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Proteoglycan loss and subsequent replenishment in articular cartilage after a mild arthritic insult by IL-1 in mice: Impaired proteoglycan turnover in the recovery phase

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Abstract. The reparative responses of articular cartilage after an arthritic insult have not been studied extensively to this day. In the present study, we injected interleukin-1 (IL-1) into knee joints of mice to provoke a mild and transient arthritic insult, and characterized both the catabolic and the subsequent recovery phase. In the catabolic phase, which lasted 2 days after IL-1 injection, proteoglycan (PG) breakdown was profoundly accelerated and PG synthesis was markedly inhibited. Sulfation and polysaccharide synthesis were not affected, yet the number of chondroitin sulfate chains was decreased. The general chondrocyte protein synthesis was not inhibited by IL-1. IL-1 injected every other day for a total of three injections prolonged this catabolic phase and resulted in frank loss of articular cartilage proteoglycans. In the recovery phase, started 3 days after IL-1, PG synthesis was enhanced (1.7 times the normal) and proteoglycans had normal hydrodynamic properties. Remarkably, PG degradation was significantly decreased (approximately 50% of the normal). Zymographic analysis demonstrated enhanced expression of gelatinolytic activities in the extracts of the articular tissues shortly after IL-1 exposure and decreased levels in the recovery phase. We found that the overshoot of PG synthesis and impaired degradation act together to facilitate full cartilage repair 7 days after the last of the three IL-1 injections.

Key words: Patella – Chondrocyte metabolism – Gelatinase-activities – Zymography – Glycosaminoglycan – Interleukin-1.

Cartilage, a highly specialized coating tissue on bones, forms the articular joint surfaces. The macromolecular structure of this tissue consists of a collagen type II fibre frame embedded in proteoglycans. The proteoglycans are large aggregates (aggrecan) of highly sulfated polysaccharides and they create a large osmotic swelling pressure [1]. Water uptake will tense the collagen network creating a resilient structure which enables smooth joint movements and can restrain compressive loading stress [2]. In contrast to collagen, the proteoglycans are continuously turned over and chondrocytes maintain their extracellular matrix by synthesis of proteoglycans. The chondrocyte metabolic activity is controlled by a range of cytokines and growth factors [3]. Cartilage PG loss can occur after a trauma, immobilization of the joint, or as a consequence of a local inflammation.

In mice, we demonstrated that an intra-articular injection of the inflammatory cytokine, interleukin-1α, induced suppression of PG synthesis and a concomitant accelerated breakdown [4, 5]. Furthermore, we recently elucidated the role of IL-1 in the antigen-induced arthritis (AIA) and collagen-induced arthritis in mice: Suppression of chondrocyte PG synthesis in AIA could be prevented by treatment with neutralizing anti-IL-1 antibodies [6, 7], implicating a key role of IL-1 in this process.

At present, the bulk of research is focused on unraveling the mechanism behind cartilage depletion and on cessation by enzyme inhibitors and cytokine antagonists [8]. Due to low cellularity, the capacity of cartilage to repair major PG loss is limited, but not refractory. The ensuing replenishment of proteoglycans is a neglected area.

In vivo reparative responses of cartilage have been examined after papain digestion of the matrix, immobilization of the joint, and after traumatizing by full-tickness cartilage defects or transection of the anterior cruciate ligament. The last mentioned is also a model of osteoarthritis (OA) in which osteophytes stabilize the joint followed by hypertrophic cartilage repair (fibrocartilage) [9].

In the present study, IL-1 action on the murine joint was examined by detailed analysis of cartilage PG metabolism, and metalloproteinases expression in tissues from the *capsula articularis* and patellae. In addition, the IL-1-induced cartilage insult was utilized as an *in vivo* model system to characterize further the subsequent recovery phase.

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Materials and methods

Animals and induction of an arthritic insult

The experiments were performed on male C57B1/6 mice aged 8–10 weeks. Mice were obtained from our Central Animal Laboratory breeding facility and were free of specific pathogenic viruses. They were fed a standard diet and tapwater *ad libitum*. Mice were injected intra-articularly along the suprapatellar ligament into joint cavity. The right knee joints received IL-1α (10 ng) and the contralateral knee joint received an equivalent volume (6 µl) of saline and served as a within animal control. We demonstrated previously that saline or 3 U (0.1 ng) of IL-1 had no significant effect on chondrocyte metabolism [4]. Murine recombinant IL-1α, cloned in Escherichia coli [10], had less than 1 ppm endotoxin in the Limulus lysate assay. Bioactivity was checked in the one-stage bioassay; a coculture of the IL-1-specific subclone of the murine thymoma cell line EL-4, designated NOB-1, which produces IL-2 and IL-4, together with the IL-2-responsive CTLL2 cells. Half-maximal proliferation was obtained at a concentration of 1 pg/ml of IL-1, and could be blocked by rabbit anti-IL-1 antibodies [6].

Analytical procedures

Assessment of proteoglycan synthesis. Patellae, with a minimal amount of adjoining soft tissue (6 pieces) were incubated for 3 h in 2 ml RPMI 1640 (Dutch modification, Flow Laboratories, Irvine, Scotland) supplemented with gentamycin (50 µg/ml), L-glutamine (2 m*M*), and 40 µCi ^{35}S-sulfate (Du Pont de Nemours, 's-Hertogenbosch, The Netherlands) at 37 °C in a humidified 5% CO_2 atmosphere. Thereafter, patellae were thoroughly washed, fixed in 10% formalin and subsequently decalcified in 5% formic acid. Patellae were punched out of the adjacent tissues, and dissolved in 0.5 ml Lumasolve (Hicol, Oud-Beijerland, The Netherlands) at 60 °C. The ^{35}S-content of each patella was measured by liquid scintillation counting (LSC). More than 95% of the radiolabel was incorporated into the proteoglycans by this method [11].

