Intra-articular injection of interleukin-4 decreases nitric oxide production by chondrocytes and ameliorates subsequent destruction of cartilage in instability-induced osteoarthritis in rat knee joints

M. Yorimitsu M.D., K. Nishida M.D., Ph.D., Associate Professor, A. Shimizu M.D., Ph.D., H. Doi M.D., S. Miyazawa M.D., Ph.D., T. Komiyama M.D., Ph.D., Y. Nasu M.D., A. Yoshida, S. Watanabe M.D., Ph.D., Associate Professor, and T. Ozaki M.D., Ph.D., Professor

Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama City, Okayama 700-8558, Japan

Department of Human Morphology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama City, Okayama 700-8558, Japan

Department of Orthopaedic Surgery, Ehime University Medical School of Medicine, Shitsukawa, Shigenobu-cho, Onsen-gun, Ehime 791-0295, Japan

Summary

Objective: To investigate the in vitro and in vivo effects of interleukin (IL)-4 on mechanical stress-induced nitric oxide (NO) expression by chondrocytes, and destruction of cartilage and NO production in an instability-induced osteoarthritis (OA) model in rat knee joints, respectively.

Materials and methods: Cyclic tensile stress (CTS; 0.5 Hz and 7% elongation) was applied to cultured normal rat chondrocytes with or without pre-incubation with recombinant rat IL-4 (rrIL-4). Inducible NO synthase (iNOS) mRNA expression and NO production were examined with real-time polymerase chain reaction and the Griess reaction, respectively. OA was induced in rat knee joints by transection of the anterior cruciate and medial collateral ligaments and resection of the medial meniscus. rrIL-4 (10, 50, and 100 ng/joint/day) was injected intra-articularly, and knee joint samples were collected 2, 4, and 6 weeks after surgery. Cartilage destruction was evaluated by the modified Mankin score and Osteoarthritis Research Society International scoring system on paraffin-embedded sections stained with safranin O. Cleavage of aggrecan and NO production were examined by immunohistochemistry for aggrecan neoepitope (NITEGE) and of nitrotyrosine (NT), respectively.

Results: rrIL-4 down-regulated CTS-induced iNOS mRNA expression and NO production by chondrocytes. The intra-articular injection of rrIL-4 gave rise to a limited, but significant amelioration of cartilage destruction, prevention of loss of aggrecan, and decrease in the number of NT-positive chondrocytes, an effect that was not dose-dependent.

Conclusion: The present study suggests that IL-4 may exert chondroprotective properties against mechanical stress-induced cartilage destruction, at least in part, by inhibiting NO production by chondrocytes.

© 2007 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

Key words: Osteoarthritis, Chondrocyte, Interleukin-4, Nitric oxide, Mechanical stress.

Introduction

Osteoarthritis (OA) is the most common form of arthritis, and is characterized by destruction of articular cartilage, osteophyte formation, subchondral bone sclerosis, and secondary synovitis. Although mechanical stimulation is essential for the maintenance of chondrocyte metabolism and homeostasis of articular cartilage, changes in matrix loading can also induce the production of various pro-inflammatory mediators from chondrocytes and promote cartilage matrix degradation. Currently, the loss of aggrecan from decreased synthesis by chondrocytes and activation of cartilage matrix degrading enzymes is recognized as one of the earliest events in the courses of OA, followed by the mechanical injury of collagen fibrils.

An inducible form of nitric oxide synthase (iNOS) expression and nitric oxide (NO) production is increased in OA cartilage. An excess amount of NO induces the destruction of cartilage by activating pro-matrix metalloproteinase (MMP) and inhibiting the synthesis of aggrecan and type II collagen by chondrocytes, and promoting chondrocyte cell death. Pro-inflammatory cytokines, such as interleukin (IL)-1α, IL-1β, and tumor necrosis factor (TNF)-α, are potent stimulators of NO production by chondrocytes. In addition, NO production by chondrocytes is reportedly influenced by mechanical stress, including intermittent compression, cyclic tensile stress (CTS), and fluid-induced...
shear stress. Several investigations have shown that treatment with NOs inhibitors reduces the progression of cartilage destruction of experimentally induced OA in animal models, suggesting that NO may be an important therapeutic target for OA. IL-4 is known as one of the anti-inflammatory cytokines that can potentially protect against severe cartilage destruction during experimental arthritis by suppression of proteolytic enzymes, up-regulation of inhibitors of these enzymes, and induction of matrix synthesis. Previous reports have demonstrated that IL-4 may have the potential to block the destruction of cartilage by antagonizing inflammatory mediators, such as IL-1. IL-4 is also known to directly inhibit the expression of iNOS mRNA and the production of NO, and indirectly by inhibiting pro-inflammatory cytokines, such as IL-1 and TNF-α in synoviocytes. Pre-incubation with IL-4 decreases IL-1 and TNF-α-induced NO production by cultured chondrocytes; however, whether IL-4 has a protective effect on the destruction of cartilage during OA is still unknown. It is also unclear whether IL-4 affects mechanical stress-induced gene expression by chondrocytes.

