Proteoglycan degradation in hemarthrosis

Intraarticular, autologous blood injection in rat knees

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We determined the degradation of articular cartilage proteoglycans in a single episode of experimental hemarthrosis in rat knees. The right knee joints of rats were injected once with autologous whole blood. Both knee joints were examined histologically. Biochemical studies of cartilage proteoglycans extracted from the knees were also conducted.

Histological examination revealed an accumulation of mononuclear cells in intraarticular fibrin clots and subsynovial layers 8 hours after the injection of blood. Accordingly, initiation of proteoglycan degradation occurred 8 hours after injection of blood, lasting from 1 day of limited degradation to 3 days; recovery then occurred within 7 days. The proteoglycan degradation could be inhibited by 1 mM phenylmethylsulfonyl fluoride, a general serine proteinase inhibitor, 0.1 M 6-aminohexanoic acid, a specific inhibitor of plasminogen activators, 10 mM EDTA, and 10^{-6} to 10^{-8} M dexamethasone, indicating that the accumulation of mononuclear cells in intraarticular fibrin clots and subsynovial layers may play an important role in cartilage damage.

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Recurrent hemarthroses in animals result in synovial cell proliferation, inflammatory cell infiltration (Roy and Ghardially 1966), and alterations in articular cartilage (Madhok et al. 1988), which lead to destructive changes in joints. In contrast, a single episode of hemarthrosis has been reported to cause only transient synovial proliferation, but apparently not to affect the articular cartilage (Roy and Ghardially 1966). Proteoglycan fragments in joint fluid are increased after injury (Lohmander et al. 1989).

We carried out histological and biochemical examinations on the effect of a single episode of hemarthrosis in the rat knee.

Animals and methods

Male Wistar rats, 6 weeks of age (mean 146 g, SD 13) were used. The rats were anesthetized with ether, followed by intraperitoneal pentobarbital.

For the histological part of the study, 0.1 mL of autologous whole blood was obtained from the tail artery or by cardiac puncture and was immediately injected into the right knee joint, without any additives. In preliminary experiments, injection of Tris-buffered saline into the left knee joint as a normal control occasionally produced substantial intraarticular or subsynovial bleeding. Therefore, the left knee joint served as a normal control, with no injection, to avoid any contamination with blood.

For the biochemical part of the study, both knee joints were injected with autologous blood. For the inhibition study, the concentrations mentioned of inhibitors were injected immediately after the mixture with autologous blood. 44 rats were used for the histological examination. 72 and 56 rats were injected bilaterally for the biochemical and the inhibition studies, respectively.

Histological examination

4 animals were killed with an overdose of ether at 1-, 2-, 4-, and 8-hour intervals and 1, 2, 3, 4, 7, 14, and 28 days after the injection of blood. Both knee joints were fixed in 10% neutral buffered formalin, decalcified with 10% EDTA, and embedded in paraffin. Sagittal sections were cut in 7 µm thickness and stained with hematoxylin and eosin, and Safranin O.

Tissue preparation

Articular cartilage was sliced off the knee joints with a scalpel, taking care not to remove any of the

underlying subchondral bone. The wet weight of this cartilage from 1 rat was determined (mean 18.5 mg, SD 4.4) and proteoglycans were extracted with 10 volumes of 4 M guanidine HCl, 50 mM sodium acetate, pH 5.8, containing 10 mM EDTA, 0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl, and 5 mM N-ethylmaleimide as proteinase inhibitors for 48 hours at 4 °C.

Agarose-polyacrylamide gel electrophoresis

Proteoglycans, extracted with 10 volumes of 4 M guanidine HCl containing proteinase inhibitors, were precipitated with 95% ethanol, centrifuged at 10,000 g for 15 min and dissolved in 0.5% sodium dodecyl sulfate (crude extracts). Equal volumes of the crude extracts from 1 rat were subjected to 0.6% agarose and 1.2% polyacrylamide gel electrophoresis in a horizontal system for submerged-gel electrophoresis as reported previously (Varelas et al. 1991, Suzuki et al. 1992). Gels were stained with 0.2 g/L toluidine blue in 3% (v/v) acetic acid and destained with 3% (v/v) acetic acid. In time course and inhibition studies, the material of each lane was derived from 1 animal. 1 experiment included a series of each lane of the studies.