Assessment of proteoglycan breakdown. Mice were injected with 50–100 µCi ^{35}S-sulfate intraperitoneally. After 24 h and at later timepoints (indicated in the legends), patellae were dissected and processed as described above. Breakdown was either expressed as a ratio of ^{35}S-content of treated over untreated contralateral joints or expressed as a percentage of the first timepoint (24 h) taken, as will be indicated in the corresponding experiments.

Glycosaminoglycan measurements of patellar cartilage. Estimation of sulfated glycosaminoglycan (GAG) content in cartilage was carried out using the colorimetric method of Farndale [12]. After dissection, patellae were fixed in ethyl alcohol (96%) overnight, and decalcified in 5% formic acid for 4 h before use. The cartilage layer was stripped from the patella with a pair of tweezers and subsequently digested with 200 µl of 5 mg/ml papain (Type IV, Sigma, St. Louis, MO) in 0.1 *M* sodium acetate pH 6.5, 10 m*M* L-cysteine and 50 m*M* disodium EDTA per patella, overnight at 60 °C. The GAG content per patella was estimated by the dimethylmethylene blue dye binding at 535 nm using shark chondroitin sulfate (Sigma) as a standard.

Measurements of hyaluronic acid synthesis. Patellae ($n=6$) were incubated for 20 h in 2 ml RPMI tissue-culture medium supplemented with 100 µCi ^{3}H-glucosamine and 30% normal mouse serum to maintain proteoglycan synthesis at the *in vivo* level. Supernatants of a papain matrix digest (see above) were applied to a cellulose acetate strip. Hyaluronic acid and chondroitin sulfate were separated by electrophoresis (60′, 10 mA, 80 V). The spots were visualized by 0.1% alcian blue in 5% acetic acid and 10% ethyl alcohol, cut out and the amount of ^{3}H-label was measured in aqualuma by LSC. As a specificity control, supernatants were dialyzed and vacuum dried and treated with 100 U of hyaluronidase (*Streptomyces*) in 0.1 *M* sodium acetate, 0.15 *M* NaCl pH 4.0. After 20 h incubation at 37 °C, the supernatant was applied to the cellulose strips.

Gel permeation chromatography of proteoglycans. Since bovine proteoglycan monomers are included more on Sephacryl S-1000 (Kav 0.38) than on Sepharose CL-2B (Kav 0.28), fractionation on Sephacryl S-1000 allowed a more clear resolution [13] and a distinct separation of proteoglycan monomers with large or small hydrodynamic volume of murine patellar cartilage [14]. Patellae were cultured for 4 h in RPMI 1640 DM medium containing ^{34}S-sulfate (10 patellae per 40 µCi/ml) followed by thorough rinsing in physiological saline. Proteoglycans were extracted immediately thereafter or the labelling interval was followed by a 48 h chase period in RPMI supplemented with hrIGF-1 (0.5 µg/ml, serum equivalence). Subsequently, the patellae were decalcified in 3.5% Na_2EDTA solution (osmolarity 270 mmol/kg) (Sigma Chemical Company, St. Louis, MO) for 4 h and the cartilage layer was stripped from the underlying bone using forceps. The cartilage layers were ground with an all-glass mortar and pestle followed by proteoglycan extraction at 4 °C for 48 h in 4 *M* guanidine-HCl (GuHCl) in 0.5 *M* sodium acetate buffer (pH 5.6) in the presence of protease inhibitors (0.01 *M* EDTA, 0.1 *M* 6-aminocaproic acid (Sigma), 0.005 *M* benzamidine hydrochloride (Sigma), 5 mg/ml trypsin inhibitor (Sigma), 0.005 *M* iodoacetate (Sigma)). This extraction procedure resulted in the extraction of over 90% of the incorporated radiolabel. Extracted proteoglycans were supplemented with 1 mg of an unlabelled bovine proteoglycan mixture as a carrier and applied to an analytical Sephacryl S-1000 column (100 × 1.6 cm). The column was equilibrated and eluted with 4 *M* GuHCl (dissociative). The column was eluted at a flow of 9 ml/h and fractions of 3 ml were collected. Aliquots of each column fraction were analyzed for glycosaminoglycans (Farndale) and radioactivity (LSC). Slices of articular cartilage obtained from the metacarpal joints of bovine were extracted with 4 *M* GuHCl, and dialyzed against 0.5 *M* GUHCl to reassociate into aggregates. To void volume (V_0) of the column was characterized by these bovine proteoglycan aggregates under associative elution conditions (0.5 *M* GUHCl) (elution position in fraction 29). Bed packing was checked with dextran blue 2000 (2×10^6 MW), and eluted with a Kav of 0.64 (dissociative running conditions). Total bed volume (V_t) was characterized by unbound ^{35}S-sulfate which eluted at fraction 67 (dissociative running conditions). All samples were analyzed on the same column and reproducibility was checked by the comparison of the elution profile of the unlabelled bovine carrier proteoglycans.

Characterization of newly synthesized chondrocyte proteins. Patellae ($n=10$ per group) were dissected with a minimal amount of surrounding tissue and incubated with 2 ml methionine-free RPMI tissue-culture medium containing 100 µCi ^{35}S-methionine plus cold L-methionine to a final concentration of 0.5 mg/L, 5% FCS and 1.5 mg/L tunicamycin, for 4 h at 37 °C in a humidified atmosphere. Patellae were then washed thoroughly and decalcified in a physiological aqueous solution of 3.5% (w/v) Na_2EDTA, glucose (1 g/L), supplemented with minimal essential amino acids (MEM 50 × amino acid solution, Whittaker M.A. Bioproducts, Walkersville, MD), 2 h at 37 °C or 4 h at 4 °C. The patellar cartilage could be removed from the bone using forceps. Chondrocytes were freed from the cartilage by enzymatic digestion of the cartilage matrix with 2000 U/ml collagenase IA (from *Clostridium histolyticum*), 20 U/ml pronase E (Protease type XIV from *Streptomyces griseus*), and 0.5 U/ml chondroitinase ABC (from *Proteus vulgaris*) and 2% BSA in HAM's F12 culture medium. After 45–60 min, the chondrocytes were centrifuged at 250 × *g* for 10 min and resuspended in 0.1% EDTA in saline. Viability was checked by trypan-blue exclusion and the cell number was determined.