In the present study, we examined the in vitro effect of recombinant rat IL-4 (rIL-4) on mechanical stress-induced iNOS and NO expression in cultured rat chondrocytes. We injected rIL-4 intra-articularly daily into rat knee joints with instability-induced experimental OA to examine the in vivo effect of IL-4. The results suggested that IL-4 may exert chondroprotective properties against excess load on articular cartilage in OA, at least in part, by inhibiting mechanical stress-induced NO production.

Materials and methods

CHONDROCYTE CULTURES AND IL-4 TREATMENT

Epiphyseal cartilage obtained from the femoral condyle of 7-day-old Wistar rats was aseptically dissected and the chondrocytes were isolated after digestion of cartilage fragments in 0.1% trypsin (Wako, Osaka, Japan) and 0.2% collagenase (Sigma, Tokyo, Japan) following the method described by Bruckner et al. Chondrocytes (5 x 10^5/mL) were seeded in six-well plates coated with type I collagen (BioFlex collagen I culture plate; Flexcell International, McKeesport, PA, USA) and cultured in 3 ml media containing a mixture of 100 U/ml penicillin and 100 µg/ml streptomycin. Chondrocytes were incubated in a 5% CO2 humidified incubator at 37°C for 3 days until cells were grown to confluence. After washing with phosphate-buffered saline (PBS), the chondrocytes were incubated with rIL-4 (0, 1, or 10 ng/mL; Diamclone Research, Cedex, France) in a 1% CO2 atmosphere for 24 h.

EXPOSURE OF CHONDROCYTES TO CTS

The cells seeded on the flexible surface of the BioFlex plates were exposed to the cyclic stress of stretch and relaxation using a Flexcell Strain Unit (Flexcell International). The vacuum induced a 7% (10 kPa) elongation of the flexible surface. The culture plate bottoms were deformed in a cyclic manner (1 s on and 1 s off; 0.5 Hz) and the cells were stimulated for 24 and 48 h. CTS did not change the viability of the cells after 24 and 48 h of stimulation.

REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Real-time PCR was performed using a LightCycler rapid thermal cycle system (Roche Diagnostics, Lewes, UK) according to the manufacturer’s instructions. Reaction components were prepared to the end-concentration indicated as follows: 9.4 µl H2O, 1.6 µl MgCl2 (3 mM), 1.0 µl forward primer (0.5 µM) and 1.0 µl reverse primer (0.5 µM), 2.0 µl LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics), and 5.0 µl sample. Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler-DNA Master SYBR Green I mix. To improve SYBR Green I quantification, a high-temperature fluorescence measurement was performed. Amplification of the cDNA encoding iNOS was performed with the following specific primers: forward primer sequence 5’-ACCAAGGCTCTAGTGCACAG-3’ and reverse primer sequence 5’-AAAGCCGACCCGAGATTC-3’.

MEASUREMENT OF THE LEVEL OF NO IN THE CULTURED MEDIA

Culture supernatants were collected for nitrite measurement. NO formation was detected by NO2− accumulation in the culture supernatants by an HPLC-Griess system (ENO-20, Eicom, Kyoto, Japan) with sodium nitrite as a standard. Optical density was measured at 540 nm. Results are expressed as µM.

PREPARATION OF THE EXPERIMENTAL OA MODEL

Eighty Wistar rats, six-week of age, were used for this study. Animals were anesthetized by intra-peritoneal injection of pentobarbital sodium (50 mg/kg). The rats’ right knee joints were incised and arthroscopically were performed using the total parapatellar approach. First, the anterior cruciate and medial collateral ligaments (ACL and MCL, respectively) were transected with surgical scissors. The medial joint space was easily opened with valgus stress. The medial meniscus was identified on the posterior corner, removed anteriorly, and resected with surgical scissors. Using this method, over two-thirds of the meniscus was resected. The left knee joints were also incised and an arthrotomy only was performed as a sham operation.

INTRA-ARTICULAR INJECTION OF rIL-4

The animals were divided into treatment (n = 59) and control (n = 21) groups. The rats in the treatment group received a daily intra-articular injection of 10 µg/ml of rIL-4 (50 nM) in 0.01 M PBS as a vehicle in the same manner. Animals were anesthetized by diethyl ether and the vehicle or rIL-4 was injected into the knee joints from just medial side of patella tendon with the knee joint flexed 90°. The medial joint space was easily identified because of the severe knee joint instability after surgery. We confirmed that the tip of the needle reached at the joint space by loss of resistance or aspiration of joint fluid. The animals were sacrificed 2, 4, or 6 weeks after surgery, and the right knee joint samples were obtained for histologic and immunohistochemical examination. The left knee joint samples of each of seven rats, 2, 4, and 6 weeks after surgery, were obtained as the sham operation group (n = 21).

