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ORIGINAL ARTICLE

Intra-articular injection of hyaluronate and indomethacin in rabbits with antigen-induced arthritis

Yow-Jen Lo · Ming-Thau Sheu · Wen-Chi Tsai · Yun-Ho Lin · Jau-Le Li · Yu-Chih Liang · Chi-Ching Chang · Ming-Shium Hsieh · Chien-Ho Chen

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Abstract Combined effects of hyaluronate and indomethacin in the treatment of rabbits with antigen-induced arthritis (AIA) were evaluated by assessing joint swelling, C-reactive protein (CRP) and prostaglandin E_2 (PGE_2) levels with periodic intra-articular (ia) injections of hyaluronate alone (HA group) and with either a low or high concentration of indomethacin (LI-HA or HI-HA group). End-point analyses included matrix metalloproteinases-3 (MMP-3) activity and macroscopic and histological joint examinations. Results demonstrated that treatment in LI-HA and HI-HA groups resulted in statistically significant suppression of CRP, PGE_2, and MMP-3 in comparison with those of HA group. Inhibition of serum CRP was only observed in LI-HA group. The order of serum MMP-3 inhibition was LI-HA>HI-HA>HA. Based on macroscopic and histological analyses of pannus formation, hyperplasia, inflammation, joint leakage and erosion, and loss of proteoglycan, the only statistically significant improvement was shown in LI-HA group compared to HA group and HI-HA group compared to control group.

Keywords Antigen-induced arthritis · Hyaluronate · Indomethacin · CRP · PGE_2 · MMP

Y.-J. Lo · M.-T. Sheu
College of Pharmacy, Taipei Medical University,
Taipei, Taiwan, ROC

W.-C. Tsai
Department of Orthopedic Surgery, Min-Sheng Healthcare,
Taoyuan, Taiwan, ROC

Y.-H. Lin
Department of Pathology, Taipei Medical University,
Taipei, Taiwan, ROC

J.-L. Li · M.-S. Hsieh
Department of Orthopedics and Traumatology,
Taipei Medical University Hospital,
Taipei Medical University,
Taipei, Taiwan, ROC

Y.-C. Liang · C.-H. Chen (✉)
School of Medical Technology, Taipei Medical University,
250 Wu-Hsing Street, Taipei, 110, Taiwan, ROC
e-mail: chenchho@tmu.edu.tw

C.-C. Chang
Department of Internal Medicine, Taipei Medical
University Hospital, Taipei Medical University,
Taipei, Taiwan, ROC

Introduction

Arthritis is a chronic multifactorial disease induced when the immune system attacks and begins degrading the body's joints. Common underlying symptoms of the above clinical manifestations include inflammation, destruction of cartilage and soft tissue, and dysfunction of the joints [1–3]. Therapeutic approaches using an elastoviscous HA solution and HA derivatives (hylans) for treatment of arthritis pain are based on the finding that long-lasting analgesia can be achieved in joints of arthritic horses by replacing pathologic synovial fluid with a highly purified HA solution of normal elastoviscosity but significantly greater concentration than that of healthy joint fluid. Hyaluronic acid (HA) is an abundant non-sulfated glycosaminoglycan component of synovial fluid and extracellular matrices. In normal human synovial fluid, the molecular weight (MW) of HA is (6–7) $\times 10^6$ Da, and the concentration is 2–4 mg/ml. HA preparations appear to be quite safe, with local reactions at the injection site (e.g., pain and swelling) generally being mild and transient [4]. As a result of this discovery, highly

purified HA and hylan solutions became available worldwide for the treatment of arthritis pain in animals and humans [5,6].

Intra-articular (ia) injections of HA as treatment for knee OA were reviewed by Brabdt et al. [4]. Despite several investigators having reported that ia injections relieve joint pain and improve function in humans with knee OA [7,8], they concluded that there was insufficient information to draw conclusions concerning the effect of this treatment, if any, on the progression of OA in humans. Nevertheless, HA has demonstrated a variety of effects on cells in vitro that may be related to its reported effects on joint disease. These include the inhibition of prostaglandin E_2 (PGE_2) synthesis induced by interleukin-1 (IL-1) [9,10] and protection against proteoglycan depletion and cytotoxicity induced by oxygen-derived free radicals, IL-1, and mononuclear cell-conditioned medium [11,12]. A recent study by Gonmis et al. further reported that elastoviscous properties of HA solutions are determining factors in reducing pain-eliciting nerve activity in both normal and inflamed rat joints [13]. Furthermore, local therapeutic effects of intra-articular injections of high-MW HA in rats with AIA were reported by Roth et al. to be biphasic, with inhibition of inflammation and cartilage damage in the early chronic phase but with promotion of joint swelling, inflammation, and cartilage damage in the late chronic phase [14].

Since their introduction, NSAIDs have commonly been prescribed by physicians for the treatment of OA by oral administration. But in the 1990s, a series of research articles cast doubt on the superior efficacy of NSAIDs compared with acetaminophen [15,16]. However, a survey of 1,799 patients with OA, RA, and fibromyalgia (FS) conducted by Wolfe et al. concluded that there was a considerable and statistically significant preference for NSAIDs compared to acetaminophen among these three groups of rheumatic disease patients. The report also stated that if safety and costs are not issues, there would hardly ever be a reason to recommend acetaminophen over NSAIDS, since patients generally preferred NSAIDS, and fewer than 14% preferred acetaminophen [17]. Furthermore, although NSAIDs are effective in reducing symptoms, they do not reduce joint damage. Because of this and their potential for serious toxicity, they should be used at the minimum effective dosage, even in patients with inflammatory joint disease [18]. Recently, Yoon et al. reported that NSAIDs, such as indomethacin, have protective effects against cartilage damage, not only by alleviating inflammation but also by inhibiting NO-induced apoptosis and dedifferentiation of articular chondrocytes. The latter effects require higher concentrations of NSAIDs that are 100- to 1,000-fold higher than those needed to inhibit prostaglandin synthesis [19]. This would imply that dosages of NSAIDs should be higher than the oral dose used for anti-inflammatory purposes via a systemic route in order to achieve optimal local concentrations at arthritic joint sites to prevent cartilage damage.

The ideal treatment for arthritis would reduce pain and inflammation, maintain function, and at the same time, be safe. The purpose of this study was to evaluate the combined effects on improving the inflammatory status, inflammatory pain, and cartilage degradation in rabbits with antigen-induced arthritis after treatment with hyaluronate alone and combined with indomethacin, which can be used as a local injection in order to avoid the serious side effects of indomethacin but increase the local concentration to a level that is sufficient to activate the mechanism which prevents cartilage damage while reducing pain and swelling at the local injection site. Also, the viscous property of the 1% HA solution in which indomethacin was dissolved was recognized to be capable of sustaining indomethacin's pharmacological activity by providing a longer period of local retention of indomethacin within the knee joints.