Chondrocytes were washed in PBS and spun-down at 300 × *g* for 5 min, and resuspended in 50 µl lysis buffer: 50 m*M* KCl, 10 m*M* NaCl, 1 m*M* EDTA, 50 m*M* potassium phosphate, 10% NP-40, and 0.25 m*M* PMSF. Nuclei were separated in an Eppendorf centrifuge at 1200 × *g*. The supernatant was diluted 1:5 with sample buffer (0.011 g/ml dithiothreitol, 0.1 g/ml SDS in 0.4 *M* Tris-HCl pH 6.8

with 45% glycerol and bromophenol blue) and heated at 100 °C for 2 min.

Gel analysis. Equal amounts of radiolabel were subjected to SDS–polyacrylamide gel electrophoresis (Daiichi 10–20% gradient gels, ISS-enprotech, Massuchusetts, USA) and run for 1 h at 40–60 mA, constant current. Gels were silver-stained according to the manufactures procedures (2-D-silver stain-II Daiichi) and afterwards submerged in an autoradiography enhancer (EN^{3}HANCE, Biotechnology Systems NEN, Massachusetts, USA) and vacuum dried. Gels were exposed to X-ray film for a defined length of time at −70 °C. All films were scanned using a Molecular Dynamics laser scanner and protein bands were quantitated using PDQ-Scan software (Protein Databases, Inc., USA). Protein bands were matched between lanes on the gels of each experiment, and apparent molecular weight assignments were made based on internal standards: Phosporylase β (95.5 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), lactoglobulin (18.4 kDa), and cytochrome C (12.4 kDa).

Isolation of metalloproteases from patellae. Patellae immediately frozen in liquid nitrogen were punched out of their surrounding soft tissue (joint capsule) without defrosting, and both were immediately extracted for 48 h at 4 °C in a 10 m*M* cacodylate buffer (pH 6) containing 0.3 *M* NaCl, 5 m*M* $CaCl_2$, 1 μ*M* $ZnCl_2$, 0.02% (v/v) NaN_3, 0.1% (v/v) Triton X-100, and 0.7 mg/l pepstatin. In some experiments, cartilage layers were cut off from the underlying bone and extracted. Samples were stored at −70 °C, and before use mixed with an equal volume of the sample buffer (10% sodium dodecyl sulfate (SDS), 4% sucrose, 0.02% NaN_3) with bromophenol blue, and subjected to zymography.

Substrate–gel analysis (zymography) of metalloproteinases. Slab gels (84 × 90 × 1.0 mm) containing the appropriate gelatin substrate (2.5 mg/10 ml, Difco Lab., Detroit, MI) were copolymerized in 7.5% polyacrylamide as described [15]. Slab gels were run at 4 °C, at a constant voltage (200 V). After electrophoresis, the slab gels were washed twice for 20 min in 50 m*M* Tris (pH 7.5), 5 m*M* $CaCl_2$, 1 m*M* $ZnCl_2$, 0.02% (w/v) NaN_3, and 2.5% (v/v) Triton X-100 at room temperature to remove the SDS. To detect gelatinase activity, the gels were incubated in 50 m*M* Tris (pH 7.5), 5 m*M* $CaCl_2$, 1 m*M* $ZnCl_2$, 0.02% (w/v) NaN_3, and 1% (v/v) Triton X-100, with or without 1 m*M* APMA, for 18–48 h at 37 °C. Gels were stained in 0.25% (w/v) Coomassie blue G250 in 50% methanol, 7% acetic acid, and destained in 40% methanol, 10% acetic acid. Enzymes were detected as a clear band within the blue background. Zymography allowed us to detect both proenzymes and active enzymes based on their apparent molecular weights and in their original ratios. For this, we activated proenzymes with APMA after the electrophoretic separation and not before.

Statistical analysis. Data are expressed as mean values ± standard deviation (SD) unless stated otherwise. Statistical significance was tested using the Mann–Whitney U test.

Results

Cartilage metabolism after a single IL-1 injection, the catabolic phase

Chondrocyte protein synthesis. Murine recombinant IL-1α (10 ng) was injected intra-articularly into the murine knee joint. Twenty-four hours later, protein synthesis of patellar chondrocytes was measured by the incorporation of ^{35}S-methionine *ex vivo*. The newly synthesized proteins were retained intracellularly by blocking the glycosylation/secretion pathway with tunicamycin [16]. IL-1 did not lead to a significant inhibition (8%) of the overall protein synthesis as compared to chondrocytes in normal cartilage. Detailed analysis of these chondrocyte-derived proteins by PAGE did not reveal significant changes in preexisting proteins, whereas of the newly synthesized proteins, the expression of a 33 kDa protein became markedly increased (Fig. 1).

