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Binding of haptoglobin, inter-α-trypsin inhibitor, and α₁ proteinase inhibitor to synovial fluid hyaluronate and the influence of these proteins on its degradation by oxygen derived free radicals

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Summary Synovial fluid from 201 normal and pathological knee joints was subjected to gel filtration by Sepharose CL-2B chromatography to separate hyaluronic acid (HA) from unbound proteins, which were retarded on this column. HA from all normal fluids was excluded from the gel and contained 1% or less bound protein. Synovial fluids taken from joints of patients with rheumatoid arthritis (RA) contained considerably more protein bound to HA. In 46% of RA samples the level of protein was >4%, whereas only one fluid examined from osteoarthritic joints contained this amount. The proteins bound to HA from RA joints were identified by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) and immunodiffusion techniques as the acute phase proteins α₁ proteinase inhibitor, inter-α-trypsin inhibitor, and haptoglobin. The average relative percentages of these proteins bound to HA were 17-6%, 32.6%, and 29.2% respectively. These HA-protein complexes could be generated in vitro by mixing normal (low protein) HA with any one of the three acute phase proteins. The HA-protein complexes formed in vitro with inter-α-trypsin inhibitor or haptoglobin, and those isolated from RA synovial fluids, were more resistant to degradation by oxygen derived free radicals (ODFR) than HA from normal fluids. From these findings we conclude that certain acute phase proteins diffusing into synovial fluid during inflammatory episodes may play an important part in protecting HA from depolymerisation by activated phagocytes.

Key words: acute phase proteins, rheumatoid arthritis.

Hyaluronic acid (HA) is the major macromolecular component of synovial fluid (SF) and accounts almost entirely for its viscoelastic properties. These remarkable properties are dependent on the HA concentration and on its molecular weight.¹ ² Previous studies have shown that both the concentration and molecular size of HA in the SF of inflamed joints are lower than those of normal SF.³ ⁴ It has been suggested that this arises from depolymerisation of HA, the most likely mechanism being via free radicals, particularly those derived from oxygen (ODFR). These high energy species are generated in significant quantities by activated polymorphonuclear leucocytes (PMNL) and macrophages that accumulate within the joints during inflammatory episodes.⁵ ⁶

The HA derived from the SF of rheumatoid joints has also been reported to bind more proteins than HA in SF of normal joints.⁷ The identity and function of these HA bound proteins, however, have not been established.

In the present study we show that the three acute phase proteins, haptoglobin, inter-α-trypsin inhibitor, and α₁ proteinase inhibitor, are bound to HA of SF from RA joints. Furthermore, two of these proteins, haptoglobin and inter-α-trypsin inhibitor, when complexed with HA afforded some protection in vitro against the degradative effects of ODFR on this polysaccharide.
Materials and methods

Sepharose CL-2B, Blue Sepharose CL-6B, Sephadex G-100, and human α1 proteinase inhibitor antisera were supplied by Pharmacia (South Seas) Pty Ltd, Sydney, Australia. Hyaluronidase (from Streptomyces hyalurolyticus (EC 4.2.99.1)), human inter-
α-trypsin inhibitor antisera, and Aquacide II were purchased from Calbiochem-Behring (Australia) Pty Ltd, Kingsgrove, NSW, Australia. Human haptoglobin antisera were obtained from Silenus Labs, Pty Ltd, Dandenong, Vic, Australia. Human haptoglobin, α1 proteinase inhibitor, trypsin type XIII: TPCK treated from bovine pancreas, N-α-carbobenzoxyl-L-arginine-p-nitroanilide HCl, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co, St Louis, MO, USA. Before use the α1 proteinase inhibitor was further purified on Blue Sepharose CL-6B and Sephadex G-100 columns. Inter-α-trypsin inhibitor was isolated and purified from human serum according to the method of Salier et al.8 All other chemicals used were of analytical grade.

Sources of hyaluronic acid (HA)

All the HA preparations used in this investigation were isolated from pathological human synovial fluids that had been aspirated aseptically from the joints of patients attending arthritis clinics at this hospital or the North Shore Medical Centre, St Leonards, NSW. Normal SF were derived from cadaveric joints and collected with six hours of death at the time of necropsy at the Royal North Shore Hospital. The cadaveric SF were only used if the pathology and clinical history showed no evidence of joint diseases. The pathological fluids were classified by one of us (PMB) as gouty, RA, or osteoarthritic using standard clinical/laboratory criteria. The particulate material and cellular content of all fluids were removed by centrifugation at 1500 g for 15 minutes. The supernatants were collected and maintained at 4°C until used. In most instances, however, they were applied to the Sepharose CL-2B column within an hour of aspiration from the joints.

