Intra-articular injection of collagenase induced experimental osteoarthritis of the lumbar facet joint in rats

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Abstract We aimed to establish an animal model to investigate primary osteoarthritis of the lumbar facet joints after collagenase injection in rats and its effects on chondrocyte apoptosis. We hypothesized that osteoarthritic-like changes would be induced by collagenase injection and that apoptosis of chondrocytes would increase. Collagenase (1, 10, or 50 U) or saline (control) was injected into the lumbar facet joints. The histology and histochemistry of

cartilage, synovium, and subchondral bone were examined at 1, 3, and 6 weeks after surgery. Apoptotic cells induced by 1 U of collagenase were quantified using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay. Degeneration of the cartilage and changes to the synovium and subchondral bone were dependent on both the doses of collagenase and the time after surgery. There were significantly more apoptotic chondrocytes in collagenase-treated joints than in control ($P < 0.001$ at 1 and 3 weeks and $P < 0.05$ at 6 weeks). Thus, lumbar facet joints subjected to collagenase developed osteoarthritic-like changes that could be quantified and compared. This model provides a useful tool for further study on the effects of compounds that have the potential to inhibit enzyme-associated damage to cartilage.

Keywords Facet joint · Zygapophysial joint · Collagenase · Osteoarthritis · Apoptosis

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Introduction

The functional spinal unit consists of two vertebrae, the intervertebral disc and the posterior elements (the facet or zygapophysial joints and ligaments). The facet joint is composed of the inferior articular process of one vertebra and the superior articular process of the adjacent vertebra. Lumbar facet joint syndrome is reported to have a prevalence of 15–40% in people with chronic low-back pain [18, 19]. Facet joint osteoarthritis and disc degeneration play an important role in degeneration of the spine [12]. Lumbar spine fusion is a commonly performed procedure in various pathological conditions of the spine, but some studies have reported the spinal fusion increased stress at adjacent levels, which may accelerate facet joint degeneration [7]. After

artificial disc replacement, some authors have reported that facet joint arthrosis was one of the causes of unsatisfactory results [23]. Facet joints are clinically important sources of chronic low-back pain, even after spinal fusion or artificial intervertebral disc implantation surgery.

The pathological lesions of osteoarthritis (OA) are characterized by clustering of chondrocytes followed by hypocellularity, destruction of the extracellular matrix with exposure of subchondral bone, and subchondral bone sclerosis with osteophyte formation. The extracellular matrix of the articular cartilage is composed of type II collagen and proteoglycans [14]. Cell–matrix interaction can also regulate chondrocyte apoptosis. Some authors have reported that the chondrocytes cannot survive in a collagen II-depleted environment [24].

An OA model of the facet joint is necessary to evaluate cartilage pathology or the effects of therapeutic agents in osteoarthritis. Although facet joint changes are usually secondary to disc degeneration (secondary facet OA) [3, 6], the timing of changes between the facet joint and disc degeneration remain controversial. Some authors have reported that no correlation could be established between facet joint damage and the intervertebral disc degeneration [9]. Swanepoel et al. [20] showed that in 20% of degenerative spines, facet degeneration preceded disc degeneration. Therefore, a model that reproduces the pathological progression of OA quickly from the early to late stages is very desirable. There are few reports on osteoarthritic changes of the facet joint in rats. In this study, our purpose was to examine the cartilaginous lesions and subchondral bone changes in the lumbar facet joint after intra-articular collagenase administration. We hypothesized that OA-like changes would be induced by collagenase injection. Furthermore, we tested if degradation of the cartilage matrix could be linked to chondrocyte apoptosis.

Materials and methods

Experimental groups

Rats (see below) were divided randomly into four groups and subjected to administration of different doses of collagenase and saline into the right side lumbar facet joint (group 1, saline control; group 2, 1 U of collagenase; group 3, 10 U; group 4, 50 U). The left facet joints were used as untreated controls.