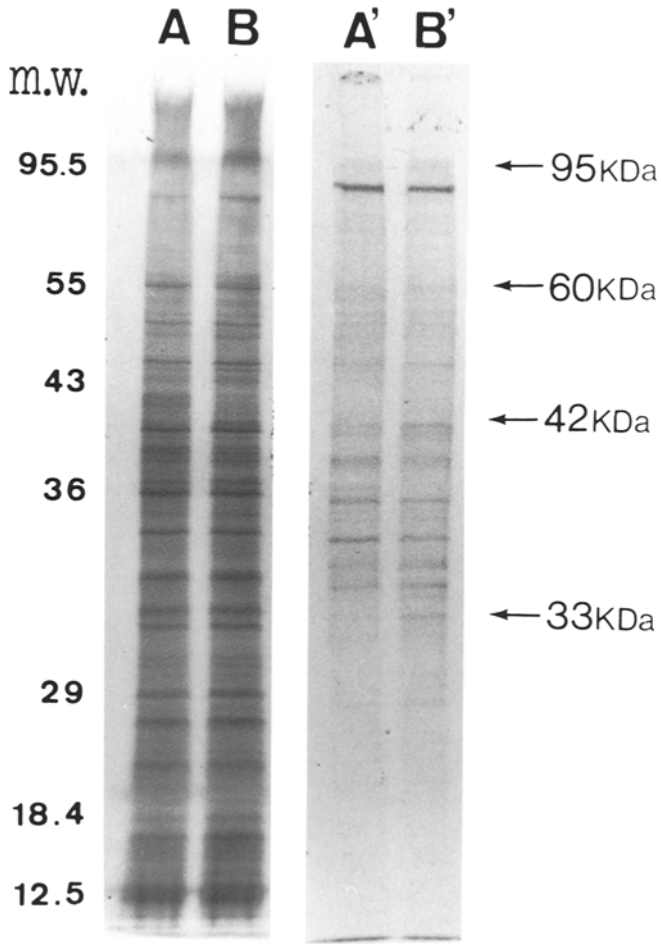

Fig. 1. Electrophoretic separation of chondrocyte intracellular proteins. The preexisting (A, B; silver-stained), and the newly synthesized proteins (A′, B′; autoradiography) were analyzed on a 10–20% polyacrylamide gel. 25.000 CPM (1–1.5 µg protein) was applied to each lane. Lane A and A′, normal chondrocytes; lane B and B′, IL-1α (10 ng)-treated cartilage (24 h). Autoradiographs were laser-scanned and apparent relative increases of matched bands between lane A′ and B′ were indicated by arrows, with their appropriate molecular weights.

Chondrocyte proteoglycan synthesis. Chondrocyte proteoglycan synthesis, as measured by ^{35}S-sulfate incorporation, was markedly suppressed after IL-1 (10 ng) exposure (Table 1). IL-1 did not cause alteration in the enzyme activities of the sulfation pathway as identical results were obtained by ^{3}H-glucosamine incorporation. Electrophoretic separation of the polysaccharide chains of the newly synthesized proteoglycans demonstrated that the amount of ^{3}H-labelled chondroitin sulfate chains was decreased, whereas the amount of ^{3}H-labelled hyaluronic

Table 1. Effect of IL-1 on the synthesis of chondroitin sulfate chains and hyaluronic acid of patellar cartilage separated by cellulose acetate electrophoresis.

	Label[b]	Radioactivity[a]		
		Contralateral	IL-1 injected	IL-1/contralateral ± SD
Patella	^{35}S-sulfate	764 ± 138	400 ± 59*	0.53 ± 0.12
Patella	^{3}H-glucose	2485 ± 511	1603 ± 304**	0.66 ± 0.15
CS	^{3}H-glucose	2067 ± 387	1182 ± 226**	0.60 ± 0.20
HA[c]	^{3}H-glucose	207 ± 161	187 ± 105[ns]	0.90 ± 0.33

[a] Whole patellae extracts, cellulose acetate spots of chondroitin sulfate (CS), and hyaluronic acid (HA) were counted and expressed as CPM ± SD or as a ratio.
[b] Patellae ($n = 6$), 24 h after a single IL-1 injection and their matched contralateral untreated joints, were incubated with ^{35}S-sulfate (20 μCi/ml) for 3 h or ^{3}H-glucosamine (glucose) (100 μCi/ml) for 20 h in serum-enriched medium.
[c] Approximately 85% of ^{3}H-glucosamine-labelled HA was hyaluronidase-degradable material.
p-values compared with the contralateral joints (Mann–Whitney): * $p < 0.001$, ** $p < 0.05$, ns, not significant.

Table 2. Breakdown of newly synthesized and the more processed proteoglycans by IL-1.

Proteoglycans evaluated	Period after IL-1 challenge (days)	Ratio ^{35}S-content[a] IL-1/contralateral (± SD)	Accelerated loss of label by IL-1 (1 − ratio) × 100 (%)
New[b]	1	0.83 ± 0.05	17*
Processed[c]	1	0.82 ± 0.04	18*
New[b]	9	0.76 ± 0.11	24*

[a] Mean ratio ($n = 6$) from ^{35}S-incorporation of IL-1 injected over contralateral kneejoints calculated from paired measurements of six mice each. Average CPM value of contralateral joints was 158 ± 28 cpm ($n = 24$).
[b] IL-1 effect on newly synthesized (IL-1 injected 2 days after ^{35}S-labelling). Two IL-1-challenge periods were studied, 1 and 9 days, over which degradation was quantitated.
[c] IL-1 effect on processed proteoglycans (IL-1 injected 10 days after ^{35}S-labelling).
* $p < 0.05$ compared with the contralateral joints (Mann–Whitney). Accelerated loss by PBS was 1 ± 3%.

acid chains remained the same after IL-1 exposure (Table 1). This excludes an altered glucosamine precursor pool in chondrocytes.

Matrix proteoglycan breakdown. Interleukin-1 injected 2 days after ^{35}S-sulfate labelling of the articular proteoglycans resulted in an accelerated breakdown of 17% within the first 24 h (Table 2). To exclude the possibility that IL-1 only induced degradation of a small subpopulation of newly synthesized proteoglycans, the effect of IL-1 on more processed proteoglycans was studied. For this, the IL-1 injection was delayed for 10 days after ^{35}S-sulfate injection. With 18% enhanced degradation, the more processed/matured proteoglycans were as susceptible to the IL-1 induced degradation. The measured cumulative loss of 24% of ^{35}S-proteoglycans 9 days after a single IL-1 injection demonstrated that the major loss occurred within the first 24 h after IL-1 injection, and that the remaining ^{35}S-labelled proteoglycans had a normal (not impaired) turnover (Table 2).