Materials and methods

Materials

Sodium pentobarbital was obtained from TCI (TOKYO, Japan). Indomethacin, ovalbumin (OVA), and Freund's complete adjuvant were obtained from Sigma (St. Louis, MO, USA). A 0.9% isotonic NaCl solution was obtained from Sington (Taipei, Taiwan). ARTZDispo®, a commercial product containing 1% sodium hyaluronate, was supplied by Seikagaku (Tokyo, Japan) and was used in combination with indomethacin.

Preparation of the hyaluronate and indomethacin intra-articular injections

Indomethacin was dissolved in isotonic buffer and mixed well with 1% sodium hyaluronate (ARTZDispo®, Seikagaku) having a weight-averaged molecular weight of (6–12) $\times 10^5$. Indomethacin was dispensed as an intra-articular (ia) injection at final concentrations of 5.6 (0.002 mg/ml) and 560 μ(0.2 mg/ml) in a 1% sodium hyaluronate solution.

Induction of AIA

The experimental disease was induced as previously published [5]. The experimental design was for arthritis to be induced by OVA in New Zealand white (NZW) rabbits with an initial weight of 2.6–3.3 kg (Fig. 1). Animal

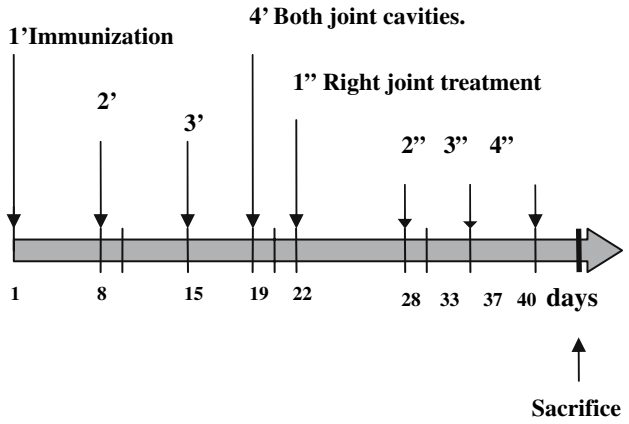

Fig. 1 Experimental design for arthritis induced by an emulsified mixture of 5 mg of ovalbumin and Freund's complete adjuvant containing 1 mg of *Mycobacterium tuberculosis* in NZW rabbits. All rabbits ($n = 5$ in each group) were immunized and treated with intra-articular injections of 0.5 ml of either 0.9% NaCl, 1% hyaluronate (the HA group), 5.6 μM indomethacin + 1% hyaluronate (the LI-HA group), or 560 μM indomethacin + 1% hyaluronate (the HI-HA group) into the right knee joint

experiments were carried out with the approval of the local and national ethics committees at the animal facility of Taipei Medical University. Sodium pentobarbital (0.2–0.3 mg/kg) was used for animal sedation. An emulsified mixture of 5 mg of OVA and Freund's complete adjuvant containing 1 mg of *Mycobacterium tuberculosis* was prepared for immunization. NZW rabbits were immunized by subcutaneously injecting this mixture into multiple sites of the shaved interscapular region on three occasions at 2-week intervals (days 1, 15, and 29). Arthritis was induced by a fourth injection of the emulsion containing 5 mg/ml of OVA in complete Freund's adjuvant into both knee joints on day 19. All procedures were performed strictly in accordance with current local regulations.

Intra-articular treatment

One group ($n = 3$) served as the normal control. Another group ($n = 5$) served as the disease control and was subjected to four treatments at intervals of 5, 4, and 3 days (i.e. receiving treatments on days 1, 6, 10, and 13), respectively, consisting of ia injections of 0.5 ml of 0.9% NaCl into the right knee joint. Three treatment groups ($n = 5$ for each group) were also subjected to the same dosing regimen using ia injections of 0.5 ml of 1% sodium hyaluronate (the HA group), 1% sodium hyaluronate plus 5.6 μM indomethacin (the LI-HA group), or 1% sodium hyaluronate plus 560.0 μM indomethacin (the HI-HA group) into the right knee joint. The left knee joint was not treated and served as the internal control. The rationale for the design of such a dosing regimen was the hypothesis that rabbits with AIA would not be completely cured by treatment with hyaluronate alone or by hyaluronate and indomethacin which might still decrease inflammation and inflammatory pain and delay arthritic progression. All animals were sedated and sacrificed on day 40 by intravenous administration of sodium pentobarbital (50–80 mg/kg).

Clinical assessments of AIA

The effects of treatment on the rabbits were monitored by analyzing weight loss, joint circumference, biochemical parameters (CRP, PGE_2, and MMP-3), and macroscopic and histological evaluations of the blood and articular cartilage [5]. The fur on the knees of all rabbits was shaved off, and the circumference of the knees was measured using a measuring tape before each injection. Measurements were taken in the anterior-to-posterior position, with the ankle extended, through the circumference of the knee.

Assay of CRP and PGE_2

The CRP reagent, in conjunction with Beckman Array Systems and Calibrator or Calibrator 5 (USA), was used for the quantitative determination of human CRP by a rate nephelometer. The method employed in the Beckman CRP test measures the rate of increase in light scattered from particles suspended in solution as a result of complexes formed during an antigen–antibody reaction. After the antibody to CRP is brought into contact with CRP in a sample, the increase in light scattering resulting from the antigen–antibody reaction is converted to a peak rate signal that is a function of the CRP concentration in the sample. Following calibration, the peak rate signal for a particular assay is automatically converted to concentration units by the analyzer.

PGE_2 production was determined by measuring the serum level of rabbits using a PGE_2 assay kit (Assay Designs, Ann Arbor, MI, USA), which was used to quantify the amount of PGE_2 according to the manufacturer's protocol. PGE_2 levels were calculated against a standard curve of PGE_2.

Quantification and Western blot analysis of MMP-3

Enzyme activity assay kits were used to quantify the amounts of the pro-form and active form of MMP-3 present in the samples (Biotrak MMP-3 activity assay system, Amersham Pharmacia Biotech, Buckinghamshire, UK) following the manufacturer's protocol. Samples were compared to a serial dilution of standards (MMP-3, 0.5–16 ng/ml). The addition of 1 mM aminophenyl mercuric acetate (APMA) was used to activate proMMPs and thus measure total MMP-3 activity (i.e., both the pro-form and active form) of

each sample. The absorbance of the colorimetric reaction product was read at a wavelength of 405 nm after incubation at $t = 0$, 1, 2, 3, 4, and 5 h. The amounts of MMP-3 activity were calculated from the serial dilution data of the standards. Data are presented as the mean ± SEM. The rate of change of MMP activity is expressed as $(Abs_{t=y} - Abs_{t=0}) \times 1000/t_y$.

Serum diluted 1:10 in PBS was used for Western blot analysis of both pro-(59/57 kDa) and active-form (48/25 kDa) MMP-3. The protein was quantified by a Bio-Rad protein assay, size-fractionated by SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. Proteins were detected using the rabbit anti-MMP3 polyclonal antibody (Calbiochem, San Diego, USA) that recognizes the ~57 kDa pro- and the ~48 kDa active forms of MMP-3. Blots were developed using a peroxidase-conjugated secondary antibody and an enhanced chemiluminescence system.