Separation of HA from non-bound synovial proteins

The synovial fluids were chromatographed individually on a precalibrated Sepharose CL-2B column (2×90 cm) maintained at 4°C. The eluting buffer was 10 mM phosphate, 0.15 M NaCl pH 7.2, and 2.5 ml fractions were collected at a flow rate of 10 ml/h. The elution of HA was detected as hexuronate by the m-hydroxydiphenyl method of Blumenkrantz and Asboe-Hansen.9 Protein was determined with bovine serum albumin as standard by Lowry's method modified by Peterson10 or by measuring the absorbance at 280 nm. The hexuronate positive fractions were pooled, concentrated by Aquacide II, and the retentate was reanalysed for hexuronate and protein. The protein bound to HA was calculated on a weight basis by the formula:

\[
\text{% complexed protein} = \frac{\text{protein in HA fraction (μg/ml)×100}}{\text{protein in HA fraction (μg/ml)×HA (μg/ml)}}
\]

Characterisation of proteins bound to HA

The pooled concentrated HA fractions eluting from the void volume of the Sepharose CL-2B column were subjected to Streptomyces hyaluronidase digestion (25 turbity reducing units/mg HA) for three hours at 37°C as described previously11 to remove HA. The identity of the HA bound proteins was established by SDS-PAGE of digests using the method of Weber and Osborn.12 Confirmation of the identity of proteins associated with HA was made in agarose gels against several commercially available antisera by Ouchterlon's method.13 The amount of individual proteins associated with HA was assessed by radial immunodiffusion as described previously.14

Generation of ODFR

Various methods were used to generate ODFR capable of degrading HA. These included ferrous ion autoxidation and PMA stimulation of polymorphonuclear leucocytes.

Ferrous ion autoxidation

A 1.0 ml reaction mixture was used consisting of (a) HA 0.5 mg/ml, (b) ferrous sulphate 30 μmol/l, (c) edetic acid 30 μmol/l (i.e., in a 1:1 ratio with ferrous ion). Under these conditions, and in the presence of atmospheric oxygen, autoxidation of ferrous ions generates hydroxyl radical, the species reported to be effective in depolymerisation of HA.15 16 The reaction was allowed to proceed for 15 minutes. The solution was then immediately fractionated on a Sepharose CL-2B column as described above, and the fractions were monitored for hexuronate by the Blumenkrantz and Asboe-Hansen method.9

PMA stimulated polymorphonuclear leucocytes

PMNL from the blood of healthy volunteers were separated by centrifugation over Ficoll-Hypaque as described by Henson et al.17 The cells were then washed three times in phosphate buffered saline. A trypan blue exclusion viability count was made immediately before each experiment, and only cell
preparations showing greater than 90% exclusion were used.

To a 1.0 ml reaction mixture consisting of PMA (250 μg/l), HA (0.5 mg/ml), edetic acid (60 μmol/l), and FeCl₃ (10 μmol/l) was added 0.5–2×10⁸ PMNL. The reaction mixture was incubated at 37°C for one hour and then the cells sedimented by centrifugation for 10 minutes at 1500 g. The supernatant was then applied directly to a Sepharose CL-2B column, which was eluted and monitored as described above.

IN VITRO BINDING OF PROTEINS TO HA FROM NORMAL SYNOVIAL FLUIDS

Protein free HA (0.5 mg/ml), isolated as the void volume (Vo) fraction from normal SF obtained at the time of necropsy by Sepharose CL-2B chromatography, was incubated for 16 hours at 4°C with an excess amount of each of the three acute phase proteins identified as binding to HA in the RA SF. At the end of the incubation periods the mixtures were immediately applied to a Sepharose CL-2B column and fractionated as described above. All the fractions were monitored for hexuronate, protein, and trypsin inhibitory activity in the case of α₁ proteinase inhibitor and inter-α-trypsin inhibitor. Trypsin inhibitory activity was determined in the following manner with 10 μl of each fraction: aliquots were incubated with 100 ng trypsin and 1 mM N-α-carbobenzoxy-L-arginine-p-nitroanilide HCl in 0.15 M NaCl for 16 hours at 37°C and the absorbance read at 405 nm.¹⁸