Cartilage proteoglycan depletion by repeated IL-1 injections, the catabolic phase

Proteoglycan turnover. Intra-articularly injected IL-1 is rapidly cleared from the joint with a half-life of 30 min though proteoglycan synthesis suppression lasted for 2–3 successive days [4]. IL-1 injected every other day for a total of three injections, inhibited proteoglycan synthesis to the same extent (≈40%) as a single IL-1 injection (Table 3). Cartilage proteoglycan breakdown was also extended after repeated IL-1 injections. A cumulative accelerated breakdown of 36% was found, as compared to 13% after a single injection (Table 3).

Glycosaminoglycan content patellae. The prolonged suppression of proteoglycan synthesis and a repeated evocation of degradation may have a profound impact on articular cartilage proteoglycan content. The patellar cartilage matrix was dissected from the bone and digested with papain. Total GAG content of patellar cartilage was determined to be 2.75 ± 0.6 μg ($n = 70$ patellae) in normal cartilage and was significantly reduced (20 ± 5%, mean of 4 experiments) in the 3 × IL-1-treated knee joint (Table 4). The IL-1-induced GAG-depleted cartilage was almost completely replenished within 7 days after the third IL-1 injection (Table 4).

Recovery of proteoglycan-depleted cartilage, the reparative phase

Proteoglycan synthesis. Both a single and three IL-1 injections caused a marked inhibition of proteoglycan

Table 3. IL-1 effect on *in vivo* proteoglycan turnover in patellar cartilage.

Number of injections[a]	PG synthesis: Ratio of IL-1/ saline joint ($\pm$ SD)[b]	Proteoglycan breakdown[c]		
		Remaining ^{35}S-PG's (%)		Accelerated loss by IL-1: (A – B)/A (ratio $\pm$ SD)
		Saline A	IL-1 B	
1 ×	0.55 $\pm$ 0.12* (n = 120)[d]	81 (n = 18)	70 (n = 18)	0.13 $\pm$ 0.07
3 ×	0.58 $\pm$ 0.08* (n = 48)	51 (n = 30)	33 (n = 30)	0.36 $\pm$ 0.08**

[a] IL-1α (10 ng) was injected intraarticularly once (day 1) or three times (on day 1, 3 and 5), and the contralateral joints received saline at the same time.
[b] PG synthesis was measured 24 h after the single (day 2) or after the third injection (day 6).
[c] ^{35}S-sulfate was injected on day 0. On day 1, the ^{35}S-sulfate content of patellae (n = 6) was measured and the content at later timepoints was expressed as a percentage of the amount on day 1. From the absolute incorporation, the accelerated loss by IL-1 was calculated and expressed as a ratio $\pm$ SD.
[d] Number of mice evaluated is stated in parentheses.
Different from saline-injected joint at * $p \leq 0.001$, at ** $p < 0.05$.

Table 4. Diminished glycosaminoglycan content of patellae after IL-1 treatment.

Time in days[a]	Effect on PG synthesis	GAG-content in µg/patella[b]		IL-1 effect (%)
		Contralateral	IL-1 injected	
2	55%	2.47 $\pm$ 0.14	1.91 $\pm$ 0.12	– 23*
7	170%	2.75 $\pm$ 0.17	2.61 $\pm$ 0.30	– 5

[a] Indicated time of days after the last of triple IL-1 injections.
[b] GAG content of the patellae was measured by the Farndale assay, and values represent the mean of four measurements consisting of two patellae each.
* $p < 0.05$, compared with the contralateral joints (Mann–Whitney).
Mean GAG loss calculated from a total of four identical experiments: day 2 (20 $\pm$ 5%); day 7 (7 $\pm$ 4%).

synthesis which was followed within 2–3 days by an overshoot of PG synthesis to supranormal levels (Fig. 2). The recovery of PG synthesis was significantly faster on day 3 of three IL-1 injections than after a single IL-1 injection ($p < 0.01$). The enhancement in PG synthesis after repeated IL-1 injections peaked on days 4–5 after the last injection with a mean stimulation index of 2.31 $\pm$ 0.59 (mean of 7 experiments) compared to the contralateral (noninjected) joints. Contralateral joints were unaffected by the IL-1 regimen when compared to joints of untreated animals there was no stimulation (1.01 $\pm$ 0.16, n = 4 experiments). Two weeks later, normalization of the PG synthesis rate occurred.

Hydrodynamic properties of proteoglycans. The hydrodynamic volume of the newly synthesized proteoglycans was analyzed by gel permeation chromatography immediately after pulse labelling and after a 48 h chase. As shown in Fig. 3A, B, the newly synthesized proteoglycans in normal cartilage and the unlabelled bulk of preexisting matrix proteoglycans monomers had a K_{av} (of $\approx$0.44 and are considerably smaller than proteoglycan monomers from bovine cartilage (K_{av} 0.38) as described by others [17, 18], with shorter glycosaminoglycan chains [9]. Proteoglycans synthesized in the recovery phase had an identical hydrodynamic volume as untreated cartilage

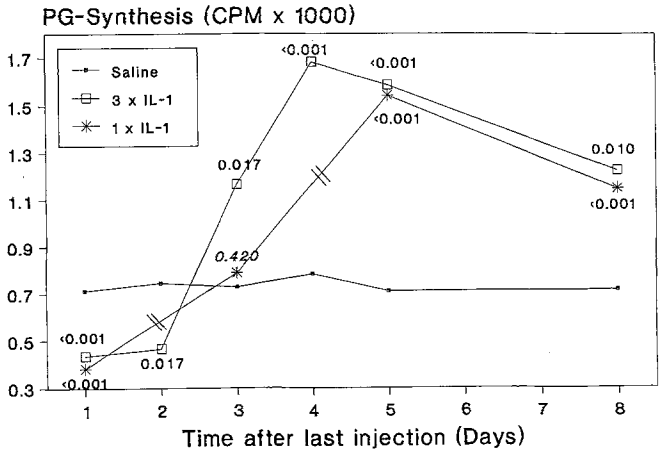

Fig. 2. Time course of patellar proteoglycan synthesis after one or three IL-1α (10 ng) injections on alternate days. Proteoglycan synthesis was measured by ^{35}S-sulfate incorporation (n=6 per group). IL-1-treated cartilage which is different from saline-treated cartilage at p-values is indicated in the figure.