TISSUE PREPARATION AND HISTOLOGIC EVALUATION

Histologic evaluation was performed on full thickness sagittal sections of cartilage in the weight-bearing area of the medial femoral condyle. The knee joint samples were dissected, fixed in 4% paraformaldehyde (PFA) for 24 h, and defatted in alcohol. Then, the knee joint samples were decalcified in 0.3 M ethylene diamine tetraacetate (EDTA; pH 7.5) for 10 days and embedded in paraffin. Sections (4.5 µm) were stained with 0.1% safranin O. Histopathologic classification of the severity of the OA lesion was graded on a scale of 0–13, using the modified Mankin scoring system and the OARSI OA cartilage histopathology assessment system. The modified Mankin score is a combined score assessing structure (0–6 points), cellular abnormalities (0–3 points), and matrix staining (0–4 points). The OARSI score is a semi-quantitative method to evaluate the histopathology of OA cartilage. In the current study, we used the recommended OA score (index of combined grade and stage, 0–24 points). The histologic evaluation was performed by two independent, blinded observers (MY and KN).

IMMUNOHISTOCHEMICAL EVALUATION OF AGGREGAN NEOEPITOPE

To evaluate the cleavage of aggrecan, we performed the immunohistochemical study on the same series of knee joint sections for neopteope sequence (NITEGE) generated by aggrecanase-mediated cleavage at Glu373延安374 of aggrecan core proteins. Deparaffinized sections were pre-treated with chondroitinase ABC (1 U/ml; SIGMA, St Louis, MO, USA) at 37°C for 2 h. The endogenous peroxidase was blocked with 3% H2O2 in PBS at room temperature for 15 min and incubated in normal goat serum at room temperature for 60 min. Rabbit anti-aggrecan neo antibody (10 µg/ml) was detected by sheep anti-rabbit IgG (Dako, Carpinteria, CA, USA) was used as the primary antibody at a 1:500 dilution. Histofine simple stain MAX PO(R) (Nichirei Co., Tokyo, Japan) was used as the secondary antibody. The reaction was visualized by diaminobenzidin (DAB; Histofine simple stain DAB, Nichirei Co., Tokyo, Japan). Histologic evaluation was performed on paraffin sections of the knee joint samples using a light microscope, and sections were classified as: 0, negative; 1, weak; 2, moderate; and 3, strong.

IMMUNOHISTOCHEMICAL EVALUATION OF NITROTYROSINE (NT)

Deparaffinized sections were pretreated with 0.1 M citrate buffer (pH 6.0) in an autoclave at 95°C for 5 min to retrieve the antigen. The endogenous...
peroxidase was blocked with 3% H2O2 in PBS at room temperature for 10 min. Rabbit anti-NT antibody (5 μg/ml; Upstate, NY, USA) was used as the primary antibody at 4°C overnight and Histofine simple stain rat MAX PO(R) (Nichirei Co., Tokyo, Japan) was used as the secondary antibody. The reaction was visualized by DAB as described above. Counterstaining was carried out with methyl green. Sections incubated with normal rabbit non-immune serum or incubated without primary antibody were used as negative controls.

In the immunostaining section of NT, chondrocyte with definite, diffusely stained cytoplasm or nuclei was regarded as positively stained. The population of NT-positive chondrocytes was quantified in the sections of femoral condyle by counting the number of chondrocytes within full thickness cartilage of 6 mm width at 200× magnifications (~100 cells/field). The number of NT-positive chondrocytes was divided by the total number of chondrocytes to calculate the positive chondrocyte ratio.

STATISTICAL ANALYSIS

All data are expressed as the mean ± standard deviation (SD). Differences among individual sample groups were statistically analyzed by Fisher’s protected least significant difference (PLSD) post hoc test. A *P* value less than 0.05 denoted a statistically significant difference.

Results

THE EFFECTS OF IL-4 ON CTS EXPRESSION OF iNOS mRNA AND NO PRODUCTION

The expression of iNOS mRNA by cultured chondrocytes was significantly increased after 24 and 48 h of CTS (*P* < 0.001). The mechanical stress-induced iNOS mRNA expression was suppressed by the pre-treatment of rrIL-4 (1 ng/ml) after 24 and 48 h of CTS. Statistical significance existed only after 48 h of CTS (*P* < 0.001). rrIL-4 (10 ng/ml) significantly suppressed mechanical stress-induced iNOS mRNA expression to the baseline level of no mechanical stress after 24 (*P* < 0.005) and 48 h (*P* < 0.001) of CTS (Fig. 1). This suppressive effect of iNOS mRNA by the treatment of rrIL-4 was dose-dependent (*P* < 0.001).