Macroscopic analysis of the joints

Opened joints were macroscopically evaluated for the extent of pannus formation as follows: 0, no involvement; 1, mild; 2, moderate; and 3, severe. The left and right normal control cartilage was referred to as the baseline in this study, and its macroscopic pannus formation and erosion were graded 0 (A and B). The left knee joint which received no treatment in treated animal in each of the four treatment groups (0.9% NaCl, HA, LI-HA, and HI-HA) was used as its own internal control, and macroscopic pannus formation of both knees was graded as well. The mean±SEM of the grading score for both knees was calculated and reported for each of the 4 treatment groups ($n = 5$ for each group).

Synovial and cartilage sample collection and histological analysis

Patellar and synovial samples from the infrapatellar, superolateral, and posterior locations were collected. Synovium was snap-frozen, or paraffin-embedded when the patellae were decalcified and embedded in paraffin. The joints of rabbit knees were also removed and fixed in 10% buffered formalin. The joints were decalcified in 5% formic acid, embedded in paraffin, sectioned at 5-μm thicknesses, and subsequently stained with hematoxylin-eosin (H&E) for general morphology examination and with toluidine blue for proteoglycan examination. Sections were examined for 5 different events: hyperplasia of cells of the synovial lining, infiltration of the sublining by mononuclear cells frequently organized in aggregates, leakage and erosion of the joint, and loss of proteoglycan [20]. Histological analysis was conducted, and the following grading scale for hyperplasia and infiltration of synovial cells was scored as 0, no changes (≤2 cell layers thick); 1, minimal hyperplasia and infiltration (2–3 cell layers thick); 2, mild hyperplasia and infiltration (5–10 cell layers thick); 3, moderate lining hyperplasia (>10 cell layers thick); and 4, marked hyperplasia and infiltration (organized pannus and lining layers). For leakage and erosion of joints, the grading scale was scored as 0, no changes; 1, minimal (size and number) erosion of hard tissues at the margins and in the central region of the joint; 2, small erosions in the notched region of the femur and the center of the tibia and marginal cartilage-pannus junctions; 3, larger deeper erosions in the same areas as in 2; 4, erosions beginning to break into the subchondral trabecular epiphyses; and 5, erosions breaking into the full depth of the epiphyses and deeply into the marginal subchondral bone. The mean±SEM of the grading scale for each event (hyperplasia, inflammation, joint leakage, erosion, and loss of proteoglycan) was calculated and reported for each of the 4 treatment groups ($n = 5$ for each group).

Inhibition of therapeutic responses and statistical analysis

The percent inhibition of clinical parameters was calculated in each rabbit using the formula: $[1 - (C - A)/(B - A)] \times 100$, where A is the mean of clinical assessments in a normal state or before joint injection with emulsion containing OVA in complete Freund's adjuvant (day 1); B is the mean of clinical assessments in the immunized state (day 22) after injection with emulsion containing OVA in complete Freund's adjuvant into both knee joints; and C is the mean of clinical assessments after various drug treatments (day 40) [21,22]. Student's t-test was used to determine the significance of the difference between values. Comparisons among multiple groups were assessed using one-way ANOVA and Bonferroni's t-test (SigmaStat). A p value of <0.05 was considered statistically significant.

Results

Development of arthritis

All rabbits had developed AIA by the end of the intra-articular injections of the antigen on day 22 as indicated by the increased serum CRP in all treatment groups as shown in Table 1. A statistically significant difference was observed between serum CRP levels before and after immunization for each of four treatment groups in the experiment. Serum levels of CRP detected on day 1 in AIA rabbits were in the range from 0.52 ± 0.14 (in the HA group) to 0.66 ± 0.03 mg/dl (in the LI-HA group). On day 22 after the final intra-articular antigen injection, the serum CRP levels were almost fourfold higher (2.08 ± 0.08 to

Table 1 Clinical assessments of the drug treatments (four intra-articular injections in the right knee joint) in rabbits with antigen-induced arthritis (AIA)

Assessment	Treatment			
	0.9% NaCl	HA	LI-HA	HI-HA
Weight (kg)				
Day 1	3.12 ± 0.11	3.09 ± 0.07	3.11 ± 0.17	3.04 ± 0.04
Day 22	3.01 ± 0.10	3.01 ± 0.06	3.01 ± 0.03	3.07 ± 0.03
Day 40	2.86 ± 0.05	2.93 ± 0.09	2.95 ± 0.06	2.98 ± 0.05
Circumference (cm) of the left joint				
Day 1	11.38 ± 0.31	11.38 ± 0.13	11.50 ± 0.20	11.88 ± 0.13
Day 22	12.50 ± 0.20§	12.50 ± 0.29§	12.63 ± 0.31§	12.88 ± 0.13§
Day 40	12.38 ± 0.13	12.25 ± 0.14	12.18 ± 0.28	12.50 ± 0.20
CRP (mg/dl)				
Day 1	0.63 ± 0.07	0.52 ± 0.14	0.66 ± 0.03	0.54 ± 0.08
Day 22	2.32 ± 0.33§	2.08 ± 0.08§	2.50 ± 0.14§	2.11 ± 0.07§
Day 40	2.18 ± 0.17	1.83 ± 0.06	1.48 ± 0.06*	1.64 ± 0.02*
PGE_2 (ng/ml)				
Day 1	9.15 ± 0.71	2.63 ± 0.15	9.47 ± 0.74	7.90 ± 0.77
Day 22	24.63 ± 1.80§	13.87 ± 0.96§	29.33 ± 1.20§	23.20 ± 2.69§
Day 40	32.75 ± 2.75*	16.87 ± 1.74	26.67 ± 0.88	17.90 ± 2.09
MMP-3 (ng/ml)				
Day 1	4.71 ± 1.19	2.27 ± 0.30	4.53 ± 0.97	4.28 ± 0.86
Day 22	13.61 ± 1.69§	13.04 ± 1.98§	12.12 ± 0.36§	11.99 ± 1.36§
Day 40	28.02 ± 3.57*	21.38 ± 3.24*	17.24 ± 1.81	17.94 ± 2.46

Changes in serum CRP, PGE_2, and MMP-3 on days 1 and 22 (before and after the intra-articular injection, respectively) were statistically analyzed using Student's *t*-test (§ $p < 0.05$). Changes in serum CRP, PGE_2, and MMP-3 on days 22 and 40 (before and after various drug treatments, respectively) were statistically analyzed using Bonferroni's *t*-test. *$p < 0.05$

2.50 ± 0.14 mg/dl) than those on day 1, with a significance level of $p < 0.05$.

Effects on joint swelling

The fur of the knee of all rabbits was shaved off, and the circumference of the knee in the anterior-to-posterior position was measured with the ankle extended; results are listed in Table 1. On day 20, AIA developed as a significant swelling of the right knee point in all treated rabbits. There were statistically significant differences in the amounts of joint swelling between days 1 and 22 (before and after immunization, at 11.56 ± 0.10 and 12.64 ± 0.11 cm, respectively, $n = 20$). However, there was a slight but statistically insignificant decrease in joint swelling in each treatment group at the end of treatment on day 40. After initiation of AIA, the swelling remained statistically significantly higher compared to the baseline levels on day 1 and had not recovered to the baseline levels at the end of any treatment.