Isolation of proteoglycan

20 animals at 1 day and 20 animals at 7 days after the injection of blood and 20 control animals were killed. Articular cartilage proteoglycans were isolated from the knees with sequential associative and dissociative CsCl density gradient centrifugation. 4 M guanidine HCl extracts of articular cartilage were dialyzed against 7 volumes of 50 mM sodium acetate, pH 5.8, containing proteinase inhibitors and centrifuged in a Hitachi SRP-70AT rotor at 35,000 r.p.m. for 48 hours at 10 °C (starting density 1.60 g/mL). The bottom two-fifths of the tubes were recovered and referred to as A1. Subsequent dissociative density gradients were established by centrifugation at 35,000 r.p.m. for 48 hours at 10 °C (starting density 1.48 g/mL). The bottom and top one-fourths were recovered and labeled A1D1 and A1 D4, respectively.

Rate zonal sedimentation on Cs_2SO_4 density gradients

Rate zonal centrifugation of preformed Cs_2SO_4 gradients (Kimata et al. 1982) was performed as reported previously (Suzuki at al. 1992). 4.34 mL linear gradients of Cs_2SO_4, 0.15–0.5 M in 0.1 M sodium acetate-0.1 M Tris-HCl buffer, pH 7.2, were prepared in polyethylene tubes on cushions of 0.33 mL of 2 M Cs_2SO_4 in the same solvent. The gradients were preformed 12 hours before centrifugation and were kept at 4 °C. After layering 0.33 mL of samples in 0.1 M sodium acetate-0.1 M Tris-HCl buffer, pH 7.2, on top, the gradients were centrifuged in a Hitachi RPS 50-2 swinging rotor at 25,000 r.p.m. for 6 hours at 11 °C. The tubes were then fractionated into approximately 0.25 mL fractions. Uronic acid contents were determined by carbazole reaction. A1D1 fractions were extensively dialyzed against 0.1 M sodium acetate-0.1 M Tris-HCl buffer, pH 7.2, and concentrated on Centricon 10 membranes (Amicon, Lexinton, MA). A1D1 fractions of 120 μg as uronic acid were analyzed by rate zonal centrifugation. For reassociation with hyaluronic acid, A1D1 monomers of 120 μg as uronic acid were incubated with 1% (w/w) of high molecular weight hyaluronic acid (Seikagaku-Kogyo, Tokyo, Japan) overnight at 4 °C, and then subjected to Cs_2 $S0_4$ rate zonal centrifugation.

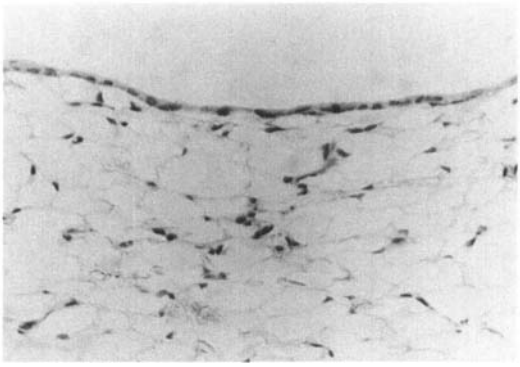
Sodium dodecyl sulfate polyacrylamide gel electrophoresis of A1D4 fractions

A1D4 fractions were extensively dialyzed against 0.2 M sodium acetate, 10 mM EDTA, pH 6.5, and concentrated on Centricon 10 membranes. Protein contents were determined, using a Coomassie protein assay reagent (Pierce, Rockford, IL). The equal protein contents of A1D4 fractions were electrophoresed on 10% sodium dodecyl sulfate slab gels in reduced condition and stained with Coomassie brilliant blue. After gel electrophoresis, some sample proteins were transferred to a nitrocellulose membrane. The membrane was incubated with monoclonal antibody 12/21/1-C-6 (anti-proteoglycan hyaluronic acid-binding region), followed by incubation with an avidin-biotinylated horseradish peroxidase complex (Vectastain ABC; Vector Laboratories, Burlingame, CA). The peroxidase reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride as substrate. The monoclonal antibody 1-C-6 was obtained from the Developmental Studies Hybridoma Bank (NICHD, Bethesda, MD).