Animal experiments

The Institutional Animal Care and Use Committees of the participating institutions approved the study. Three-month-

old Sprague Dawley rats, weighing 250–270 g, were used. Animals were maintained in accordance with the NIH “Guide for the Care and Use of Laboratory Animals”. The collagenase type 2 (from *Clostridium histolyticum*, enzyme activity 321 U/mg, Worthington Biochemical Corporation, Lakewood, NJ, USA) was dissolved in sterile phosphate-buffered saline (pH 7.4) to provide the indicated concentrations (0.2, 2.0, and 10 U/ μ L). All of the surgical procedures were performed under sterile operating conditions with the rats under intraperitoneal anaesthesia (50 mg/kg ketamine hydrochloride and 5 mg/kg xylazine hydrochloride). A 6 cm midline incision was made on the back and the paraspinal muscle was dissected to expose the lumbar facet joints bilaterally. Collagenase or saline was injected slowly into the right lumbar facet joint in a final volume of 5 μ L with a 34 gauge blunt NanoFil needle (WPI, Inc., Sarasota, FL, USA) at a rate of 2 μ L/min controlled by an infusion pump. The injection level of the facet joint was chosen randomly from L3/4 to L5/6. The left side lumbar facet joints were used as untreated normal controls. After injection, the fascial layer of the muscles and skin was sutured. The rats were euthanized with CO₂ and the lumbar spines were then removed at 1, 3, and 6 weeks post-surgery ($n = 8$ for each group at each time point).

Histology and histochemistry

The lumbar spines were fixed in 10% neutral buffered formalin for 48 h, decalcified, and then embedded in paraffin. Five micrometre sections from the facet joints were obtained for haematoxylin and eosin (H&E) and Safranin O staining. The articular cartilage was graded according to the criteria of the Osteoarthritis Research Society International (OARSI) system (score range 0–24, from normal to most severe degeneration) [17]. The synovium was evaluated according to Yoshimi’s histological grading (score range 0–18, from normal to most severe reaction) [25]. Two independent examiners assessed the histological findings obtained from these specimens in a blind manner. Each data point was the mean of scores graded by these two readers.

Apoptosis assay

Apoptotic chondrocytes were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assays. The sections used for TUNEL analyses were from samples taken 1, 3, and 6 weeks after injections of 1 U of collagenase, and from the saline control group. After deparaffinization and rehydration, specimens were then permeabilized by heating in a

microwave oven in 0.1 M citrate buffer (pH 6.0) for 1 min. Reaction, labelling, and detection of all samples were performed using In Situ Cell Death Detection Kits, AP (Roche, Indianapolis, IN, USA) according to the manufacturer's protocols. Fragmented DNA was detected by an alkaline phosphatase-conjugated anti-fluorescein antibody. The color reaction was developed by adding nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Sigma-Aldrich, St Louis, MO, USA) to stain TUNEL-positive cells black. Total cell number was determined by counterstaining nuclei with Safranin O. Cells in apoptosis were quantified using the method proposed by Diaz-Gallego [5]. The apoptotic chondrocyte ratio (%) was measured as the ratio between the total number of TUNEL-positive nuclei and the total number of cells present in six randomly chosen microscope fields (400 $\times$ magnification). Positive nuclei got stained black and negative ones red.

Statistical analysis

All data are presented as the mean $\pm$ standard deviation. One-way ANOVA with Fisher's post hoc tests for multiple comparisons was performed on the normalized data using SPSS (release 10.0, SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Histology of the lumbar facet joint

The histology of the facet joint cartilage of the normal control group and the collagenase-injected group were compared in the same section for each sample (Fig. 1a). The structure of normal articular cartilage of the facet joint is shown in Fig. 1b.

Histology of the articular cartilage

In the saline-injected group, the cartilage had never degenerated at any interval studied. The articular surface was smooth and the matrix was densely stained red with Safranin O (Fig. 2a, b, c). In the group injected with 1 U collagenase, all eight rats showed superficial fibrillation of articular cartilage with chondrocytes clustering in the superficial zone at week 1 (Fig. 2d). Vertical fissures into the deep zone were observed in six of eight rats at week 3 (Fig. 2e). At week 6, localized deformed articular surfaces, focal hypocellularity of chondrocytes, reduction of Safranin O staining in the matrix adjacent to the fissure, and

Fig. 1 **a** An axial section of the facet joint from L5/6 stained with Safranin O. *Left* normal control and *right* 50 U collagenase-injected joint at 1 week. The facet joint is formed by the inferior articular process (IAP) of L5 and the superior articular process (SAP) of L6. IVD intervertebral disc and VB vertebral body. Bar = 1.0 mm. **b** Normal articular cartilage of the facet joint stained with Safranin O. Bar = 100 μ m

fibrocartilage repair were observed in seven rats (Fig. 2f). In the group injected with 10 U collagenase, articular cartilage changes included surface irregularities (all eight rats) and branched fissures extended into the mid zone (seven rats), hypocellularity of chondrocyte in the superficial and mid zones (all eight rats), and reduction of Safranin O staining in the deep zone (six rats) at week 1 (Fig. 2g). By 3 weeks, irregular surfaces with reduced Safranin O staining and clustering of chondrocytes in the superficial zone were observed in all eight rats (Fig. 2h). Denudation of articular surface and fissures extending into the deep zones were found in seven rats at 6 weeks (Fig. 2i). In the group injected with 50 U collagenase, seven rats showed degeneration of facet joints characterized by denudation, with matrix loss extending to the calcified cartilage interface starting at 1 week (Fig. 2j, k). Deformation of the articular surface and interposition of

fibrocartilaginous tissue occurred in all eight rats by 6 weeks (Fig. 2l).