Effects on serum CRP, PGE_2, and MMP-3 levels

Clinical assessment of serum levels of CRP, PGE_2, and MMP-3 was conducted at the initiation of AIA induction (on day 1), and before and after treatment (on days 22 and 40), and results are illustrated in Table 1. As shown in Table 1, there was a statistically significant increase in serum CRP levels after the final AIA induction on day 22 (0.59 ± 0.04 and 2.25 ± 0.10 mg/dl, respectively) for all treatment groups. At the end of treatment on day 40, there were statistically significant decreases in serum CRP levels compared with those before treatment on day 22 for the LI-HA and HI-HA groups (2.50 ± 0.14 vs. 1.48 ± 0.06 mg/dl and 2.11 ± 0.07 vs. 1.64 ± 0.02 mg/dl, respectively), whereas slight but statistically insignificant decreases were recorded for the control group and HA group (2.32 ± 0.33 vs. 2.18 ± 0.17 mg/dl and 2.08 ± 0.08 vs. 1.83 ± 0.06 mg/dl, respectively). There were statistically significant increases in serum PGE_2 levels at the end of AIA induction on day 22 in all treatment groups (7.46 ± 0.78 vs. 22.92 ± 1.69 ng/ml, $n = 20$). At the end of treatment on day 40, serum PGE_2 levels had continually increased in the control and HA groups, whereas they had dropped in the LI-HA and HI-HA groups. There were statistically significant increases in serum MMP-3 levels on day 22 in all treatment groups compared to respective values on day 1 (12.69 ± 0.66 vs. 3.95 ± 0.48 ng/ml). At the end of treatment on day 40, serum MMP-3 levels had continually increased in comparison to values on day 22 in all treatment groups.

Inhibition of therapeutic responses

Therapeutic responses of each treatment were compared by the percent inhibition (%) of serum levels of CRP,

Table 2 Inhibition (%) of drug treatments (four intra-articular injections in the right knee joint) on antigen-induced arthritis (AIA) in rabbits in comparison to the therapeutic response on day 40

Inhibition (%)	0.9% NaCl[a]	HA[a]	LI-HA[a]	HI-HA[a]
CRP	5.0 ± 9.5	2.2 ± 0.8	54.6 ± 0.17*	30.3 ± 9.2*
PGE_2	−53.2 ± 10.6	−26.0 ± 6.1	13.3 ± 2.9*	35.9 ± 3.3*
MMP-3	−160.0 ± 11.8	−81.7 ± 26.7*	−64.6 ± 12.0*	−75.8 ± 5.0*

[a] The right joints of rabbits with AIA were injected with 0.5 ml 0.9% NaCl, hyaluronate, LI-HA, or HI-HA. Values are the mean ± SEM

* $p < 0.05$ versus the baseline for the variables that decreased by one-way ANOVA

PGE_2, and MMP-3 calculated as defined in "Methods", and the results are displayed in Table 2. The order of serum CRP inhibition after treatment was the LI-HA group (54.6 ± 0.17%) > HI-HA group (30.3 ± 9.2%) > HA group (2.2 ± 0.8%) ≈ control group (5.0 ± 9.5%). Statistically significant inhibition of serum CRP levels was only observed in the LI-HA and HI-HA groups, while treatment with HA alone demonstrated an insignificant effect on serum CRP similar to that treated with 0.9% NaCl. The treatment with 0.9% NaCl (−53.2 ± 10.6%) or HA alone (−26.0 ± 6.1%) was not able to inhibit the formation of serum PGE_2, whereas the inhibition of serum PGE_2 levels was parallel to indomethacin concentrations with the percent inhibition in the LI-HA group (13.3 ± 2.9%) lower than that in the LI-HA group (35.9 ± 3.3%). No significant inhibition but an increasing serum level of MMP-3 was observed in all treatment groups as indicated by the negative values. However, statistically significant retardation of the increase in serum MMP-3 levels in the HA, LI-HA, and HI-HA groups was found when compared to the control group treated with an injection of 0.9% NaCl. The order of serum MMP-3 retardation of the increase was the LI-HA group (−64.6 ± 12.0%) > HI-HA group (−75.8 ± 5.0%) > HA group (−81.7 ± 26.7%). MMP-3 protein expression levels after the 4 treatments were also assessed by Western blot analysis, and results are shown in Fig. 2. There was no significant change in the serum pro-form MMP-3 protein (calculated as the total intensity of two bands) in the normal group (no immunization) during the treatment course, but there were continual increases at a lower extent than the normal group in serum levels of the pro-form of the MMP-3 protein (calculated as the total intensity of two bands) in all treatment groups (immunization) from days 1 to 40.

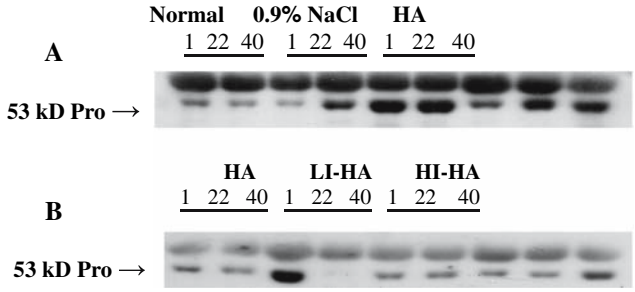

Fig. 2 MMP-3 protein-expression after the various drug treatments assessed by Western blot analysis. *A* For pro-form MMP-3 detection, rabbits were neither immunized nor treated in the normal group ($n = 3$), and were treated with 0.9% NaCl ($n = 5$) or 1% hyaluronate ($n = 5$) in the immunized groups. *B* For pro-form MMP-3 detection, rabbits were treated with 1% hyaluronate alone (HA, $n = 5$), 5.6 μM indomethacin + 1% hyaluronate (LI-HA, $n = 5$), or 560 μM indomethacin + 1% hyaluronate (HI-HA, $n = 5$) in the immunized groups. Day 1 was before immunization; day 22 was the immunized state after injection with 0.5 ml of an emulsified mixture into both knee joints; and day 40 was the immunized state after the various drug treatments. The results shown are representative of four independent experiments

Macroscopic analysis of the joints

After three intervals (5, 4, and 3 days) of ia treatment, rabbits were sacrificed, and a macroscopic examination revealed that all treated animals had developed typical arthritic lesions (Fig. 3). The left and right normal control cartilage was used as references in this study, and macroscopic pannus formation and erosion shown in Fig. 3 were graded 0 (A and B). In the group treated with an injection of 0.5 ml 0.9% NaCl, macroscopic pannus formation of the left knee joint as the internal control which received no treatment was graded 2–3 (Fig. 3C), whereas that for the right knee joint was graded 3 (Fig. 3D). In the HA group, macroscopic pannus formation of the left knee joint as the internal control which received no treatment was graded 3 (Fig. 3E), whereas that for the right knee joint was graded 2−3 (Fig. 3F). In the LI-HA group, macroscopic pannus formation of the left knee joint as the internal control which received no treatment was graded 3 (Fig. 3G), whereas that for the right knee joint was graded 2−3 (Fig. 3H). In the HI-HA group, macroscopic pannus formation of the left knee joint as the internal control which received no treatment was graded 3 (Fig. 3I), whereas that for the right knee joint was graded 3 (Fig. 3J). Table 3 summarizes the overall grading scores for all treated animals ($n = 5$) in each treatment group. There were no obvious differences in macroscopic pannus formation between the left and right knee joints for the treatment group injected

