in both the HA and the saline groups (H&E staining), the degree of degradation in the HA group (Fig. 5A) was less severe than in the saline group (Fig. 5B).

Electron Microscopic Findings

Samples were collected from high stress areas of the tibia. Increased glycogen granules,

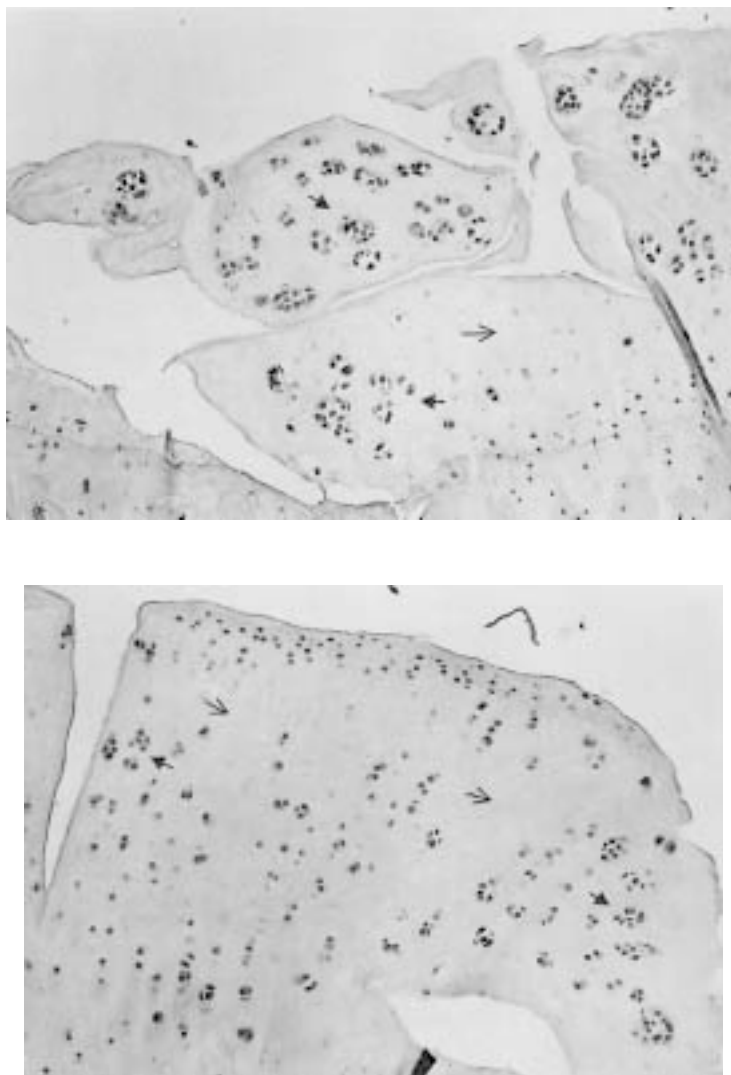


Fig. 5. Histology of the cartilage (Hematoxylin and Eosin staining; original magnification, $\times 100$). (A) HA treatment group. (B) Saline treatment group. Cartilage fibrillation (\diagup), chondrocyte clustering (\blacktriangledown), and abnormal disposition of chondrocytes were seen in both groups. The degree of degeneration in the HA group was less severe.

HYALURONAN IN OSTEOARTHRITIS

vacuolization, and abnormal endoplasmic reticulum were seen. The HA treated cartilage showed less degeneration, and resembled normal cartilage (Fig. 6A). In the saline group, cartilage degeneration was seen (Fig. 6B).