NO production from the cultured chondrocytes was not affected following 24 h of CTS, but was significantly increased following 48 h of CTS (*P* < 0.001). The treatment of rrIL-4 (10 ng/ml) significantly suppressed the increased production of NO3 (*P* < 0.005; Table I).

Table I  
NO3 concentration in the cultured media of normal rat chondrocytes after CTS with or without IL-4 pre-incubation

<table>
<thead>
<tr>
<th>Hours</th>
<th>No stress</th>
<th>CTS (0.5 Hz and 7% elongation)</th>
<th>IL-4 (10 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.49±0.36</td>
<td>11.19±0.83</td>
<td>10.43±1.36</td>
</tr>
<tr>
<td>24</td>
<td>10.58±0.32</td>
<td>11.19±0.83</td>
<td>10.43±1.36</td>
</tr>
<tr>
<td>48</td>
<td>10.85±0.56</td>
<td>12.89±2.61</td>
<td>9.19±0.44</td>
</tr>
</tbody>
</table>

*P* < 0.005 vs no mechanical stress, |P| < 0.05 vs 24 h of mechanical stress without IL-4 treatment, **P* < 0.001 vs 48 h of stress without IL-4 treatment.

HISTOLOGIC EVALUATION

On the safranin O-stained sections of the femoral condyle, mild progression of cartilage destruction was observed 2 weeks after surgery with surface fibrillation, decreased number of chondrocytes, and decreased staining of safranin O in the superficial layer. The cartilage lesions were more severe 4 weeks after surgery, with absence of the superficial layer, surface fibrillation, cluster formation, and decreased staining of safranin O in the deeper layer. In some sections, the full thickness of joint cartilage disappeared and the subchondral bone was exposed, so-called eburnation. Six weeks after surgery, the cartilaginous lesions were further developed (Fig. 2).

The sections of femoral condyles from the sham operation group of rats showed minimum changes in the modified Mankin scores and OARSI score through the subsequent time course following capsulotomy. The modified Mankin scores of the control group sections 2, 4, and 6 weeks after surgery were 2.00 ± 1.73, 4.71 ± 1.49, and 6.00 ± 3.20, respectively, and significantly higher 4 and 6 weeks after the surgery than those of the sham operation group sections (*P* < 0.001; Table II). The OARSI score of the control group sections 2, 4, and 6 weeks after surgery were 5.14 ± 2.85, 9.25 ± 4.89, and 12.29 ± 4.50, respectively, were significantly higher than those of the sham operation group sections at 2 (*P* < 0.005), 4 (*P* < 0.001) and 6 weeks (*P* < 0.001) after surgery (Table IV). In the control group, histologic scores gradually increased with time after surgery. There were statistically significant differences in the histologic scores between 2 and 4 weeks, but there were no significant differences between 4 and 6 weeks. OARSI score is more sensitive to evaluate the early phase of OA progression than modified Mankin score, similar results were obtained using both scoring systems. The histologic scores of the treatment group sections are shown in Tables II and IV. The intra-articular injection of rrIL-4 significantly ameliorated cartilage destruction 4 and 6 weeks after surgery; the histologic scores of the treatment group sections were significantly higher than those of the sham operation group sections. A dose-dependent effect of IL-4 on the inhibition of cartilage destruction did not occur in the present sets of concentrations of rrIL-4.

The proteoglycan staining in the sections seemed to be predominant abnormality seen in this rat model of OA. Among the sub-categories of the modified Mankin scoring
System, matrix staining is shown in Table III. In the control group sections, a statistically significant loss of safranin O staining was observed 4 and 6 weeks after surgery. The matrix staining scores were significantly improved by the intra-articular injection of rrIL-4. A dose-dependent effect of IL-4 on the prevention of proteoglycan loss did not occur in the present sets of concentrations of rrIL-4. These results indicate that IL-4 prevented the loss of proteoglycan from the articular cartilage in this model.

**IMMUNOHISTOCHEMICAL EVALUATION OF AGGRECAN NEO**

Immunohistochemical staining for NITEGE neoepitope demonstrated the distribution and relative amount of G1 fragment of aggrecan core protein in the rat knee joint cartilage from the control and treatment groups. In general, staining intensity appeared to increase with time after surgery and degree of cartilage damage. The cartilage of control group at 2 weeks showed mild staining at superficial and middle layer, and intense staining throughout the matrix at 4 weeks. However, the cartilage of control group at 6 weeks showed weak matrix staining and intense cellular and pericellular matrix staining. In contrast, the cartilage of treatment group at 2 and 4 weeks showed weak staining for the neoepitope at superficial, and superficial and middle layer, respectively, when compared with those of OA control. The cartilage of treatment group at 6 weeks showed intense matrix staining throughout the matrix (Fig. 3).