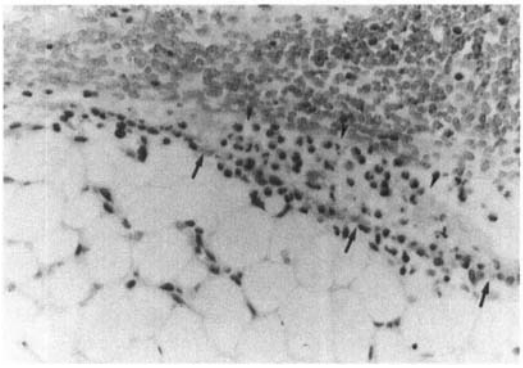
Inhibition study of proteoglycan degradation

To determine the possible factors involved in proteoglycan degradation in experimental hemarthrosis, one-tenth of the total volume of injected blood was added to a proteinase inhibitor dissolved in Tris-buffered saline, pH 7.2, mixed with autologous blood, and immediately injected into the knee joints. After 1 day of injection, articular cartilage was collected to extract proteoglycans, which were subsequently submitted to agarose-polyacrylamide gel electrophoresis. After gel electrophoresis, each lane was plotted

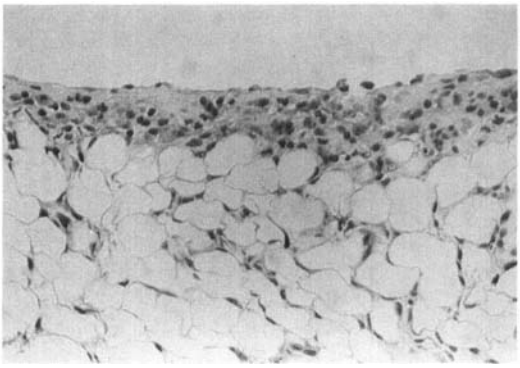
Figure 1. Photomicrographs of synovial tissues from rat knees in experimental hemarthrosis (HE, ×400).



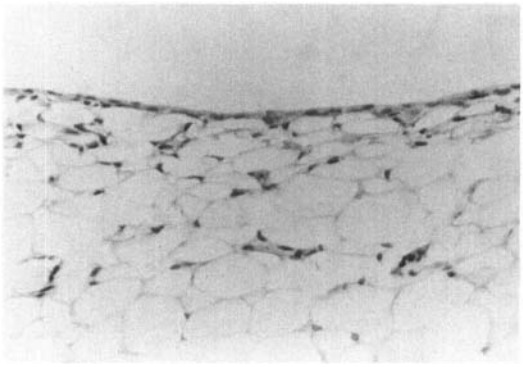
Normal infrapatellar synovial tissues in the non-injected control knee.



The infrapatellar region of the injected knee 8 hours after injection of blood. An intraarticular fibrin clot containing abundant mononuclear cells and polymorphonuclear leukocytes (arrowheads) was adherent to the synovial lining cells (arrows). Note that a number of mononuclear cells are also present in the subsynovial layers. Proliferation of synovial lining cells was not obvious at this stage.



The infrapatellar region of synovial tissues 2 days after the injection of blood. Note the distinct proliferation of the synovial lining cells. Very few mononuclear cells accumulated in the sublining layers.



The infrapatellar synovial tissues 7 days after the injection of blood.

with NIH Image 1.52 and the relative molecular size of the larger proteoglycans to that of the control was expressed as the control of 1. The significance of the results were analyzed by the Student's *t*-test in 4 serial experiments. Final concentrations of inhibitors in injected blood were as follows: 1 mM phenylmethylsulfonyl fluoride, 0.1 M 6-aminohexanoic acid, 5 mM N-ethylmaleimide, 10 mM EDTA, 10^{-6} to 10^{-9} M dexamethasone, and 50 mM Tris-HCl, 0.15 M sodium chloride, pH 7.2, as the negative control.