Histology of the synovium

The synovium in the saline-injected group appeared the same as the normal synovium (Fig. 3a–c). In the group injected with 1 U collagenase, all eight rats showed hypertrophy of subsynovial tissue and infiltration of few inflammatory cells. These changes were most severe at week 1 and became weaker with time (Fig. 3d–f). In the group injected with 10 U collagenase, infiltration of inflammatory cells in subsynovial tissue was noted in all eight rats by week 1 (Fig. 3g). All eight rats showed hypertrophy of synovial lining cells and subsynovial granulation tissue formation by 3 weeks after surgery (Fig. 3h, i). In the group injected with 50 U collagenase, subsynovial tissue infiltrated by inflammatory cells was severe at week 1 and eventually replaced with granulation tissue in all eight rats (Fig. 3j–l). The predominant inflammatory cells were polymorphonuclear neutrophils at week 1 (Fig. 3g–i) and lymphocytes at weeks 3 (Fig. 3h–l) and 6 (Fig. 3i–l).

Subchondral bone changes and osteophyte formation

In all collagenase-treated groups, subchondral bone changes had developed immediately beneath the damaged cartilage by 1 week. Proliferation of fibrous tissue including active fibroblasts with prominent nuclei and abundant cytoplasm and new vessels resulted in discontinuity of subchondral bone trabeculae (three rats in the 1 U group, six in the 10 U group, and seven in the 50 U group; Fig. 4a). Multinucleated osteoclasts had proliferated along the junction between damaged cartilage and subchondral bone. The mean numbers of osteoclasts found per high power field (400 $\times$) on tissue sections in the normal control, saline-injected, and collagenase-injected groups were 2.2 ± 0.8 , 2.3 ± 0.5 , and 5.3 ± 1.4 , respectively. Some adjacent trabeculae were lined by osteoblasts. By week 3, subchondral bone destruction with mesenchymal tissue proliferation was observed (four rats in the 1 U group, six in the 10 U group, and all eight rats in the 50 U group; Fig. 4b). New bone formation was present between damaged cartilage and subchondral bone only in small areas (no rats in the 1 U group, two in the 10 U group, and two in the 50 U group). In these focal areas, bone marrow elements had been replaced with loosely arranged spindle

Fig. 2 Photomicrographs of facet joint cartilage stained with Safranin O in the saline-injected controls (a, b, c) and the collagenase-injected groups (d, e, f 1 U collagenase; g, h, i 10 U; j, k, l 50 U) at 1, 3, and 6 weeks after surgery. Bar = 200 μ m

Fig. 3 a, b, c Histology of synovium stained with H&E in the saline-injected control group and the collagenase-injected groups (**d, e, f** 1 U collagenase; **g, h, i** 10 U; **j, k, l** 50 U) at 1, 3, and 6 weeks after surgery. Bar = 100 μ m. The predominant inflammatory cells were polymorphonuclear neutrophils at week 1 (**g-1** arrow) and lymphocytes by weeks 3 (**h-1** arrow) and 6 (**i-1** arrow). Bar = 10 μ m

cells in a fibrous stroma. By 6 weeks, all rats in the 10 and 50 U collagenase-injected groups showed irregularity of the bony architecture of the inferior articular process of the vertebra. Osteophytes were present at the margin of the facet joint or the dorsal aspect of the inferior articular process of the vertebra (Fig. 4c). Thickening of subchondral bone trabeculae and new bone formation were present in focal areas (five rats in the 1 U group, all eight rats in both the 10 and the 50 U groups; Fig. 4d).