**IMMUNOHISTOCHEMICAL EVALUATION OF NT**

NO reacts with superoxide radicals to form peroxynitrite. Addition of peroxynitrite to biological fluid leads to nitration of aromatic amino acid residues (i.e., NT). Measurement of NT is useful for demonstrating NO-mediated tissue damage in vivo. Representative appearances of immunohistochemical

---

**Table II**

<table>
<thead>
<tr>
<th></th>
<th>Modified Mankin score</th>
<th>No. of rats</th>
<th>Modified Mankin score</th>
<th>No. of rats</th>
<th>Modified Mankin score</th>
<th>No. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 Weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.28 ± 0.48</td>
<td>7</td>
<td>0.42 ± 0.53</td>
<td>7</td>
<td>0.42 ± 0.53</td>
<td>7</td>
</tr>
<tr>
<td>OA control</td>
<td>2.00 ± 1.73</td>
<td>7</td>
<td>4.71 ± 1.49*,††</td>
<td>7</td>
<td>6.00 ± 3.20*,††</td>
<td>7</td>
</tr>
<tr>
<td>IL-4 (10 ng/joint/day)</td>
<td>1.28 ± 1.25</td>
<td>7</td>
<td>2.80 ± 0.44**</td>
<td>5</td>
<td>3.42 ± 1.13**,††</td>
<td>7</td>
</tr>
<tr>
<td>IL-4 (50 ng/joint/day)</td>
<td>0.85 ± 0.69</td>
<td>7</td>
<td>2.66 ± 1.21**,††</td>
<td>6</td>
<td>2.14 ± 2.26††</td>
<td>7</td>
</tr>
<tr>
<td>IL-4 (100 ng/joint/day)</td>
<td>1.66 ± 1.21</td>
<td>6</td>
<td>2.57 ± 3.91**,††</td>
<td>7</td>
<td>4.00 ± 2.20**,††</td>
<td>7</td>
</tr>
<tr>
<td><strong>4 Weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.28 ± 0.48</td>
<td>7</td>
<td>0.42 ± 0.53</td>
<td>7</td>
<td>0.42 ± 0.53</td>
<td>7</td>
</tr>
<tr>
<td>OA control</td>
<td>2.00 ± 1.73</td>
<td>7</td>
<td>4.71 ± 1.49*,††</td>
<td>7</td>
<td>6.00 ± 3.20*,††</td>
<td>7</td>
</tr>
<tr>
<td>IL-4 (10 ng/joint/day)</td>
<td>1.28 ± 1.25</td>
<td>7</td>
<td>2.80 ± 0.44**</td>
<td>5</td>
<td>3.42 ± 1.13**,††</td>
<td>7</td>
</tr>
<tr>
<td>IL-4 (50 ng/joint/day)</td>
<td>0.85 ± 0.69</td>
<td>7</td>
<td>2.66 ± 1.21**,††</td>
<td>6</td>
<td>2.14 ± 2.26††</td>
<td>7</td>
</tr>
<tr>
<td>IL-4 (100 ng/joint/day)</td>
<td>1.66 ± 1.21</td>
<td>6</td>
<td>2.57 ± 3.91**,††</td>
<td>7</td>
<td>4.00 ± 2.20**,††</td>
<td>7</td>
</tr>
<tr>
<td><strong>6 Weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.28 ± 0.48</td>
<td>7</td>
<td>0.42 ± 0.53</td>
<td>7</td>
<td>0.42 ± 0.53</td>
<td>7</td>
</tr>
<tr>
<td>OA control</td>
<td>2.00 ± 1.73</td>
<td>7</td>
<td>4.71 ± 1.49*,††</td>
<td>7</td>
<td>6.00 ± 3.20*,††</td>
<td>7</td>
</tr>
<tr>
<td>IL-4 (10 ng/joint/day)</td>
<td>1.28 ± 1.25</td>
<td>7</td>
<td>2.80 ± 0.44**</td>
<td>5</td>
<td>3.42 ± 1.13**,††</td>
<td>7</td>
</tr>
<tr>
<td>IL-4 (50 ng/joint/day)</td>
<td>0.85 ± 0.69</td>
<td>7</td>
<td>2.66 ± 1.21**,††</td>
<td>6</td>
<td>2.14 ± 2.26††</td>
<td>7</td>
</tr>
<tr>
<td>IL-4 (100 ng/joint/day)</td>
<td>1.66 ± 1.21</td>
<td>6</td>
<td>2.57 ± 3.91**,††</td>
<td>7</td>
<td>4.00 ± 2.20**,††</td>
<td>7</td>
</tr>
</tbody>
</table>