Results

Histological examination (Figure 1)

8 hours after the injection of blood, marked accumulation of mononuclear cells and polymorphonuclear leukocytes was observed in the subsynovial layers of the infrapatellar region and intraarticular fibrin clots. Proliferation of synovial lining cells was not yet detectable at this stage. Although minimal at 4 hours after injection, mononuclear cell infiltration in subsynovial layers was prominent at 8 hours, persisted for 2 days, and subsided within 4 days. On the other hand, proliferation of synovial lining cells appeared at day 1, then became noticeable on day 2 sections, where a fibrin layer adherent to the synovial lining cells accompanied by abundant mononuclear cells was often indistinguishable from the proliferated lining cells, and subsequently returned to normal appearance within 7 days. Massive coagulations were frequently observed within 8 hours, infrequently at 2 days, then cleared away from the joint cavity within 4 days. These findings were not

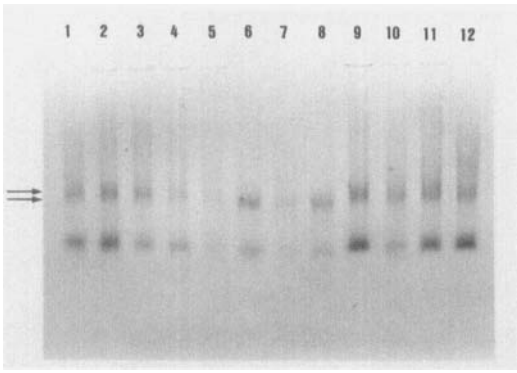


Figure 2. Time course of degradation of articular cartilage proteoglycans on agarose-polyacrylamide gel electrophoresis after a single injection of autologous blood into the articular space. Lane 1, no injection as control; lanes 2, 3, 4, and 5: 1, 2, 4, and 8 hours, respectively, after injection of blood; lanes 6, 7, 8, 9, 10, 11, and 12: 1, 2, 3, 4, 7, 14, and 28 days, respectively, after injection of blood. Control level, upper arrow; day 1, lower arrow.

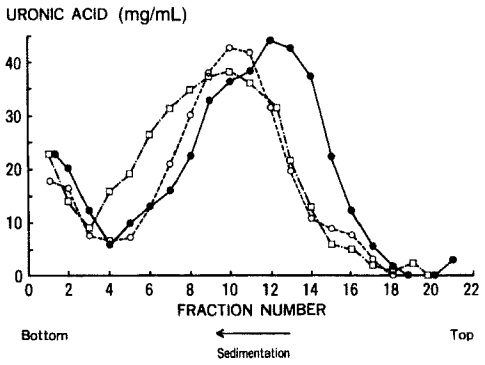


Figure 3. Distribution of uronic acid in Cs_2S0_4 rate zonal gradients of A1D1 fractions. A1D1 fractions of proteoglycans of injected knees after 1 day and 7 days as well as in control knees that were not injected were analyzed by Cs_2S0_4 rate zonal centrifugation. ○ control, ● 1 day, □ 7 days.

observed in the control knees. Depletion of proteoglycans was not detectable on sections stained with Safranin O.

Time course of proteoglycan degradation

Faster migrating bands were observed on agarose-polyacrylamide gel electrophoresis 8 hours after the injection of blood. The limited degradation was attained at day 1 (lower arrow) and persisted for 3 days, then returned to the control level (upper arrow) on the fourth day through the twenty-eighth day (Figure 2). Similar results were obtained consistently in 6 of 7 serial experiments.

Cs_2S0_4 rate zonal centrifugation of proteoglycan A1D1 fractions

The distribution of uronic acid in the gradients of the A1 D1 fraction of the 1-day sample migrated slower than that of the control, yielding a somewhat bimodal profile with an increase in the slower migrating fraction (Figure 3). Uronic acid distribution of the A1 D1 fraction of the 7-day sample returned to the control level, although a faster migrating fraction increased with a somewhat broad distribution. Similar results were obtained in duplicate in 2 experiments.