OARSI and synovium scores

The OARSI scores are shown in Table 1. The OARSI scores differed significantly between the saline control and the collagenase-injected groups at each time point studied ($P < 0.001$). In the groups injected with 1 and 10 U

collagenase, the scores increased significantly with time ($P < 0.001$). However, the scores were no different between weeks 1 and 3 in the group injected with 50 U collagenase. The synovium scores are shown in Table 2. Scores in the collagenase-injected groups were significantly greater than that in the saline groups at each time point ($P < 0.05$). In the group injected with 1 U collagenase, the synovium score at week 3 was significantly higher than at weeks 1 and 6 ($P < 0.05$). By contrast, in the groups injected with 10 and 50 U of collagenase, the synovium score at week 1 was significantly higher than at weeks 3 and 6 ($P < 0.001$).

Apoptosis of chondrocytes

Apoptotic chondrocytes were observed using the TUNEL assay (Fig. 5a, b, c, d, e). The mean percentages of

Fig. 4 Photomicrographs of osteophyte formation and subchondral bone changes in facet joints stained with H&E. **a** Subchondral bone changes 1 week after administration of 10 U of collagenase included focal fibrous tissue proliferation (*open arrow*), increase of osteoclasts along the junction between damaged cartilage and subchondral bone (*short arrow*), and some adjacent trabeculae lined by osteoblasts (*long arrow*). Bar = 100 μ m. **b** Joint injected with 50 U collagenase by

3 weeks. Subchondral bone destruction with mesenchymal tissue proliferation was noted (*arrow*). Bar = 100 μ m. **c** Joint injected with 10 U collagenase by 6 weeks. The osteophytes were located at the margin of the facet joint (*arrow*) and the dorsal aspect of the inferior articular process of the vertebra (*open arrow*). Bar = 1 mm. **d** Joint injected with 50 U collagenase by 6 weeks. New bone formation was present (*open arrow*). Bar = 100 μ m

apoptotic chondrocyte in the saline-injected control group at weeks 1, 3, and 6 were 1.6 ± 0.4 , 1.5 ± 0.4 , and $1.1 \pm 0.3\%$, respectively. However, the mean percentage in

the group injected with 1 U collagenase at weeks 1, 3, and 6 were significantly increased to 11.5 ± 2.7 , 15.0 ± 2.9 , and $6.7 \pm 1.1\%$, respectively ($P < 0.001$ at 1 and 3 weeks;

Table 1 Results of the OARSI scores

Weeks after operation	Saline	Collagenase (U)		
		1	10	50
1	0.4 ± 0.5	1.9 ± 0.6	4.9 ± 1.6	16.0 ± 2.1
3	0.5 ± 0.5	5.1 ± 1.2^a	11.3 ± 2.5^c	18.4 ± 2.2
6	0.8 ± 0.5	$10.9 \pm 1.6^{a, b}$	$19.1 \pm 1.8^{c, d}$	21.3 ± 3.2^e

Values are mean $\pm$ standard deviation

^a $P < 0.01$, versus the value at week 1 in the group injected with 1 U collagenase

^b $P < 0.01$, versus the value at week 3 in the group injected with 1 U collagenase

^c $P < 0.01$, versus the value at week 1 in the group injected with 10 U collagenase

^d $P < 0.01$, versus the value at week 3 in the group injected with 10 U collagenase

^e $P < 0.01$, versus the value at week 1 in the group injected with 50 U collagenase

Table 2 Results of the synovium scores

Weeks after operation	Saline	Collagenase (U)		
		1	10	50
1	1.5 ± 0.5	4.1 ± 0.8	7.9 ± 1.4	8.0 ± 0.8
3	1.3 ± 0.5	5.5 ± 1.5^a	5.1 ± 1.0^c	5.5 ± 0.5^d
6	1.3 ± 0.5	3.4 ± 0.5^b	5.0 ± 0.8^c	$3.5 \pm 0.5^{d, e}$

Values are mean $\pm$ standard deviation

^a $P < 0.05$, versus the value at week 1 in the group injected with 1 U collagenase

^b $P < 0.05$, versus the value at week 3 in the group injected with 1 U collagenase

^c $P < 0.001$, versus the value at week 1 in the group injected with 10 U collagenase

^d $P < 0.001$, versus the value at week 1 in the group injected with 50 U collagenase

^e $P < 0.001$, versus the value at week 3 in the group injected with 50 U collagenase

Fig. 5 Photomicrographs of TUNEL stain showing **a** the saline-injected control group and the collagenase-injected group (1 U) after **b** 1, **c** 3, and **d** 6 weeks. Bar = 100 μ m. **e** A higher magnification of the rectangular outlines in **c** demonstrates the TUNEL-positive cells (arrow). **f** Quantitative evaluation of chondrocyte apoptosis in both groups. * $P < 0.05$ and ** $P < 0.001$

$P < 0.05$ at 6 weeks). In this group, the apoptotic chondrocyte ratios at weeks 1 and 3 were significantly greater than that measured at week 6 (Fig. 5f).

