Methylprednisolone acetate induced release of cartilage proteoglycans: determination by high performance liquid chromatography

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Abstract
A high performance liquid chromatography (HPLC) procedure suitable for the simultaneous determination of the molecular size and concentration of macromolecular hyaluronate and proteoglycans in synovial fluid has been developed. Irrigation of the equine tarsocural joint with 20 ml physiological saline (PSS) caused a mild inflammation with an increase of proteoglycans in the synovial fluid over the baseline arthrocentesis control sample. Proteoglycan and hyaluronate in the synovial fluid did not interact to form hyaluronate-proteoglycan aggregates, but separated as distinct chromatographic peaks. This suggests that the cartilage derived proteoglycans in synovial fluid in the inflamed joint have been proteolytically cleaved from the non-covalent aggregates containing link protein and hyaluronate. Hyaluronidase digestion completely abolished the hyaluronate peak without affecting the proteoglycans. This seems to indicate that proteoglycan in synovial fluid is unable to interact with hyaluronate in synovial fluid to form cartilage type aggregates.

Proteolytic degradation and the time dependent release into the synovial fluid of such digested proteoglycan also resulted from the intra-articular injection of methylprednisolone acetate into normal tarsocural joints and joints irrigated with PSS. These proteoglycans were insensitive to hyaluronidase but may consist of a protein moiety with attached glycosaminoglycans, as suggested by their sensitivity to proteinase and keratanase/chondroitinase digestion. These observations with cartilage treated with methylprednisolone acetate and mildly stimulated articular cartilage are inconsistent with earlier work on osteoarthritic and rheumatoid articular cartilage and have interesting implications for the pathogenesis and for the therapeutic action of intra-articular corticosteroids. A rapid HPLC procedure applicable to unprocessed small volume samples of synovial fluid gives information simultaneously on hyaluronate and proteoglycan in synovial fluid which is not attainable with immunoradiometric or isotope tracer techniques. It therefore appears to be useful for the analysis of cartilage turnover and destruction in health and disease.

Intra-articular injections of glucocorticoids have been widely used in the treatment of arthritis. Although their beneficial effect of relieving the acute inflammatory response is well documented, reports of their influence on the metabolism of cartilage proteoglycans are still contradictory. Articular cartilage extracellular space is composed of at least four different proteoglycan populations, some of which are able to form aggregates with hyaluronate. In proteoglycan aggregates numerous proteoglycan subunits are non-covalently bound to a central strand of hyaluronate. The interaction is further stabilised by link proteins. The proteoglycan subunit consists of a central core protein to which glycosaminoglycan side chains are covalently bound. The core protein contains different regions for the attachment of chondroitin sulphate and keratan sulphate chains. The N terminal globular domain of the core protein possesses the ability to bind hyaluronate. The unique viscoelastic properties of the articular cartilage are mainly due to the strong water retention capacity of polyanionic proteoglycan aggregates. In addition, collagen fibres tend to oppose the considerable swelling pressure of hydrated proteoglycan aggregates.

The proteoglycan content of cartilage has been determined by various histochemical staining methods. Proteoglycan synthesis has been studied by monitoring the incorporation of $^{35}$SO$_4$ into the cartilage proteoglycans and its breakdown by following the appearance of labelled proteoglycans in the culture medium. Immunological methods have been developed for the determination of proteoglycans in synovial fluid, including several enzyme linked immunosorbent assays (ELISAs). Whether the effect of corticosteroids on the metabolism of proteoglycans in cartilage is anabolic or catabolic seems to be dependent on the experimental model systems or proteoglycan assay method, and is still largely uncertain.

The aim of this work was to develop a reproducible method for the simultaneous determination of large proteoglycans and hyaluronate in synovial fluid samples without any preceding sample preparation. It was intended to apply this method to determine the in vivo effects of intra-articular corticosteroids on cartilage glycosaminoglycan metabolism, using the equine tarsocural joint as a model system.

Materials and methods
EFFECT OF INTRA-ARTICULAR Methylprednisolone Acetate
One tarsocural joint in five horses (Nos. 1, 2, 3, 4 and 7) was injected at day 0 with 20 ml physiological saline (PSS) to induce a slight inflammatory reaction. Twenty four hours after irrigation (day 1), the same joint was injected...
with 100 mg (2·8 ml) methylprednisolone acetate (MPA). Each injection was performed after taking a sample by the aseptic arthrocentesis technique. In addition, samples of synovial fluid were collected 2, 3, 4, 7, and 11 days after irrigation with PSS (table 1).