In the interaction of proteoglycan monomers with hyaluronic acid, 63, 79, and 65 percent of proteoglycans in the control, the 1-day, and the 7-day samples, respectively, were able to aggregate with hyaluronic acid (Figure 4). In contrast, 37, 22, and 35 percent of proteoglycans in the control, the 1-day, and the 7-day samples, respectively, could not aggregate with

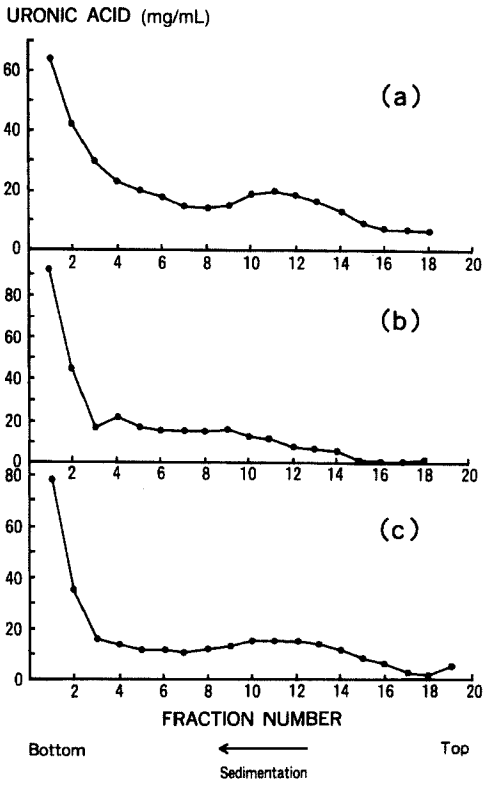


Figure 4. Distribution of uronic acid in the interaction of A1D1 fractions with high molecular weight hyaluronic acid analyzed by Cs_2S0_4 rate zonal centrifugation. (a) A1D1 fraction of control knees + 1 % hyaluronic acid; (b) A1D1 fraction of 1 day sample + 1 % hyaluronic acid; (c) A1D1 fraction of 7 day sample + 1 % hyaluronic acid.

Table 1. Inhibition study, n 4

	Control	Hemarthrosis	Tris-buffered saline	Hemarthrosis withTris-buffered saline	6-amino-hexanoic acid	Phenylmethyl-sulfonyl fluoride	N-ethyl-maleimide	EDTA
Mean	1	0.923 [a]	1.017	0.935[a]	1.04	0.973	0.988	0.99
SD	0	0.034	0.064	0.035	0.04	0.056	0.06	0.38

[a] $P < 0.05$ versus Control, using the Student's t-test.
Relative molecular size of proteoglycans to the control was determined by plotting with NIH Image 1.52 after gel electrophoresis. The values were expressed as the control of 1

Table 2. Inhibition study by dexamethasone, n 4

	Control	Hemarthrosis	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Mean	1	0.916[a]	1.025	0.997	1.026	0.943[b]
SD	0	0.028	0.022	0.05	0.027	0.024

[a] $P < 0.001$ versus Control, using the Student's t-test
[b] $P < 0.05$ versus Control, using the Student's t-test
Relative molecular size of proteoglycans was expressed as the control of 1

hyaluronic acid (Figure 4). Similar results were obtained in 2 experiments.

Inhibition study of proteoglycan degradation

The relative molecular size of proteoglycans was significantly smaller in hemarthrosis and hemathrosis with Tris-buffered saline ($P < 0.05$, using the Student's *t*-test). The proteoglycan degradation was inhibited by 1 mM phenylmethylsulfonyl fluoride, 0.1 M 6-aminohexanoic acid, 5 mM N-ethylmaleimide, and 10 mM EDTA. Injection of Tris-buffered saline without blood failed to yield degradation (Table 1). Furthermore, 10^{-6} to 10^{-8} M dexamethasone, which are physiological concentrations, were also effective in inhibiting the degradation (Table 2).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of isolated A1D4 fractions and subsequent immunoblotting

On sodium dodecyl sulfate polyacrylamide gel electrophoresis of A1D4 fractions and subsequent immunoblotting with monoclonal antibody 1-C-6 directed against the hyaluronic acid-binding region from Swarm rat chondrosarcoma, a 67 kDa band of the 1-day postinjection sample was more distinct than that of the control sample and returned to a comparable level in 7 days, but it was still more intense than that of the control sample (Figure 5). A 42 kDa band of the link protein (Faltz et al. 1979) was not altered in size.