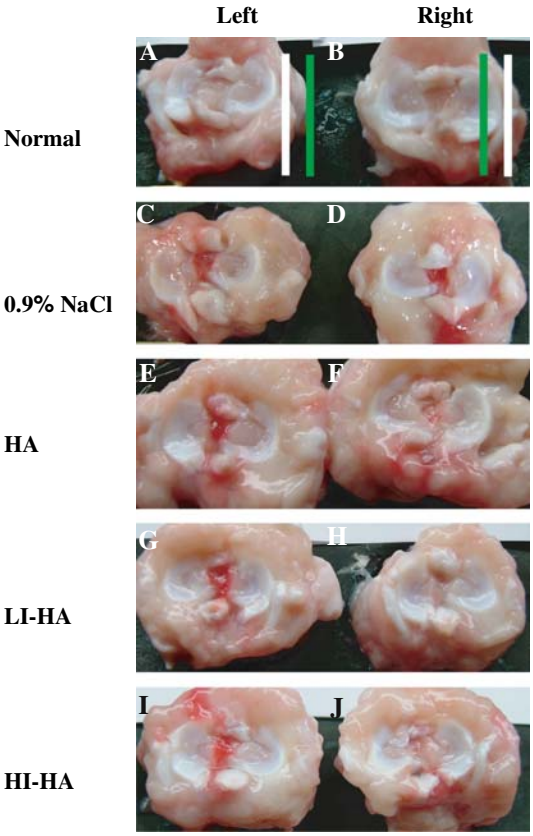

Fig. 3 Macroscopic appearance of an opened joint with graded scales as follows for pannus formation and erosions: 0, none; 1, mild; 2, moderate; and 3, severe involvement. The left and right normal control cartilage was used in this study, and macroscopic pannus formation and erosion were graded 0 (*A* and *B*). The left knee joint as the internal control received no treatment and macroscopic pannus formation was graded 2−3 (*C*), while the right knee joint was injected with 0.5 ml of 0.9% NaCl and macroscopic pannus formation was graded 3 (*D*). The left knee joint as the internal control received no treatment and macroscopic pannus formation was graded 3 (*E*), while the right knee joint was injected with 0.5 ml of 1% hyaluronate (HA group) and macroscopic pannus formation was graded 2−3 (*F*). The left knee joint as the internal control received no treatment and macroscopic pannus formation was graded 3 (*G*), while the right knee joint was injected with 0.5 ml of 5.6 μM indomethacin + 1% hyaluronate (LI-HA group) and macroscopic pannus formation was graded 3−4 (*H*), while the left knee joint as the internal control received no treatment and macroscopic pannus formation was graded 3 (*I*), while the right knee joint was injected with 0.5 ml of 560 μM indomethacin + 1% hyaluronate (HI-HA group), and macroscopic pannus formation was graded 3 (*J*). Only one typical image of the left and the right knees for each treatment group is shown

with 0.9% NaCl or for the HA group. However, there was obviously improved efficiency when the overall grading of macroscopic pannus formation of the right knee joint was compared to that of the left knee joint in the LI-HA and HI-HA groups with statistically insignificant differences between these two groups (89.6 ± 17.8% for the LI-HA vs. 98.3 ± 14.5% for the HI-HA group).

Histological analysis of the joints

Figures 4 and 5 show the microscopic features of synovial tissues and joint cartilage, respectively, after staining with hematoxylin and eosin for general morphology examination and staining with. Briefly, these alterations can be grouped into 5 different events: hyperplasia of cells of the synovial lining, infiltration of the sublining by mononuclear cells frequently organized into aggregates, leakage and erosion of the joint, and loss of proteoglycan. The mean ± SEM of grading scale scores on both knees and the ratio of the right to the left knee for each animal calculated for 5 different events in the 4 treatment groups are summarized in Table 3. There were greater extents of lining hyperplasia and inflammatory cell infiltration in all treatment groups than in the normal synovium ($p < 0.05$). In comparisons among the 4 treatment groups, a greater extent of hyperplasia in the treatment group injected with 0.9% NaCl was noted (2.00 ± 0.41 for the right knee vs. 1.75 ± 0.25 for the left knee). Nevertheless, improved efficiency in retardation of the progression of hyperplasia was seen in the LI-HA group (1.67 ± 0.58 for the right knee vs. 1.83 ± 0.29 for the left knee) or at least no further deterioration in the extent of hyperplasia for the HA and HI-HA groups (2.0 ± 0.0 for both knees in these 2 groups). Regarding inflammation, no difference was shown between the 2 knees in the group treated with 0.9% NaCl, whereas a slight decrease in the extent of inflammation in the treated knee was observed for the other 3 groups in the order of LI-HA (75.0 ± 8.3%) ≈ HI-HA (80.0 ± 8.2%) > HA (91.7 ± 8.3%). There were greater extents of leakage and erosion of the joints of both knees in all treatment groups than in the normal joints ($p < 0.05$) after treatment. In comparisons between the internal control left knee and the treated right knee, however, all 4 treatments demonstrated improvements in joint leakage and erosion but to different extents in the order of the LI-HA group (72.2 ± 14.7% and 61.1 ± 5.6%) ≈ HI-HA group (76.7 ± 10.0% and 66.7 ± 11.8%) > HA group (87.5 ± 12.5% and 66.7 ± 33.3%) > NaCl group (91.7 ± 8.3% and 91.7 ± 8.3%). Similarly, the loss of proteoglycan examined after staining with toluidine blue (data not shown) was greater for both treated and untreated knees in all treatment groups than for the corresponding site of normal joints ($p < 0.05$). However, comparisons between the untreated left knee as the internal control and the treated right knee demonstrated no improvement in the loss of proteoglycan in the HA group, whereas statistically significant decreases in the loss of proteoglycan were observed for the other 3 treatment groups, with that for the LI-HA group (62.5 ± 12.5%) being greatest.