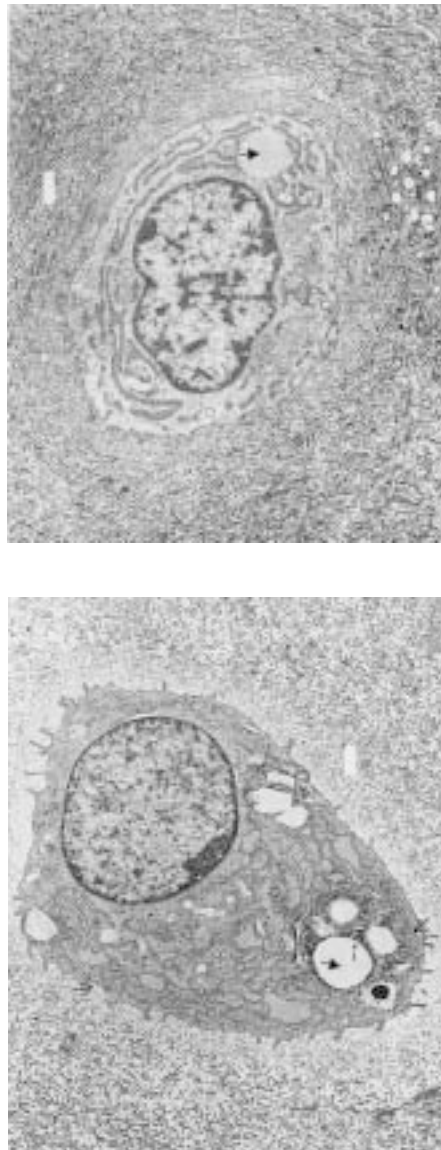


Fig. 6. Ultrastructure of cartilage as examined by transmission electron microscopy of high stress areas of the tibia (original magnification, $\times 5000$). Increased glycogen granules ($/$), vacuolization (\blacktriangleright), and abnormal endoplasmic reticulum ($*$) were seen. The HA treatment group showed less degeneration. (A) HA treatment group. (B) Saline treatment group.

DISCUSSION

Various types of experimental OA models have been described.²⁵⁾ In OA produced in rabbits by resetting the ACL, PCL, medial collateral ligament, and medial meniscus,⁹⁾ cartilage degeneration proceeds rapidly. These significant destructive cartilage changes are similar to those observed in human OA. This model has been used previously in many studies;^{11,25,33)} the authors found severe lesions within 2 weeks after surgery. HA is a type of glycosaminoglycan that is widespread in connective tissue and is a major ingredient of synovial fluid. It is useful in the absorption of mechanical impact, joint lubrication and preservation of articular cartilage.²¹⁾

The effect of HA on cartilage degeneration after synovectomy in rabbits has been reported by Toyoshima.²⁸⁾ Beneficial effects of HA on cartilage degeneration induced by papain injection have also been reported by Kitoh *et al.*¹²⁾ Wigren *et al.*³⁰⁾ have studied the effect of HA on cartilage degeneration induced by joint immobilization. Abatangelo *et al.*¹⁾ and Schiavinato *et al.*²⁴⁾ reported that the intraarticular injection of HA has a beneficial effect on cartilage degeneration induced by resection of the knee joint ACL. It has been described that the HA content decreases before proteoglycan in the initial stage of OA.¹⁵⁾

The elevated level of dermatan sulfate proteoglycans reported in OA cartilage arises from enhanced synthesis of dermatan sulfate proteoglycans by chondrocytes subjected to mechanical overload. In the early stages of OA, a change in chondrocyte phenotypic expression occurs in which increased catabolism of aggrecan is accompanied by increased synthesis of dermatan sulfate proteoglycans.⁴⁾

In this study, uptake of [³⁵S] sulfate into cartilage in the HA group was significantly higher than in the saline and normal groups. It suggests that cartilage proteoglycan synthesis was increased by HA. Moreover, uptake of chondroitin 4 and 6 sulfate in the HA treatment group was also higher than in the other groups, while the saline group revealed a higher uptake than the normal group. It can therefore be concluded that HA has more of an enhancing effect than saline on cartilage proteoglycan biosynthesis.

The proteoglycans can be degraded by MMP-3 and other proteases. In normal cartilage, there is a close balance between the activities of MMPs and the level TIMPs. In OA, the protease activities are higher than the level of TIMPs, leading to a presumptive excess of metalloproteinase.¹⁹⁾ In OA tissue, the increase in active MMPs is associated with an increase in plasmin in the absence of concomitant increase in TIMPs, a strong indication that cartilage degeneration in OA is related to an imbalance in the regulation of MMPs.¹⁴⁾

Such an imbalance between inhibition and promotion of enzymatic activity leads to an increase in the amount of active proteases, which combined with suppression of matrix synthesis results in increased degeneration of the cartilage.¹⁸⁾ Although Yasui *et al.*³¹⁾ found that 2×10^6 Da molecular weight of HA enhanced TIMP-1 production in cultured house bovine chondrocytes, the authors did not observe an increase of TIMP-1 mRNA. In this study, HA had the same inhibitory effects on MMP-3 and TIMP-1 which exhibited a parallel decrease in mRNA level expression. The authors did not find a relative suppression of MMP-3 expression compared with TIMP-1 expression in the current study. HA did not have a marked effect on MMP-1 at the mRNA level. These results suggest that HA may act by suppressing cartilage response activities resulting in a protective function during the early stages of OA.

The mechanisms of HA action in preventing cartilage degeneration are unknown. Sakamoto *et al.*²³⁾ have characterized the distribution of fluorescent labeled HA injected into papain induced arthritic cartilage: deep intrusion into the arthritic cartilage tissue was seen, but with no intrusion into normal cartilage tissue was evident. Two possible mechanisms have been proposed to explain this effect: either a barrier against the diffusion of inflammatory enzymes into

HYALURONAN IN OSTEOARTHRITIS

the arthritic cartilage, or a protective effect of HA on the cartilage proteoglycan macromolecule itself.¹⁸⁾

In the present study, the effects of HA on the development of early stage OA changes suggest that HA affects anabolism and catabolism to prevent the progress of OA.

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