(Fig. 3C). A 48 h *in vitro* chase did not alter the hydrodynamic volume of these ^{35}S-labelled proteoglycans (Fig. 3B–D).

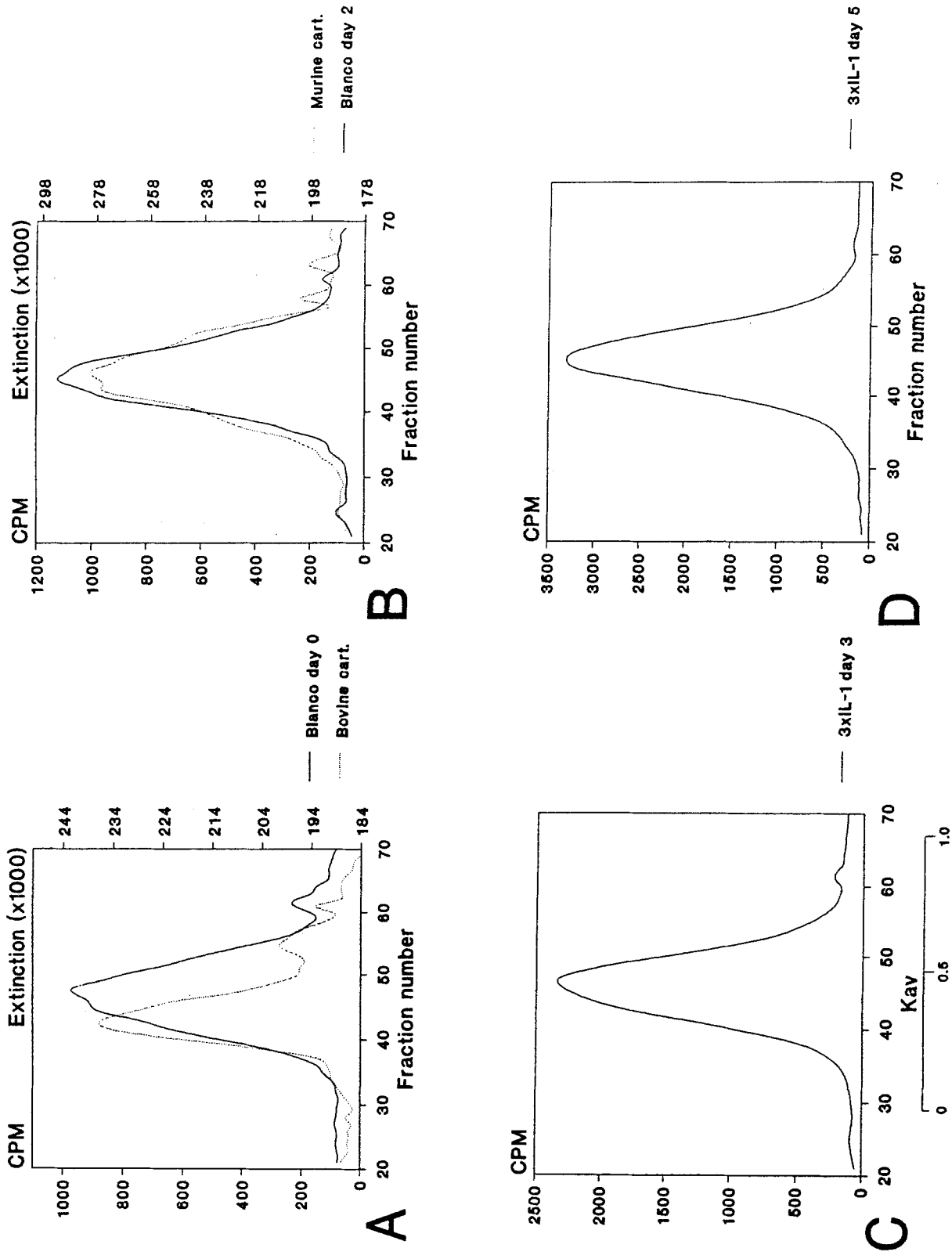

Fig. 3. Hydrodynamic volume of murine proteoglycan monomers. 4M-GuaHCl extracted patellar proteoglycan monomers were analyzed on a Sephacryl S-1000 column. Patellae were pulse labelled with ^{35}S-sulfate and proteoglycans were extracted either directly ($n = 10$ patellae, A and C) or after an *in vitro* chase period of 48 h ($n = 20$ patellae, B and D). Solid lines represents ^{35}S-labelled proteoglycan monomers: Figs. A and B, normal-untreated patellae; Figs. C and D, patellae from day 3 after 3 × IL-1. Elution profiles of unlabelled preexisting proteoglycan monomers are indicated by the dashed lines: Fig. A, from bovine cartilage; Fig. B, from murine patellar cartilage.

Breakdown of newly synthesized proteoglycans. Degradation of proteoglycans synthesized on day 5 after the last IL-1 injection, at the time of supranormal proteoglycan synthesis, was markedly reduced: 23% compared to 60% loss over a 48 h chase period in control patellae (Table 5). This circumstantial evidence of impaired breakdown of the newly synthesized proteoglycans was further substantiated *in vivo*. Mice were injected with ^{35}S-sulfate on day 3 after the last IL-1 injection. Proteoglycan synthesis in the IL-1-treated knee was two times higher than in the normal contralateral knee joint (Table 6). Total loss of ^{35}S-proteoglycans labelled in the recovery phase was 33% after 6 days in the IL-1-treated knee and this was a significant reduction compared to the 53% loss in the contralateral normal cartilage (Table 6).