Table 3 Macroscopic appearance and histological score analysis of the knee joint after various drug treatments (four intra-articular injections in the right knee joint) on antigen-induced arthritis (AIA) in rabbits

Variables	Normal	0.9% NaCl	HA	LI-HA	HI-HA
Pannus formation					
Left joint	0.00 ± 0.00	2.25 ± 0.48	2.50 ± 0.29	3.50 ± 0.29	3.00 ± 0.32
Right joint	0.00 ± 0.00	2.25 ± 0.48	2.50 ± 0.29	3.00 ± 0.41	2.80 ± 0.20
Right/left ratio (%)	0.00 ± 0.00	100.0 ± 0.00	100.0 ± 0.00	89.6 ± 17.8	98.3 ± 14.5
Synovial hyperplasia					
Left joint	0.00 ± 0.00	1.75 ± 0.25	2.00 ± 0.00	1.83 ± 0.29	2.00 ± 0.00
Right joint	0.00 ± 0.00	2.00 ± 0.41	2.00 ± 0.00	1.67 ± 0.58	2.00 ± 0.00
Right/left ratio (%)	0.00 ± 0.00	112.5 ± 12.5	100.0 ± 0.00	87.5 ± 12.5	100.00 ± 0.00
Inflammation					
Left joint	0.00 ± 0.00	3.00 ± 0.41	2.25 ± 0.25	3.00 ± 0.00	2.80 ± 0.20
Right joint	0.00 ± 0.00	3.00 ± 0.41	2.00 ± 0.00	2.50 ± 0.29	2.20 ± 0.20
Right/left ratio (%)	0.00 ± 0.00	100.0 ± 0.00	91.7 ± 8.33	75.0 ± 8.33	80.0 ± 8.16
Joint leakage					
Left joint	0.33 ± 0.33	2.25 ± 0.63	1.75 ± 0.63	2.33 ± 0.33	2.60 ± 0.51
Right joint	0.00 ± 0.00	2.00 ± 0.41	1.50 ± 0.65	1.67 ± 0.33	1.80 ± 0.20
Right/left ratio (%)	0.00 ± 0.00	91.7 ± 8.33	87.5 ± 12.5	72.2 ± 14.7	76.7 ± 10.0
Erosion					
Left joint	0.00 ± 0.00	2.00 ± 0.41	1.00 ± 0.41	2.67 ± 0.33	2.50 ± 0.85
Right joint	0.00 ± 0.00	1.75 ± 0.25	0.75 ± 0.48	1.67 ± 0.33	1.50 ± 0.29
Right/left ratio (%)	0.00 ± 0.00	91.7 ± 8.33	66.7 ± 33.3	61.1 ± 5.56	66.7 ± 11.8
Loss of proteoglycan					
Left joint	0.00 ± 0.00	7.50 ± 1.44	7.50 ± 1.44	8.75 ± 1.25	9.00 ± 1.00
Right joint	0.00 ± 0.00	5.00 ± 0.00	7.50 ± 1.44	5.00 ± 0.00	6.00 ± 1.00
Right/left ratio (%)	0.00 ± 0.00	75.0 ± 14.4	100.0 ± 0.00	62.5 ± 12.5	80.0 ± 12.5

Values are the mean ± SEM score. The macroscopic appearance and histological analyses of the knee joint (Right/left ratio, %) after the various drug treatments were statistically analyzed using Bonferroni's *t*-test. $*p < 0.05$

Discussion

The effects after treatments of ia injections with HA alone and combined with 2 levels of indomethacin on alleviating inflammation and improving cartilage degradation in rabbits with antigen-induced arthritis were evaluated by examining the CRP level for the resolution of inflammation, the PGE_2 level for the reduction of inflammatory pain, and the serum MMP-3 level and macroscopic and histological analyses of the joints for protection against cartilage damage. Results indicated that treatment by an ia injection with HA alone (the HA group) was able to inefficaciously decrease the CRP serum level, to only moderately suppress the serum level of PGE_2, to slightly but significantly inhibit the sustained elevated serum level of MMP-3, and to protect against cartilage damage only to some extent as evidenced by no difference in macroscopic pannus formation, no further deterioration in the extent of hyperplasia, a slight decrease in the extent of inflammation, some improvement in joint leakage and erosion, but no improvement in the loss of proteoglycan. As expected, treatments with an ia injection of HA in combination with 2 levels of indomethacin (in the LI-HA and HI-HA groups) were both more beneficial to arthritic therapy than HA alone as evidenced by statistically significant improvements in the responses of all parameters examined, with the low concentration of indomethacin (the LI-HA group) being more effective than the high concentration of indomethacin (the HI-HA group).

CRP is produced by the liver in response to circulating IL-6, tumor necrosis factor α (TNFα), or IL-1, and is a traditional marker of systemic inflammation, including that originating in joints [23]. CRP has been employed since the 1950s as a laboratory marker of the inflammatory response [24]. The serum CRP level was also found to be a good parameter for prognostic purposes and for monitoring the treatment effect if the influences of other stimuli of the acute phase response are excluded [25]. Results in Table 2 show that there were statistically significant decreases in serum CRP levels in the LI-HA (54.6 ± 0.2%) and HI-HA groups (30.3 ± 9.2%) compared to the HA group (2.2 ± 0.8%), whose effect was limited and little different from the control group injected with 0.9% NaCl (5.0 ± 9.5%). This indicates that an ia injection of HA alone was insufficient for alleviating inflammation in rabbits with AIA, and the combination of HA with indomethacin, regardless of the level, was able to enhance alleviation of inflammation. However, the alleviating effect on inflammation by the low concentration of indomethacin was more profound than that by the high concentration of indomethacin as indicated by the greater extent of suppression of serum CRP levels. Since serum CRP is released by hepatoma