*P < 0.001, **P < 0.05, ***P < 0.005 vs sham operation group, †P < 0.05, ††P < 0.01, †††P < 0.001 vs control OA group at the week that correspond, respectively, †††P < 0.005 vs control OA group at 2 weeks.
sections for NT are shown in Fig. 4(A). NT expression occurred in normal cartilage and the population of NT-positive chondrocytes in the sham operation group sections was 17.1 ± 6.7, 13.4 ± 13.3, and 9.3 ± 9.1%, 2, 4, and 6 weeks after surgery, respectively. No difference was seen in the population of NT-positive chondrocytes between each week’s sections. In the control group sections, the population of NT-positive chondrocytes was significantly higher than in the sham operation group and was 33.4 ± 8.1 (P < 0.005), 39.9 ± 5.6 (P < 0.001), and 45.2 ± 8.2% (P < 0.001) 2, 4, and 6 weeks after surgery, respectively. The intra-articular injection of rrIL-4 significantly decreased the population of NT-positive chondrocytes at 4 and 6 weeks. In the treatment group (IL-4 50 ng/day), the population of NT-positive chondrocytes was 32.1 ± 12.8 (n.s.), 28.6 ± 4.8 (P < 0.05), and 28.8 ± 7.6% (P < 0.05), 2, 4, and 6 weeks after surgery, respectively [Fig. 4(B)].

Discussion

The types and magnitude of mechanical stresses are known to considerably alter chondrocyte metabolism. Indeed, application of shear stress to OA chondrocytes increases the release of pro-inflammatory mediators and NO and decreases aggrecan and type II collagen expression, whereas hydrostatic pressure increases matrix molecule expression. We have previously reported that CTS (0.5 Hz and 7% elongation) loaded by a Flexercell Strain Unit suppresses the expression of aggrecan and type II collagen mRNA in cultured rat normal chondrocytes, suggesting that the present set of mechanical stress seemed to be rather catabolic for chondrocyte metabolism in vitro. In the current study, it was demonstrated that CTS (0.5 Hz and 7% elongation) up-regulated iNOS expression and increased NO production by rat normal chondrocytes. In vivo, chondrocytes in the arthritic joint are exposed to the high amount of NO in the synovial fluid. Farrell et al. reported that the nitrite concentration in the synovial fluid from RA patients was 0.91 μM, which was higher than that in the synovial fluid from OA patients (0.354 μM). In vitro, two of the authors of the current study reported excessive mechanical stress (CTS 0.5 Hz and 7% elongation) decreases synthesis of matrix components in chondrocytes (aggrecan and type II collagen) through a NO-regulated pathway, probably by increased production of ~5 μM of NO2/NO3 after the stimulation by CTS. Thus, an increase of 2 μM of NO3 at 48 h after CTS seen in the current study appears to be biologically important. Strikingly, pre-incubation with rrIL-4 for 24 h down-regulated iNOS mRNA expression in a dose-dependent manner and treatment with 10 ng/ml of rrIL-4 suppressed NO production to the baseline level 24 and 48 h after CTS. The molecular mechanism for IL-4 action against mechanical stress-induced NO production is still unclear. It has been demonstrated that high levels of IL-4 (100 ng/ml) exhibit an inhibitory effect on NO release enhanced with pro-inflammatory cytokines, such as IL-1 or TNF-α. Nishikawa et al. demonstrated that IL-4 inhibits an IL-1-evoked increase in intra-cellular Ca2+ ion concentration in chondrocytes, an important IL-1-induced downstream signaling. As high magnitude tensile load of 17 kPa at a frequency of 30 cycles/min (0.5 Hz) for 12 h increases the mRNA levels of IL-1β and TNF-α, it might be reasonable to consider that IL-4 inhibited NO production through the regulation of pro-inflammatory cytokine signaling induced by relatively severe mechanical stress.