Expression of gelatinases in both phases of IL-1-treated articular tissues

The accelerated degradation of cartilage proteoglycans coincide with enhanced expression of gelatinolytic activities in both patellae and tissues of the joint capsule (Fig. 4). Extracts were prepared from the joint capsule tissue, the whole patellae and cartilage slices on days 1 and 5 after the last of triple IL-1 injections. These tissue extracts were subjected to gelatin–substrate PAGE and gelatinolytic proteases were demonstrated as clear bands in the dark-stained gel.

In the extracts of whole patellae, consistent expression of four distinct gelatinolytic bands were found: two small clearing bands of approximately 218 and 242 kDa, a moderate band of 59 kDa and a major band of 97 kDa (Fig. 4). After IL-1 exposure, markedly enhanced expression was seen and additional minor bands arose. No clearing bands emerged in gelatin gels incubated in the presence of 1 m*M* EDTA, or using irrelevant substrates, e.g., serum albumin, and fibrinogen, suggesting that the gelatinolytic bands could originate from metalloproteinase activity. When the cartilage was stripped from the patellae, the pattern was identical, although less pronounced due to the minimal amount of cartilage isolated with this procedure.

In the tissues of the joint capsule, 59 kDa appeared to be the major band. Shortly after phosphate-buffered saline (PBS) injection, the 97 kDa band was as prominent, whereas after IL-1 exposure a similar set of bands was noted as compared to the patella. In contrast, this expression returned to the baseline on day 5, whereas moderately enhanced expression was still present in the patella.

Table 5. Reduced breakdown of proteoglycans synthesized in the recovery phase during an *in vitro* chase.

Chase *in vitro*[a]	^{35}S-sulfate content	
	Contralateral (CPM ± SD)	IL-1 injected (CPM ± SD)
0 h	10166 ± 1540	18153 ± 5323
48 h	4070 ± 702* (60%)	14011 ± 1236** (23%)

Interleukin-1 (10 ng) was injected on three alternate days. 5 days later, patellae ($n=5$) were dissected and labelled with 40 µCi ^{35}S-sulfate for 4 h.

[a] After thorough rinsing, patellae were either counted directly or cultured for another 48 h in RPMI supplemented with 0.5 µg/ml hr IGF-1.

Loss of incorporated radiolabel in the 48 h time interval is indicated in parentheses. In a second experiment, ^{35}S-PG loss was 46% for contralateral joints, and 25% for the IL-1-treated joints.

p-values compared with its proper control (0 h chase) (Mann–Whitney): * $p<0.001$, * $p<0.01$.

Table 6. Reduced breakdown of proteoglycans synthesized in the recovery phase *in vivo*.

Days after ^{35}S[a]	Days after IL-1[b]	^{35}S-sulfate content	
		Contralateral (CPM ± SD)	IL-1 injected (CPM ± SD)
1	4	929 ± 253	1713 ± 255
3	6	573 ± 119* (38%)[c]	1424 ± 417 (17%)
7	10	437 ± 106** (53%)	1153 ± 241* (33%)

[a] ^{35}S-sulfate (150 µCi) was injected intraperitoneally one day 3 after the IL-1 treatment, $n=6$ per group.

[b] IL-1α was injected on three alternate days. This caused a 41% loss of ^{35}S-prelabelled proteoglycans ($p=0.125$), and a significant inhibition of PG synthesis of 50% on day 1 after the last injection.

[c] Percentage in parentheses represents the cumulative loss of ^{35}S-labelled proteoglycans as calculated from the ^{35}S-sulfate content on day 4 after IL-1 injection. In a second experiment, ^{35}S-PG loss was 42% for the contralateral joints and 26% for the IL-1-treated joints between day 4 and 10.

p-values compared with the contralateral joints or the IL-1-injected joint on day 4 (Mann–Whitney): * $p<0.05$; ** $p<0.01$.

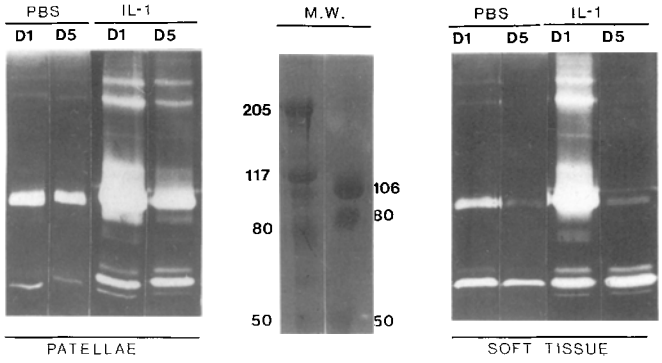

Fig. 4. Zymography of cacodylate extracts of patellae and joint capsules (soft tissue). Interleukin-1α (10 ng) or phosphate-buffered saline (PBS) was injected intra-articularly on three alternate days. Proteoglycan synthesis, 1 day (D 1) and 5 days (D 5) after the last IL-1 injection were 0.53 times and 1.89 times the saline-injected group, respectively. Extracts were normalized based on the dry weights of the tissues. Clear bands on the dark gel indicated gelatinolytic activities. Two middle lanes: prestained molecular weight markers.

Discussion

The objective of this study was to analyze the reparative responses of articular cartilage after an arthritic insult as

induced by intra-articular injections of IL-1 into murine knee joints. IL-1 was used for a number of reasons: first, we recently demonstrated that IL-1 plays a key role in cartilage destruction in several experimental arthritis models in mice [6, 7]; second, IL-1 was rapidly cleared from the joint; third, IL-1 caused only a mild and transient joint inflammation [4]. Detailed analysis of initial cartilage proteoglycan depletion was performed to provide a basis to study the reparative response.