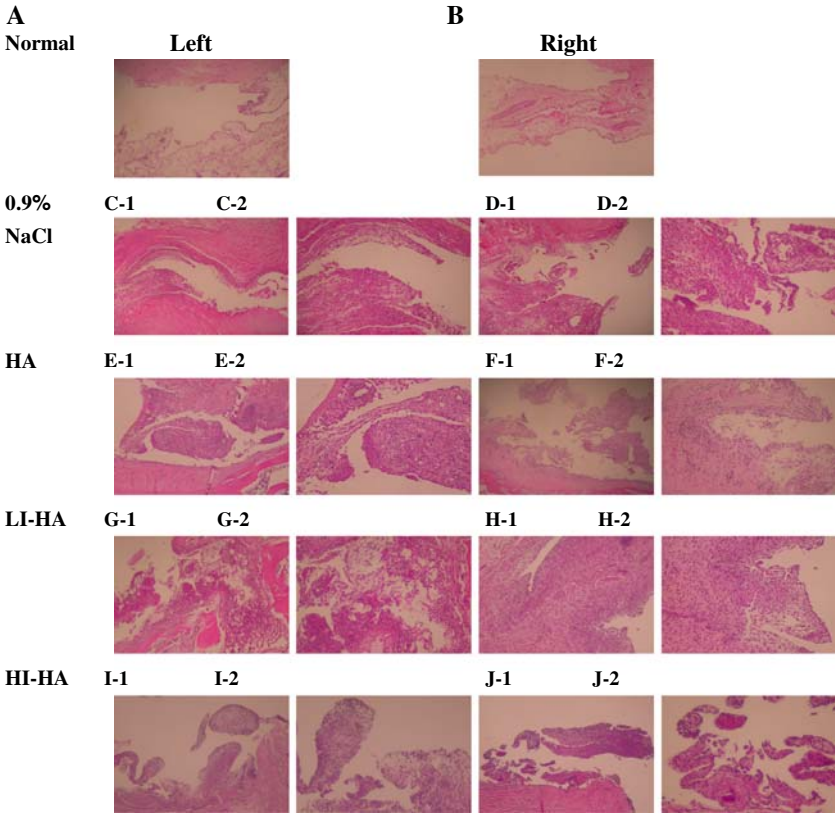

Fig. 4 Histological analysis of the synovial membrane after various drug treatments by intra-articular injections in the right knee joint of rabbits with antigen-induced arthritis (AIA). A hematoxylin-and-eosin (H&E)-stained 5-μm longitudinal section (see Fig. 3, *green section*) through the knee joint of a normal rabbit and a similar section in a rabbit exhibiting arthritis are shown for general morphology examination. The arthritic joint has increased proteinaceous fluid and abundant neutrophils in the joint space. The synovial lining is thickened with an increase in type II synovial epithelium and adjacent infiltrates of lymphocytes and plasma cells. The left and right normal control cartilage was used in this study (*A* and *B*). The left knee joint as the internal control received no treatment (*C*), while the right knee joint was injected with 0.5 ml of 0.9% NaCl (*D*). The left knee joint as the internal control received no treatment, while the right knee joint was injected with 0.5 ml of 1% hyaluronate (HA group, *F*). The left knee joint as the internal control received no treatment (*G*), while the right knee joint was injected with 0.5 ml of 5.6 μM indomethacin + 1% hyaluronate (LI-HA group, *H*). The left knee joint as the internal control received no treatment (*I*), while the right knee joint was injected with 0.5 ml of 560 μM indomethacin + 1% hyaluronate (HI-HA group, *J*). Only one typical image of the left and the right knees at two magnifications (*-1: ×25 and *-2: ×30−80) for each treatment group is shown

cells in response to the induction of IL-6 secreted by macrophages during the acute-phase response, the serum level or receptor expression of the IL-6 receptor should correspondingly have been more-strongly suppressed in the LI-HA group than in the HI-HA group [26].

The increasing serum level of PGE_2 was moderately suppressed in the HA group ($-26.0 \pm 6.1\%$) in comparison to that in the control group injected with 0.9% NaCl ($-53.2 \pm 10.6\%$). This reveals that an ia injection of HA alone is able to inhibit the formation of PGE_2 but not to a sufficient extent. Indomethacin, a NSAID, is therapeutically used for its ability to block COX activity resulting in a smaller amount of PGE_2 being formed. As expected, there was more-efficient reduction in the serum PGE_2 level in the HI-HA group ($35.9 \pm 3.3\%$) than in the LI-HA group ($13.3 \pm 2.9\%$) since a higher concentration of indomethacin was combined with HA in the former group. Therefore, the higher concentration of indomethacin in the HI-HA group did produce greater suppression of the serum PGE_2 level, but less suppression of the serum CRP level, whereas the opposite was true for the low concentration of indomethacin in the LI-HA group. It has been reported that PGE_2 is a potent downregulator of IL-6 receptor expression in the NFS-60 cell line and mediates its effects through an EP2-receptor-mediated cAMP signaling pathway [27]. Thus, this may provide a mechanistic explanation for the increased serum level of PGE_2 possibly downregulating IL-6 receptor expression in hepatoma cells leading to a greater reduction in the extent of CRP secretion in the LI-HA group than in the HI-HA group. Therefore, it was concluded that an ia injection of HA combined with either a low or high concentration of indomethacin is beneficial for the treatment of AIA, with the low concentration of indomethacin capable of suppressing the formation of CRP to

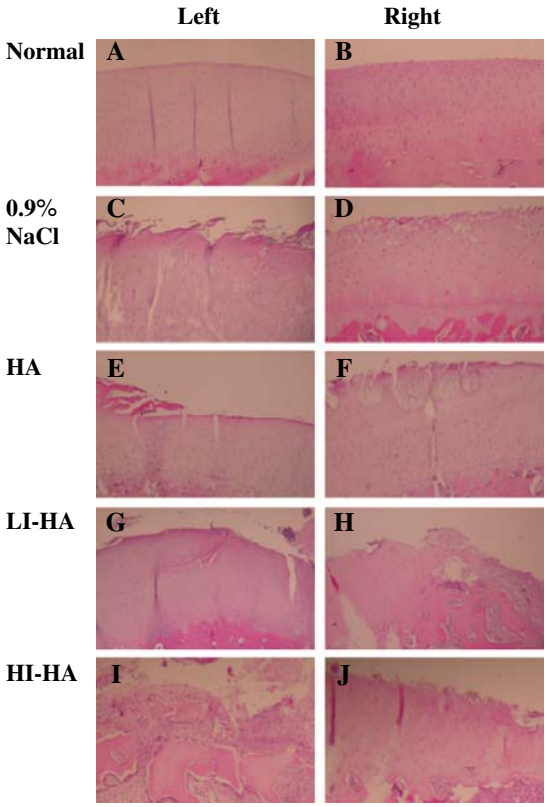

Fig. 5 Histological analysis of knee joint damage after the various drug treatments by intra-articular injections in the right knee joint of rabbits with antigen-induced arthritis (AIA). A hematoxylin-and-eosin (H&E)-stained 5-μm longitudinal section (see Fig. 3, *green section*) through the knee joint (articular surface of the lateral coudyle of femur) of a normal rabbit and a similar section in a rabbit exhibiting arthritis are shown for general morphology examination. Left and right normal control cartilage was used in this study (*A* and *B*). The left knee joint as the internal control received no treatment (*C*), while the right knee joint was injected with 0.5 ml of 0.9% NaCl (*D*). The left knee joint as the internal control received no treatment (*E*), while the right knee joint was injected with 0.5 ml of 1% hyaluronate (HA group, *F*). The left knee joint as the internal control received no treatment (*G*), while the right knee joint was injected with 0.5 ml of 5.6 μM indomethacin + 1% hyaluronate (LI-HA group, *H*). The left knee joint as the internal control received no treatment (*I*), while the right knee joint was injected with 0.5 ml of 5.6 μM indomethacin + 1% hyaluronate (HI-HA group, *J*). Only one typical image (×20−40 magnification) of the left and the right knees for each treatment group is shown.

alleviate inflammation and decrease complement activation [28] and the high concentration capable of inhibiting the formation of PGE_2 to reduce the inflammatory pain.

In contrast to CRP, a marker of systemic inflammation, MMP-3 is produced in the joint in response to local IL-6, TNFα, and IL-1 and is a more-specific marker of synovial inflammation [23]. Elevation of MMP-3 serum levels might be restricted to inflammatory diseases associated with joints, as it is intensively expressed in the rheumatoid synovium. Since MMP-3 has potent activity of degrading the proteoglycan of cartilage [29], serum levels of MMP-3 have previously been shown to be correlated with radiological damage in RA patients [29–31]. The results of this study demonstrate that the serum level of MMP-3 was elevated and sustained (Table 1) in the group treated by injecting 0.9% NaCl, whereas elevation of MMP-3 serum levels was significantly suppressed in the other 3 treatment groups (Table 2), especially in the 2 treatment groups that combined HA with indomethacin at a low and high level. Therefore, it is expected that indomethacin at both levels in combination with HA would be beneficial in the prevention of cartilage damage, as reflected by further suppression of serum levels of MMP-3, with the low concentration of indomethacin exhibiting a greater extent of suppression than the high concentration.