Discussion

Traumatic hemarthrosis is common and may modify the disease process in arthritic disorders. Hemophilic arthropathy is secondary to recurrent bleeding, although the mechanisms of the joint changes are not well understood (Madhok et al. 1988). The pathological changes after repeated episodes of experimental hemarthrosis have been intensively investigated.

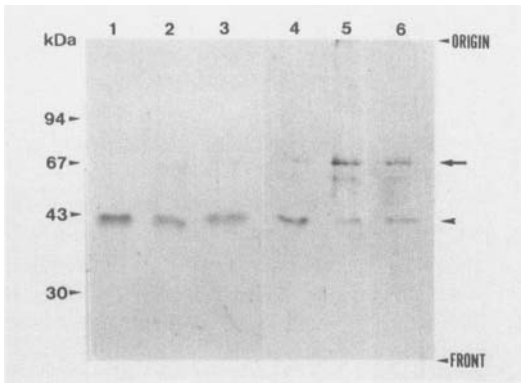


Figure 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of A1D4 fractions and immunoblotting with monoclonal antibody 1-C-6. A1D4 fractions of no injection as control (lanes 1, 4), 1 day after injection of blood (lanes 2, 5) and 7 days after injection of blood (lanes 3, 6). Gels were stained with Coomassie brilliant blue (lanes 1, 2, 3) or immunoblotted (lanes 4, 5, 6). Molecular size markers are indicated on the left. Arrow shows the 67 kDa band of the hyaluronic acid-binding region. Arrowhead indicates the 42 kDa band of the link protein.

They include proliferation of synovial cells, infiltration of inflammatory cells, and cartilage changes after pannus formation (Madhok et al. 1988). In contrast, few pathological changes have previously been described after a single episode of experimental hemarthrosis. It has been reported that a single injection of blood resulted in a mild, transient synovial proliferation (Roy and Ghardially 1966), whereas no essential changes in articular cartilage could be observed. In this regard, we also detected no cartilage changes on the histological examination.

Proteoglycan fragments in joint fluid are increased after injury (Lohmander et al. 1989, Dahlberg et al. 1992). In our study, we demonstrated the degradation of proteoglycans in experimental hemarthrosis using a single injection of autologous whole blood. On agarose-polyacrylamide gel electrophoresis, 2 bands which correspond to large and small proteoglycan monomers (Fellini et al. 1981) were observed, although the density of the bands varied. This variation in the density might be derived from the variation in the wet weight of the extracted cartilage or the extractability of proteoglycans from the cartilage. Nonetheless, faster migrating bands indicate proteoglycan degradation qualitatively (lower arrow in Figure 2). In analytical Cs_2S0_4 rate zonal gradients of A1D1 fractions, distribution of uronic acid of the 7-day sample exhibited a somewhat broader distribution profile with an increase in the faster migrating fraction than that in the control, indicating that the large, newly synthesized proteoglycans (Fellini et al. 1981) following the degradation may be attributed to the increase in the faster migrating fraction. It has previously been shown that enzymatic depletion of the extracellular matrix of cartilage is followed by a marked stimulation of proteoglycan synthesis (Bosmann 1968). These results, therefore, indicate that the limited degradation of proteoglycans was followed by a rapid recovery by means of stimulated proteoglycan synthesis.