In the present study, we also examined the effect of intra-articular injection of IL-4 on in vivo destruction of cartilage and NO production using an instability-induced OA model in rat knee joints. We used the same animal model of OA which was reported by Hayami et al.; the transection of the ACL and the MCL and a partial medial meniscectomy showed a relatively rapid and severe destruction of cartilage. To evaluate the efficacy of IL-4 treatment, it was critical to determine the amount and interval of IL-4 as used in the intra-articular injections. van Lent et al. demonstrated that the local over-expression of IL-4 prevents the activation of pro-MMPs during immune complex-induced arthritis in mice. Cartilage destruction reaches

<table>
<thead>
<tr>
<th>Modified Mankin score</th>
<th>No. of rats</th>
<th>Modified Mankin score</th>
<th>No. of rats</th>
<th>Modified Mankin score</th>
<th>No. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.00 ± 0.00</td>
<td>7</td>
<td>0.00 ± 0.00</td>
<td>7</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>OA control</td>
<td>0.42 ± 0.78</td>
<td>7</td>
<td>2.16 ± 0.83*</td>
<td>7</td>
<td>2.00 ± 1.15*</td>
</tr>
<tr>
<td>IL-4 (10 ng/joint/day)</td>
<td>0.14 ± 0.37</td>
<td>7</td>
<td>1.40 ± 0.54*</td>
<td>5</td>
<td>1.14 ± 0.90*,†</td>
</tr>
<tr>
<td>IL-4 (50 ng/joint/day)</td>
<td>0.42 ± 0.53</td>
<td>7</td>
<td>0.83 ± 0.75**,†</td>
<td>6</td>
<td>0.71 ± 0.95**,†</td>
</tr>
<tr>
<td>IL-4 (100 ng/joint/day)</td>
<td>0.50 ± 0.54</td>
<td>6</td>
<td>0.57 ± 0.78‖</td>
<td>7</td>
<td>1.25 ± 0.70‖</td>
</tr>
</tbody>
</table>

*P < 0.001, **P < 0.005 vs sham operation group, †P < 0.05, ‡P < 0.001 vs control OA group at the week that correspond, respectively.

<table>
<thead>
<tr>
<th>Modified Mankin score</th>
<th>No. of rats</th>
<th>Modified Mankin score</th>
<th>No. of rats</th>
<th>Modified Mankin score</th>
<th>No. of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.14 ± 0.38</td>
<td>7</td>
<td>0.57 ± 1.13</td>
<td>7</td>
<td>0.14 ± 0.38</td>
</tr>
<tr>
<td>OA control</td>
<td>5.14 ± 2.85*</td>
<td>7</td>
<td>9.25 ± 4.89*,‡</td>
<td>7</td>
<td>12.29 ± 4.50*,‡‡</td>
</tr>
<tr>
<td>IL-4 (10 ng/joint/day)</td>
<td>4.00 ± 2.00***</td>
<td>7</td>
<td>5.14 ± 2.48*,‡</td>
<td>5</td>
<td>4.29 ± 2.93***,‡</td>
</tr>
<tr>
<td>IL-4 (50 ng/joint/day)</td>
<td>4.57 ± 2.15***</td>
<td>6</td>
<td>4.50 ± 1.97***,‡</td>
<td>6</td>
<td>5.71 ± 5.09***,‡</td>
</tr>
<tr>
<td>IL-4 (100 ng/joint/day)</td>
<td>4.43 ± 1.62***</td>
<td>6</td>
<td>6.57 ± 5.16*,‡</td>
<td>7</td>
<td>5.56 ± 3.38*,‡</td>
</tr>
</tbody>
</table>

*P < 0.001, **P < 0.005, ***P < 0.005 vs sham operation group, †P < 0.05, ‡P < 0.01, ‡‡P < 0.001 vs control OA group at 2 weeks.
MMP activity in chondrocytes is partly regulated by NO10,11. Enzymolytic activity by MMPs and aggrecanases; it is known that the synthesis of type II collagen and aggrecan by chondrocytes and the subsequent increased proteolytic activity by MMPs and aggrecanases; it is known that down-regulation of NO production by rrIL-4 may contribute to the prevention in MMP-induced aggrecan loss and subsequent loss of aggrecan. These results suggest that down-regulation of NO production by rrIL-4 may contribute to the prevention in MMP-induced aggrecan loss in the cartilage treatment group rats at 4 and 6 weeks. In addition, IL-4 was reported to play a role in the regulation of a disintegrin and metalloproteinase thrombospondin motif (ADAMTS)-4 and -9 in chondrocytes43, although the precise mechanism has not yet been elucidated. Further study is needed to explore whether the chondroprotective effects of IL-4 demonstrated in the current study relate to the regulation of aggreganases by chondrocytes.