Treatment with an ia injection of HA alone was expected to be efficacious in the prevention of cartilage damage as reflected by a reduction in serum MMP-3 levels. MMP-3 is also recognized as an enzyme which plays a part in the destruction of cartilage and bone in rheumatoid arthritis (RA) [32–33]. Patients with RA have increased serum levels of MMP-3, which is thought to originate from the synovium [34,35] and strongly suggests that it reflects synovial inflammation [25]. In addition, serum levels of MMP-3 are correlated with the number of joints affected and are decreased after an ia injection of steroids [36]. Therefore, serum MMP-3 levels were expected to be elevated after induction of AIA and be continuously sustained during the treatment period for the control group (which received an ia injection of a 0.9% NaCl solution in the right knee), whereas increases in the serum levels of MMP-3 were suppressed in the 3 other treatment groups (HA, LI-HA, and HI-HA) since their arthritic right knee joints were efficaciously treated. It has been documented that ia administration of HA in the rabbit knee inhibits MMP-3 and TIMP-1 production at the mRNA level in cartilage and synovium [37]. It was also reported that one mechanism of the therapeutic effect of HA by ia injection into the ACLT (anterior cruciate ligament transection) of rabbit knees is downregulation of MMP-3 and IL-1β in the synovium [38,39]. An in vitro study by Sasaki et al. revealed that HA inhibits the expression and production of MMP-1 and MMP-3 in IL-1β-stimulated human synovial cells [40]. A similar effect of inhibiting MMP-3 synthesis induced by IL-β in human OA chondrocytes was disclosed for both chondroitin sulfate and HA (500−730 kDa) [41]. Therefore, it was concluded that the observed suppression of increasing MMP-3 serum levels by an ia injection of HA was due to the inhibition of MMP-3 expression and production in the synovium and possibly in chondrocytes.

Indomethacin in combination with HA is beneficial for preventing cartilage damage by further suppressing serum levels of MMP-3 with the low concentration of indomethacin producing a greater extent of suppression than the high concentration. It was reported by Sadowski and Steinmeyer that

when tested at a concentration of 10 μM in bovine articular chondrocytes, indomethacin inhibited MMP-3 expression [42]. This finding is in agreement with a previous study by Yamada et al. in which indomethacin reduced MMP-3 production by human chondrocytes [43]. Further, both studies demonstrated that the addition of exogenous PGE_2 did not reverse the effect of indomethacin, and it was noted that MMP-3 inhibition by this drug is independent of PGE_2 synthesis. Further, oral administration of nimesulide, a COX-2 selective inhibitor, significantly reduced serum levels of MMP-3, whereas that of ibuprofen, a COX-1/COX-2 inhibitor, moderately but significantly increased the serum concentrations of MMP-3 in patients with OA [44]. It was also found that indomethacin (a COX-2/COX-2 inhibitor) and NS-398 (a COX-2 selective inhibitor) enhanced IL-1α-induced MMP-3 production in human PDL (periodontal ligament) cells, but both agents completely inhibited IL-1α-induced PGE_2 production. Since exogenous PGE_2 reduced IL-1α-induced MMP-3 production in a dose-dependent manner and both EP2 and EP4 agonists significantly inhibited IL-1α-induced MMP-3 production, it was concluded by this study that COX-2-dependent PGE_2 downregulates IL-1α-elicited MMP-3 production by c-AMP-dependent pathways via EP2/EP4 receptors in human PDL cells [45]. However, although this fact is in conflict with the results disclosed in the above study, it provides a possible mechanistic explanation for how the low or high concentration of indomethacin influences serum levels of MMP-3. A higher serum level of PGE_2 as a result of giving a low dose of indomethacin in the LI-HA group should have reduced MMP-3 production following the mechanism discussed above to a greater extent than that by the low serum level of PGE_2 resulting from administering a high dose of indomethacin in the HI-HA group.

Furthermore, MMP-3 serum levels are reported to be correlated with parameters of inflammation including the erythrocyte sedimentation rate (ESR), CRP, and interleukin (IL)-6 levels [46–48]. This is consistent with the results demonstrated by the low serum level of MMP-3 correlating with the low level of CRP in the LI-HA group and the high serum level of MMP-3 with the high level of CRP in the HI-HA group in this study. As discussed above, the change in CRP serum levels might also have been mediated by PGE_2 through the EP2-receptor-mediated cAMP signaling pathway to downregulate the receptor expression of IL-6 that is responsible for secretion of CRP in hepatoma cells. Hypothetically, PGE_2 might play a determining role in the correlation between serum levels of MMP-3 and CRP. However, this could create a dilemma in the choice of which level of indomethacin to combine with HA to produce effective improvement in antiarthritic therapy, since a low dose of indomethacin could lead to a higher serum level of PGE_2 but a low level of MMP-3 and vice versa for a high dose of indomethacin. If destruction of cartilage and bone by MMP-3 plays the determining role in the disease progression of arthritic joints, a low dose of indomethacin might be the best choice in combination with HA for ia administration.

For the macroscopic and histological analyses, a statistically insignificant difference in most of those parameters examined was found when comparing the treated groups with the control group. This could be attributed to either a high variability of macroscopic and histological analyses or the treatment period not being long enough to allow significant improvements to develop. Since differences in the serum levels of CRP, PGE_2, and MMP-3 were statistically determined to have sufficient power with this sample size, it would be reasonable to accept the former. Although there were no statistically significant differences in most of the parameters examined by macroscopic and histological analyses in the 3 treatment groups (HA, LI-HA, and HI-HA) in comparison with the control group (0.9% NaCl), there was a little more improvement in the therapeutic efficiency in the LI-HA group in comparison to the HA group in terms of pannus formation, synovial hyperplasia and inflammation, leakage and erosion of the joint, and loss of proteoglycan (Table 3). As discussed above, correspondingly lower serum levels of CRP and MMP-3 in the LI-HA treatment group in comparison with those in the HA and HI-HA groups might have been responsible for this.

Conclusion

In conclusion, it was found in this study that hyaluronate combined with a low dose of indomethacin (5.6 μM) might provide substantially more clinical benefits to arthritic rabbits based on suppression of the serum levels of CRP and MMP-3 with correspondingly improved therapeutic efficiency observed in the macroscopic and histological analyses. The results of this study demonstrated that MMP-3 was elevated and that this elevation was sustained (Table 1) in all drug treatment groups, which suggests that MMP inhibition may be a potential therapeutic strategy. MMP inhibitors have frequently exhibited toxicity in clinical trials with systemic administration. A local injection combined with NSAIDs and an MMP inhibitor in a hyaluronate solution might be another appropriate strategy for arthritis therapy.

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