CONTROLS
In each of these horses, the contralateral joint was irritated at day 0 and, instead of MPA, was injected on day 1 with 2·8 ml PSS as a control. Three horses (Nos. 5, 6, and 8) had one tarsocrural joint subjected to repeated sampling only as an arthrocentesis control. In two of these (Nos. 6 and 8) the contralateral joint was irritated with 20 ml PSS on day 0 and injected with 2·8 ml PSS on day 1 for comparison with similarly treated joints in horses injected with MPA. Three horses (Nos. 9, 10 and 11) were injected in one tarsocrural joint with 100 mg of MPA without a preceding PSS injection to differentiate between the effects of PSS irrigation and injection.

CLINICAL SIGNS
The horses were observed for signs of lameness; exercise was restricted and they were kept at rest in their stalls. Clinically identifiable gross changes in the joints were evaluated at each synovial sampling by visual and manual examination.

COLLECTION OF SYNOVIAL FLUID
Synovial fluid samples were obtained using an aseptic technique before the injections (day 0), on day 1 (24 hours after irrigation) and on days 2, 4, 7, and 11 after injection. Each sample was immediately transferred into a sterile test tube and centrifuged at 1500 g for 10 minutes to pellet the cells. The synovial fluid samples were stored without preservatives at –20°C for HPLC analysis.

The synovial fluid volume was evaluated by forced aspiration into the syringe at each sampling time; 5 ml was taken for further studies and the rest was carefully injected back into the joint. Total protein was determined by the method of Lowry et al6 and the leucocyte count was performed on a 1:20 dilution using a standard haemocytometer (Haemocytometer Counting Chamber, American Optical Corporation).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
The HPLC determination was performed using an LKB 2150 chromatographic pump (with a sample loop of 50 µl) coupled with an LKB 2151 online UV detector.10 12 Hyaluronate and proteoglycan in synovial fluid were detected at a wavelength of 206 nm and the chromatograms were recorded with an LKB 2220 recording integrator. The elution buffer was 50 mM sodium phosphate, pH 6·50, and the constant flow rate was 1·0 ml/min. TSK 5000 PW (30 cm×7·5 mm) and TSK 6000 PW (60 cm×7·5 mm) size exclusion columns were obtained from Toyo Soda.

Synovial fluid samples were diluted to 1:20 to 1:40 with elution buffer, and 50 µl was immediately injected into the HPLC apparatus.

CALIBRATION OF THE COLUMNS
For determination of the concentration of hyaluronate, a calibration graph was obtained using Healon (Pharmacia Diagnostica, S-75182) with the appropriate dilutions. The molecular weight calibration was performed as described by Saari.13 Proteoglycan concentrations in synovial fluid were calculated using Ariefaron, a commercial proteoglycan mixture obtained from Luitpold-Werk.

The detection limits for hyaluronate and proteoglycans in synovial fluid were less than 5 µg/ml.

CHEMICALS
Hyaluronidase from Streptomyces hyalurolyticus (type IV), chondroitinase ABC from Proteus vulgaris and keratanase from Pseudomonas species (EC 3.2.1.103) were purchased from Sigma. MPA (Depo-Medrol 40 mg/ml) was from Upjohn. Sodium chloride, 9 mg/ml, was obtained from Orion Oy. All other chemicals were of the highest commercial purity available.

RESULTS
DETERMINATION OF HYALURONATE AND PROTEOGLYCAN IN SYNOVIAL FLUID WITH HPLC
A single injection of MPA (100 mg) into the equine tarsocrural joint induced a progressive release of proteoglycan into the synovial fluid (fig 1, day 0–11). At the same time the hyaluronate content of the synovial fluid increased, but not to the same degree as the proteoglycan content. The peaks of hyaluronate and proteoglycan were easily separated from

### Table 1: Experimental protocol for 105 synovial fluid samples

<table>
<thead>
<tr>
<th>Day</th>
<th>Horse (leg)</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9, 10, 11</th>
</tr>
</thead>
<tbody>
<tr>
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<td>PSS 20</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<td>S</td>
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</tr>
<tr>
<td>1</td>
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<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<td></td>
<td>2·8</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

Abbreviations: PSS 20=20 ml of physiological saline solution; MPA 100=100 mg of methylprednisolone acetate; PSS 2·8=2·8 ml of physiological saline solution; S=sample, arthrocentesis only.
proteoglycan treatment of the synovial fluid sample (obtained at day 11) resulted in the complete disappearance of the hyaluronate containing peak, whereas the proteoglycan peak (retention time 15-52 minutes) was not affected by hyaluronidase treatment. If digestion was continued with protease or with a combination of chondroitinase ABC and keratanase, the proteoglycan peak was also degraded (lower curve in fig 2A).