Discussion

In this study, we demonstrated that intra-articular administration of collagenase induced osteoarthritic changes including degradation of cartilage, synovial inflammation, remodelling of subchondral bone, and osteophyte formation in the facet joints of rats.

Although secondary osteoarthritic changes of the facet joints have been observed by creating disc degeneration in dog or by removal of the annular rim of the disc in sheep [8, 13], advanced degenerative changes of the facet joints cannot be observed in all experimental animals in these

models. We used a microinjection method to create primary OA of the facet joint not secondary to disc degeneration. Accurate insertion of the needle into the facet joint and adequate infusion rate are critical for such a study. The inserted depth of the needle is too shallow if the needle is inserted from the lateral side. On the other hand, we observed that it is easy to access the joint space and control the inserted depth of the needle by insertion through the synovium from the inferior portion of the facet joint.

The collagen type II network is extremely stable. The collagenase used in this study was bacterial collagenase. This enzyme degraded the collagen and proteoglycan in the cartilage matrix. Such damage to the matrix can lead to the production of sufficient degradation products that can cause further degradation, resulting in chondrocyte dedifferentiation, and eventually matrix mineralization and cell death [16]. When intra-articular injection of collagenase

was used in rabbits to induce experimental OA of the knee [11], an inflammatory response in the synovium was also noted. Anti-collagenase antibodies were not found in the serum, so the synovial reaction was not an immunological response. In our study, inflammation of the synovial tissue became weaker with time during the course of this study. This may have been an early response to collagenase. Repeated mechanical stress to the injured articular cartilage can cause secondary synovitis, stimulate chondrocytes and synovial cells, produce various proteases [10], and alter chondrocyte metabolism [15]. This could explain why collagenase injection into the facet joint could not only directly destroy the cartilage but also induce an inflammatory reaction of the synovium and increased cartilage degradation. Subchondral bone changes developed from 1 week after collagenase injection. These changes were consistent with the initiation of bone remodelling. Micro-damage to the subchondral bone is closely related to the progression of cartilage damage [2]. In one human-cadaveric study, osteophyte of the lumbar facet joint was present in up to 30% of studied specimens (mean age 80.1 ± 11.2 years) [22]. In our present study, osteophytes were observed at the margin of the facet joint and the dorsal aspect of the inferior articular process of the vertebra by 6 weeks after collagenase administration. The latter effect may have resulted from the stimulation of periosteal or synovial membranes with appositional bone formation. In our study, injection of collagenase into the facet joint-induced hypocellularity of chondrocytes, fibrillation and loss of cartilage, and formation of osteophytes; all are characters of OA. Different severity of OA could be observed from the different concentrations of collagenase used (dose dependent) and different time courses (time dependent). Injection of 1 U of collagenase induced early OA-like changes and 50 U of collagenase induced severe OA-like changes 1 week after surgery. However, intra-articular administration of 50 U of collagenase caused rapid and extensive cartilage damage. This situation is not similar to the time course of progression of OA. Administration of a low dose of collagenase (<10 U) is thus appropriate in this model.

Collagen–chondrocyte interactions are mediated through β_1 -integrin and these interactions may protect chondrocytes from apoptosis [4]. The TUNEL stain is generally considered an indicator of apoptosis, which has been reported as a marker for osteoarthritis [1]. Apoptosis of chondrocytes is positively associated with the degree of cartilage matrix damage [21]. In this study, chondrocytes in the superficial zone and middle zone of cartilage were lost in extensive areas because of breakdown of cartilage into the deep and calcified zones in the groups injected with 50 U of collagenase by week 1. The same results were observed at week 6 in the group treated with 10 U of collagenase. However,

we wanted to evaluate chondrocyte apoptosis throughout the entire thickness of the articular cartilage. For this reason, the TUNEL assay was only carried out in the group treated with 1 U of collagenase; apoptosis was observed to increase significantly by as early as 1 week.

Conclusion

To our knowledge, this is the first study of articular cartilage in the lumbar facet joint after collagenase injection using a small animal model. It is a rapid and economic method to induce primary OA-like lesions in the lumbar facet joint. This will provide a useful tool for the evaluation of potential therapeutic modalities for the treatment of OA of the facet joint.

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