The 67 kDa protein band has been previously reported to be the hyaluronic acid-binding region of the proteoglycan core protein, which is often present in small amounts in A1D4 fractions (Faltz et al. 1979, Heinegård and Hascall 1979). It was further confirmed by immunoblotting with monoclonal antibody 1-C-6 directed against the hyaluronic acid-binding region from Swarm rat chondrosarcoma, although non-specific binding to the 42 kDa band of the link protein remained. Therefore, the hyaluronic acid-binding regions of proteoglycan monomer increased in the 1-day sample. In the interaction of the A1D1 fractions with exogenous hyaluronic acid, the non-aggregating proteoglycan monomers decreased in the 1-day sample. These changes showed recovery to comparable levels in 7 days. Since cleavage adjacent to the hyaluronic acid-binding region is one of the preferred sites of degradation (Hughes et al. 1991), the proteolytic degradation of proteoglycan monomer yields the hyaluronic acid-binding region and the glycosaminoglycan-bearing regions. The former can be recovered in the A1 fraction and subsequent A1D4 fraction, whereas most of the latter fails to be recovered in the A1 fraction (Heinegård and Hascall 1979). Therefore, proteolytic degradation of proteoglycans may be responsible for the increased accumulation of the hyaluronic acid-binding regions in the A1D4 fraction of the 1-day sample. In addition, the decrease in the non-aggregating proteoglycan monomers in the 1-day sample may be ascribed to the decreased recovery of non-aggregating proteoglycan monomers in the A1D1 fraction as a result of proteolytic degradation. Indeed, the interaction of D1 fractions, which had been isolated in direct dissociative CsCl density gradients, with hyaluronic acid showed an increase in the non-aggregating proteoglycan monomers in the 1-day sample compared to those monomers in the control (results not shown). These results, therefore, indicate that, in the sequential associative and dissociative density gradients, proteolytically-cleaved proteoglycans unable to aggregate with hyaluronic acid have not been recovered in the A1 fraction, consequently, the A1D1 fraction contains fewer proteoglycan monomers capable of binding to hyaluronic acid than the directly isolated D1 fraction. Despite the recovery from the degradation in 7 days, the hyaluronic acid-binding regions in the A1D4 fraction of the 7-day sample are more abundant than in that of the control, indicating that there are still more abundant hyaluronic acid-binding regions which remain to be bound to hyaluronic acid in 7 days. Therefore, proteoglycan degradation in hemarthrosis may be responsible for the increased proteoglycan fragments in joint fluid after injury (Lohmander et al. 1989).

The initiation of the proteoglycan degradation at 8 hours after the injection of blood coincided with the accumulation of mononuclear cells in sublining layers and intraarticular fibrin clots, where secretion products of stimulated mononuclear cells may be implicated in the degradation of proteoglycans. It should be noted that the degradation of proteoglycans could be inhibited by 1 mM phenylmethylsulfonyl fluoride, a general serine proteinase inhibitor, 0.1 M 6-aminohexanoic acid, a specific inhibitor of plasminogen activators (Werb 1989), 10 mM EDTA, and physiological concentrations of dexamethasone. Plasminogen activators are serine proteinases con-

verting plasminogen into plasmin, which plays an important role in the dissolution of fibrin clots (Saksela 1985) and can degrade cartilage proteoglycan (Mochan and Keler 1984). According to some authors, coagulation products, such as platelet factor-4 (Deuel et al. 1981), thrombin (Bar-Shavit et al. 1983), and fibrinogen-derived peptides (Richardson et al. 1976), are chemotactic for monocytes, whereas plasminogen activator is secreted by stimulated macrophages (Unkeless et al. 1974) and synoviocytes stimulated by mononuclear supernatants (Hamilton and Slywka 1981), and its secretion is inhibited by dexamethasone (Werb 1978, Hamilton et al. 1981). Therefore, it is possible that accumulated macrophages and stimulated synovial lining cells are implicated in the degradation of proteoglycans and serine proteinases, in particular, the plasminogen activator may be responsible for the proteoglycan degradation in experimental hemarthrosis.

Interleukin-1, which is produced by stimulated monocytes and synovial cells themselves, can mediate the degradation of cartilage proteoglycan (Ratcliffe et al. 1986). The secretion of stromelysin and collagenase, neutral metalloproteinases which are synthesized and secreted by chondrocytes, is increased in systems undergoing enhanced turnover, for example during interleukin-1 treatment (Hughes et al. 1991). It is, therefore, conceivable that metalloproteinases such as stromelysin and collagenase may be implicated in the proteoglycan degradation, since 10 mM EDTA is also capable of inhibiting the degradation.

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