The proteoglycan fraction was collected after hyaluronidase treatment from the terminal end of the HPLC capillary tube and further chromatographed on a TSK 5000 PW column. Figure 2B shows the chromatogram obtained for the native, large molecular size proteoglycan (retention time 8-34 minutes) and the degradation products after subjection to chondroitinase ABC and keratanase (retention time 12-14 minutes). The lower curve shows chromatograms of pure chondroitinase ABC and keratanase enzymes; the results indicate that no marked interference with the determination of proteoglycans occurred.

**EFFECT OF ARTHOCENTESIS, PSS IRRIGATION AND MPA ON THE CONCENTRATIONS OF HYALURONATE AND PROTEOGLYCAN IN SYNOVIAL FLUID**

The injection of PSS into the joint induces a slight inflammatory reaction\(^4\) with concomitant leukocyte infiltration and an increase in the protein content in synovial fluid (table 2). In this model there was a slight increase of proteoglycans in the synovial fluid over the baseline concentration and arthrocentesis controls. Figure 3A shows that this returned to the baseline concentration in seven days. The intra-articular injection of 100 mg MPA on day...
1 did not reverse the effect of PSS irrigation, but caused an increased concentration of proteoglycan in the synovial fluid, this increase being at a maximum as late as 11 days after PSS injection. Interestingly, the injection of MPA without preceding irrigation with PSS produced an even higher and immediate appearance of large molecular size proteoglycans in the synovial fluid. The effect of MPA was at a maximum on day 7, after which it began to diminish. Figure 3B shows that in all experiments the effect of MPA on the hyaluronate concentration in synovial fluid was fairly similar to the effect on proteoglycan, although the extent of the response was less (Fig 3B). These results show that MPA induces a degradative action on cartilage aggregates of proteoglycans.

Corticosteroids may cause rapid dehydration of the swollen joint. We therefore calculated the total concentrations of proteoglycan and hyaluronate in synovial fluid by taking into account the volume of synovial fluid.

Figure 4 shows the total concentrations of proteoglycan and hyaluronate in synovial fluid when the volume corrections were made. The PSS and PSS plus MPA induce an initial release of proteoglycans at nearly the same magnitude. When MPA was present, the proteoglycan content of synovial fluid remained at the constant high value throughout the observation period. MPA alone drastically increased the total proteoglycan concentration in synovial fluid, which reached its maximum value in four days, with a slight decrease towards the end of the follow up period.

The corresponding total determinations of hyaluronate in synovial fluid gave very different results. PSS and PSS plus MPA caused a moderate increase in hyaluronate in synovial fluid, but the hyaluronate concentration began to decrease at day 1 (MPA) and day 2 (PSS plus MPA), reaching the original value during the sample collection period. MPA alone caused an increase in the total hyaluronate concentration, which remained constant until day 7, whereafter it fell and reached the original level on day 11. The increase of total hyaluronate in synovial fluid with PSS irrigation seems mainly to be due to the increased volume of synovial fluid (from a mean of 12.0 ml to a maximum volume of 19.5 ml at day 2) and the simultaneous slight increase in hyaluronate concentration (0 mg/ml at day 0 and 0.59 mg/ml at days 2 and 3). At the same time the protein content and leucocyte count increased in synovial fluid samples and decreased in parallel with the total hyaluronate concentration (table 2). Thus, it is reasonable to suggest that the slight inflammatory condition
obtained with the injection of PSS causes first the stimulation of hyaluronate synthesis in synovial fluid, which then causes the retention of water in the joint resulting in an increased volume of synovial fluid. This was then followed by the reduction of hyaluronate synthesis to its basal level with a parallel volume depletion and decrease of total hyaluronate in synovial fluid to near the original value. In the experiments where MPA was injected after PSS the volume reduction occurred faster. Table 2 summarises the results obtained with this experimental method.

Discussion
Subunits of proteoglycan are released from articular cartilage into the synovial fluid from large hyaluronate-proteoglycan aggregates by the action of enzymes which digest the hyaluronate binding globular region from the proteoglycan core proteins. Two distinct metalloproteases, able to degrade proteoglycans,15 have been isolated from human articular cartilage. These matrix metalloproteases are secreted in the extracellular space in thezymogen form. They can be activated by organomercury compounds or by trypsin and their production is stimulated by interleukin 1.16 They are zinc enzymes which require calcium for full activity. Neutral proteoglycan degrading enzymes from human articular cartilage have been identified as stromelysin.17 These cartilage matrix metalloproteinases may be involved in the MPA induced release of proteoglycan into the synovial fluid from normal or mildly irritated cartilage. This conclusion is indirect and is based on the inability of proteoglycan in synovial fluid spontaneously to aggregate with hyaluronate in synovial fluid and is supported by evidence provided by the insensitivity of the proteoglycan HPLC peak to hyaluronidase action.