A single IL-1 injection increased the degradation of ^{35}S-PG loss by 1.55-fold within the first 24 h. Three repeated IL-1 injections did not further increase the rate of loss, but rather extended the time period during which the cartilage was in a catabolic state. We measured an additional loss of 36% over normal. Although both newly synthesized and the more processed proteoglycans were as sensitive for IL-1-induced degradation, the measured loss of unlabelled glycosaminoglycans was only 20%. This underestimates cartilage depletion since only the superficial layer, physically unseparable from the calcified cartilage layer, became depleted after IL-1 exposure [4, 5]. IL-1-induced suppression of chondrocyte proteoglycan synthesis also contributes to the cartilage proteoglycan depletion. With the use of radiolabels and electrophoretic separation, we demonstrated that the synthesis of chondroitin sulfate was suppressed, whereas the synthesis of hyaluronic acid chains, aggrecan backbone, was not affected. This further indicates that polysaccharide synthesis in general was not affected. Benton and Tyler [20], using explants of pig articular cartilage, and Morales and Hascall [21], using bovine articular cartilage, showed that IL-1 down-regulated proteoglycan synthesis at the level of core-protein. This suggests that the synthesis of core-protein and chondroitine sulfate chains were linked. Total chondrocyte protein synthesis was not affected by IL-1 and we found no dramatic changes which could be used as markers for IL-1 signalling of chondrocytes. Analysis of the first phase, the catabolic phase, set the parameters to be studied in the recovery phase: proteoglycan synthesis and degradation.

Articular cartilage fully recovers from the IL-1-induced arthritic insult within 7 days after the last IL-1 injection. Recovery was accelerated by an overshoot to enhanced chondrocyte proteoglycan synthesis for a prolonged period of time. In rabbits it was also demonstrated that part of the driving force to recuperate from an IL-1 insult was an overshoot to enhanced PG synthesis [22]. Proteoglycan recovery after an IL-1 insult occurred earlier in mice than in rabbits. This is consistent with the high rate of cartilage proteoglycan turnover (ca. 10–15% per day) in young mice, and the high chondrocyte content per wet weight of cartilage ($1.75 \pm 0.76 \times 10^5$ cells per 70 μg wet-weight per patella) compared with other species [23]. Recovery of the cartilage of rats after an IL-1 insult was not accompanied by supranormal PG synthesis. Moreover, the ability to recover was impaired after a second IL-1 injection [24]. This was probably due to the much higher IL-1 dose used in rats (150 μg) as compared to our 10 ng injection in mice. We found that the recovery was not impaired. It even reached significantly higher synthesis at an earlier time point after the last of the three IL-1 injections as compared to a single injection.

In vivo replenishment of PGs after papain-induced depletion was preceded by an increased activity of chondrocyte oxidative metabolism (2-fold) and an increased activity of uridine diphosphoglucose dehydrogenase (UDPGD) (1.5-fold), an enzyme directly linked to PG synthesis [25]. This may also provide part of the driving force for the supernormal PG synthesis in the reparative phase of IL-1-affected cartilage.

In addition, we found evidence for a second, highly relevant recovery mechanism, which may substantially contribute to the replenishment of articular matrices after an IL-1 insult. Degradation of proteoglycans was significantly decreased during the recovery phase and was reduced to rates of about 50% of those found in normal cartilage. To our knowledge, this novel mechanism has not been described before. In normal murine costal cartilage, two populations of ^{35}S-labelled, newly synthesized proteoglycan monomers are found, a major one (85%) of large hydrodynamic size and a minor one (15%) of small hydrodynamic size with half-lives of 180 and 247 h, respectively [17]. It was interesting to find that the calculated half-lives of ^{35}S-labelled proteoglycans in normal patellae and from patellae in the IL-1 recovery phase were 144 and 216 h, respectively. We found no evidence that the impaired PG breakdown was related to a shift towards predominant synthesis of small hydrodynamic size PG monomers. The newly synthesized PGs (95%) in the recovery phase have a hydrodynamic volume identical to that of proteoglycans either newly synthesized or preexisting in normal, untreated cartilage. A 48 h chase period did not shift the PG monomers synthesized in the recovery phase from large to small hydrodynamic size (processing).

Decreased degradation of proteoglycans could be a result of reduced enzymatic activity. The preferable cleavage site of many enzymes, including stromelysin, collagenases and the gelatinases (72 and 95 kDa) is between the G1 and G2 domain of the core-protein of proteoglycans [26, 27]. It remains to be defined which enzyme is responsible for the cleavage of proteoglycans. Based on sequence data, the IL-1-induced cleavage of core-protein appears not to be at one of the above-mentioned or other known enzymes [28]. For this, we examined extracts from tissues of the joint capsule, patellae and articular cartilage by zymography. Using copolymerized gelatin as a substrate allowed us to detect a broad range of enzymes, e.g., gelatinases, collagenases, cathepsins, plasmin, and stromelysin. On day 1 after the third IL-1 injection, all articular tissues expressed enhanced gelatinolytic activities and the 97 kDa protein became the dominant gelatinase. Lefebre et al. [29] reported that IL-1 enhanced expression of proMMP9 (91 kDa type IV collagenase) and 55 kDa, either proMMP3 and/or proMMP1 (57 kDa interstitial type 1 collagenase) in rabbit articular chondrocytes. In the recovery phase, gelatinolytic levels subsided but were still above baseline. This did not correlate with the markedly impaired degradation of the newly synthesized proteoglycans. Two possibilities exist: first, the gelatinases were not responsible for the PG loss, although capable of doing so; second, gelatinase activity was antagonized by inhibitors, e.g., tissue inhibitor of metalloproteinases (TIMP) [30].

In this study, changes in proteoglycan synthesis and degradation coincided in both phases of the IL-1-induced insult. This does not necessarily have to mean that both processes were linked. Arner and Pratta [31] found in IL-1-challenged bovine nasal cartilage, that both processes were mediated by independent post-receptor mechanisms. We demonstrated that the recovery of IL-1-induced arthritic insult in mice was facilitated by enhanced proteoglycan synthesis and impaired degradation of proteoglycans. Studies are in progress to understand the mechanism of decreased proteoglycan degradation.

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