On the other hand, the intra-articular injection of IL-4 did not completely control NO production from articular cartilage and the destruction of cartilage in vivo. These results suggested that in this OA model, mechanical stress loaded to the articular cartilage was too strong and IL-4 was not sufficient to neutralize the mechanical stress-induced NO production and cartilage destruction. Another possible cause of the in vivo limited prevention of cartilage destruction by the intra-articular injection of rrIL-4 was a change in the reaction of chondrocytes to IL-4 in OA cartilage. IL-4 shows different responses on normal and OA cartilages. In human normal chondrocytes, IL-4 signals through the type II IL-4 receptor and causes a membrane depolarization response in a dose-dependent manner. In chondrocytes from OA cartilage, on the other hand, IL-4 signals through the type I IL-4 receptor and causes a membrane depolarization response. In addition, normal chondrocytes show an increase in aggrecan mRNA and a decrease in MMP-3 mRNA following mechanical stimulation, and these changes are blocked by IL-4 antibody. In contrast, chondrocytes isolated from OA cartilage show no change in aggrecan or MMP-3 mRNA levels following mechanical stimulation. It was considered that in

maximal values around day 4 and is almost completely blocked by IL-4 at concentrations of 650–750 pg/ml21. Allen et al.23 demonstrated that the intra-peritoneal injection of recombinant murine IL-4 (100 ng/rat) prevents the chronic phase of arthritis induced by streptococcal cell wall fragments by as much as 60–70%. Because of its short half-life, IL-4 should be administrated continuously23,41. In the current study, we administered 10, 50, and 100 ng/joint/day of rrIL-4 into the knee joint of the OA rat model and the results showed an approximately 33–64% reduction of cartilage damage by 6 weeks, as evaluated by the modified Mankin scoring system (Table II). Similar size of reduction (54–65%) of cartilage damage was also confirmed by OARSI scoring system. Owing to its short half-life in vivo, IL-4 treatment has to be given by repeated daily injection or continuous administration, obviously former is not practical for treatment in the clinical setting. The fact that no statistical significance existed in the prevention of cartilage destruction between different doses of rrIL-4 suggested that the intra-articular injection of rrIL-4 has a limited impact on the destruction of cartilage. Recent reports suggest the possible application of adenoviral vector-mediated over-expression of IL-421 or dendritic cells genetically engineered to express IL-4 in the treatment of murine collagen-induced arthritis42 and subsequent cartilage destruction. Further studies using more chronic and mild OA model systems would be required to test the therapeutic efficacy of continuous administration of IL-4 for cartilage destruction.

In the OA model used in the current study, a significant loss of safranin O staining and structural cartilage damage were noted 4 and 6 weeks after surgery (Table III), probably due to the decreased synthesis of type II collagen and aggrecan by chondrocytes and the subsequent increased proteolytic activity by MMPs and aggrecanases; it is known that MMP activity in chondrocytes is partly regulated by NO10,11. NO production was monitored by the number of NT-positive chondrocytes. The number of NT-positive chondrocytes was higher in cartilage from the control group rats than in the sham operation group rats 2 weeks after surgery, whereas only minimal structural changes were noted in the cartilage of both groups. Treatment with IL-4 did not affect the number of NT-positive cells, probably because the increase of NT protein at 2 weeks may have been correlated with relatively severe post-operative inflammation which could not be sufficiently inhibited by the present amount of rrIL-4. At 4 and 6 weeks, it was demonstrated that the intra-articular injection of rrIL-4 significantly suppressed the increase in the number of NT-positive chondrocytes and subsequent loss of aggrecan. These results suggest that down-regulation of NO production by rrIL-4 may contribute to the prevention in MMP-induced aggrecan loss in the cartilage treatment group rats at 4 and 6 weeks. In addition, IL-4 was reported to play a role in the regulation of a disintegrin and metalloproteinase thrombospondin motif (ADAMTS)-4 and -9 in chondrocytes43, although the precise mechanism has not yet been elucidated. Further study is needed to explore whether the chondroprotective effects of IL-4 demonstrated in the current study relate to the regulation of aggreganases by chondrocytes.

Fig. 3. Representative results of immunohistochemistry for aggrecan neoepitope (NITEGE) in femoral condylar cartilage of knee joints of OA control (a–c) and IL-4 treated (d–f) animals at 2 weeks (a and d), 4 weeks (b and e), and 6 weeks (c and f) after surgery. Original magnification: 200×.
OA cartilage the phenotype of chondrocytes changes and the response of the chondrocyte to the anti-inflammatory properties of IL-4 is decreased, and therefore IL-4 only in part ameliorates the destruction of cartilage in the later phase of OA.

To summarize, we examined the effect of rrIL-4 on cartilage destruction using a relatively severe instability-induced OA knee in rats. Our results suggested that the intra-articular injection of rrIL-4 seemed to be effective for the early phase of cartilage destruction partly by prevention of proteoglycan loss and promotion of matrix synthesis through inhibition of NO production. On the other hand, the present protocol of IL-4 administration may not be sufficient to prevent NO production and proteoglycan loss leading to the structural damage of cartilage in later stages of cartilage degeneration in this model of OA.

**Conflict of interest**

The authors have no conflict of interest.
Osteoarthritis and Cartilage Vol. 16, No. 7

Acknowledgment

The authors would like to thank Miss Ann Nakato for secretarial help.

References