In all joints treated with MPA the proteoglycan concentration in synovial fluid stayed considerably high during the sample collection period, indicating that the continuous degradation of cartilage matrix proteoglycan aggregates occurs during this time. The effects of MPA on the cartilage may occur over a long time, as methylprednisolone, the main metabolite of MPA, could be detected in synovial fluid even several weeks after a single intra-articular injection of MPA.18

To calculate the total amounts of hyaluronate and proteoglycan in synovial fluid, it was necessary to consider their concentrations with respect to the volume of synovial fluid on different days of sample aspiration. We used the forced aspiration of synovial fluid with simultaneous measurement of the synovial fluid volume. When measured with an isotopic technique, the mean equine tarsocrural joint fluid volume is 40 ml.19 Forced aspiration is not the most reliable method with which to measure synovial fluid volumes, because about 40% of the synovial fluid still remains in the joint cavity.20 However, if care is taken to aspirate at the same time of day, careful estimations of the volume of synovial fluid can be obtained.

Various histochemical staining methods have been used to assess the degenerative changes in the articular cartilage. For example, tissues have been stained using haematoxylin, eosin, toluidine blue and safranine O.21-23 As a result, the formation of ulcers, loss of metachromasia, and cyst formation were observed as signs of proteoglycan degradation.21 The appearance of cartilage derived proteoglycans in the synovial fluid has been studied using several immunological techniques, including the use of antibodies against different molecular structures of proteoglycans. ELISA methods have been developed for the determination of proteoglycan core protein hyaluronate binding region8 and for extracted articular cartilage proteoglycans.8 Monoclonal antibodies have been used to determine keratin sulphate epitopes from synovial fluid.25 Many factors may influence the immunological methods, and these can affect the specificity and hamper the reliable determination of proteoglycans released in synovial fluid. Also, they do not give any information on the molecular size of the determined proteoglycan. We describe here a rapid HPLC procedure for simultaneous assessment of the concentration and molecular size and form of hyaluronate and proteoglycan in synovial fluid. As this assay can be performed on unprocessed samples and less than 5 μl of native synovial fluid is required, it is useful for the study of cartilage metabolism and destruction.

The HPLC chromatogram (fig 1) shows that the hyaluronate and proteoglycan fractions in the synovial fluid do not interact stably, as seen in cartilage, as their chromatographic peaks are eluted separately. This is probably due to the digestion of the hyaluronate binding region of the core protein by MPA induced matrix metalloproteases, which have been shown to be capable of degrading proteoglycan aggregates.

The insensitivity of the proteoglycan peak to hyaluronidase treatment supports this suggestion. The proteoglycan peak may consist of proteoglycan subunits lacking the hyaluronate binding core protein domain. It is suggested that MPA in vivo causes the healthy equine articular cartilage to express proteolytic enzyme activity, which cleaves the hyaluronate binding region from proteoglycan aggregates and subsequently causes the release of proteoglycan subunits into the synovial fluid. This also suggests that normal cartilage turnover may be a protease mediated process.

In various tissue culture experiments corticosteroids have been shown to depress the degradation of cartilage derived from patients with osteoarthritis and rheumatoid arthritis by inhibiting the production of various inflammatory mediators and decreasing the activity of proteolytic enzymes.5 26-29 Corticosteroids have also been shown to decrease the concentration of proteoglycans in synovial fluid in the treatment of inflammatory joint diseases, being indicative for the decreased breakdown of cartilage proteoglycan aggregates.8 30

Using HPLC we were able to see the rapid release of large molecular size proteoglycans into synovial fluid during PSS induced irritation. MPA alone or given intra-articularly at
day 1 after PSS irritation surprisingly increased the cartilage degradation as measured in terms of proteoglycan and hyaluronate concentrations in synovial fluid. We used the tarsocural joint irritated with PSS as an experimental model and there might be species differences in the action of MPA, but this seems unlikely. Considerable ethical reasons do not allow similar experiments to be performed on humans. The discrepancies between cartilage in normal subjects and patients with osteoarthritis or rheumatoid arthritis implies different modes of action of MPA. In patients with osteoarthritis and rheumatoid arthritis, MPA may induce lipomodulin gene expression and suppress the expression of, for example, the interleukin 1 gene in the well described conventional way. This might decrease the intrinsic and extrinsic degradation of cartilage extracellular matrix. In contrast, according to our findings, MPA increases the release of proteoglycan and hyaluronate into synovial fluid from normal or mildly stimulated cartilage. Whether this is due to some hitherto unrecognised alternative signal transduction pathway or is based on physicochemical properties of the MPA suspension is at present unknown.