Results

FRACTIONATION OF SYNOVIAL FLUIDS BY SEPHAROSE CL-2B CHROMATOGRAPHY

Fractionation of all cell free SF samples by Sepharose CL-2B chromatography separated the high molecular weight HA from most of the proteins (Fig. 1A). In all fluids examined the HA eluted at the Vo of the column, the exclusion limits of this gel not permitting resolution of HA of molecular size >1×10⁶ daltons. With the highly sensitive Peterson modification of the Lowry assay¹⁰ it was possible to show the presence of small amounts of protein in the Vo fractions of all SF examined. Fluids from joints of patients with RA, however, generally contained much larger amounts of protein, which eluted in the Vo fraction with HA (Fig. 1B). Table 1 shows the percentage of protein in Vo fractions from SF from various sources. It was found that 24% of RA fluids contained >7% protein and 46% >4% protein. In contrast, none of the other pathological fluids had HA bound protein levels exceeding 3–4%. As the largest synovial fluid protein, α₂ macroglobulin (mol. wt 780 000 daltons), was well

contained in the Sepharose CL-2B column we assumed that those proteins eluting at Vo could only be bound to HA.

IDENTIFICATION OF PROTEINS BOUND TO HA

The HA of concentrated (Aquacide II) pooled Vo fractions from the Sepharose CL-2B chromatograms of (A) normal and (B) RA synovial fluids eluted (10 ml/h) with 10 mM phosphate, 0.15 M NaCl pH 7-2 at 4°C. Fractions (2.5 ml) were monitored for hexuronate acid at 520 nm (-----) and for protein¹⁰ at 750 nm (-----) as described in ‘Materials and methods’. Note, for the RA synovial fluids, the protein (hatched) firmly associated with the hexuronate acid containing fractions that eluted in the void volume (Vo) of the column.

Table 1  Protein content of Vo fractions from Sepharose CL-2B chromatography of synovial fluids aspirated from pathological and normal joints

<table>
<thead>
<tr>
<th>Joint</th>
<th>Protein content (as percentage of HA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-1</td>
</tr>
<tr>
<td>Normal</td>
<td>15</td>
</tr>
<tr>
<td>Osteoarthritic</td>
<td>24</td>
</tr>
<tr>
<td>Gouty</td>
<td>9</td>
</tr>
<tr>
<td>Rheumatoid</td>
<td>59</td>
</tr>
</tbody>
</table>

Fig. 1  Sepharose CL-2B chromatograms of (A) normal and (B) RA synovial fluids eluted (10 ml/h) with 10 mM phosphate, 0.15 M NaCl pH 7-2 at 4°C. Fractions (2.5 ml) were monitored for hexuronate acid at 520 nm (-----) and for protein¹⁰ at 750 nm (-----) as described in ‘Materials and methods’. Note, for the RA synovial fluids, the protein (hatched) firmly associated with the hexuronate acid containing fractions that eluted in the void volume (Vo) of the column.
graphy of RA SF was completely removed by digestion with proteinase free Streptomyces hyaluronidase. These digests, when subjected to SDS-PAGE using the method of Weber and Osborn, showed four major bands corresponding in mobility to α proteinase inhibitor, inter-α-trypsin inhibitor, and haptoglobin (Fig. 2). The identity of these proteins was confirmed using commercially available monospecific antisera, particularly to those proteins known to be increased in the inflamed joint. With the Ouchterlony technique only antisera...
raised against the acute phase proteins, \( \alpha_1 \) proteinase inhibitor, inter-\( \alpha \)-trypsin inhibitor, and haptoglobin, produced precipitin lines with the HA bound proteins from RA SF (Fig. 3). Antisera to other acute phase proteins, such as C reactive protein, caeruloplasmin, \( \alpha_2 \) macroglobulin, etc, were found to be inactive against the HA bound proteins using this immunological assay.

Relative amounts of the individual acute phase proteins associated with HA, in the total Vo fractions of RA fluids, were calculated from the diameters of the precipitin rings produced against various concentrations of each standard protein antigen in the radial immunodiffusion assay (Fig. 4). The mean (SD) values for haptoglobin, \( \alpha_1 \) anti-trypsin, and inter-\( \alpha \)-trypsin inhibitor were found to be 29.2 (12.7), 17.6 (11.2), and 32.6 (11.3)% respectively. These results are expressed as percentages of the total protein in the Vo fractions.

**IN VITRO COMPLEX FORMATION BETWEEN ACUTE PHASE PROTEINS AND HA ISOLATED FROM NORMAL JOINT SF**

In these experiments HA in the Vo fractions from chromatography of normal SF was incubated separately with each of the three acute phase proteins identified as binding to HA in the RA synovial fluids. The presence of bound proteins in the Vo fractions was determined by UV absorption at 280 nm and by the trypsin inhibitory assay for \( \alpha_1 \) proteinase inhibitor and inter-\( \alpha \)-trypsin inhibitor. Binding of these three acute phase proteins to protein ‘free’ HA was demonstrable both by the appearance of protein positive material eluting at Vo and by the inhibitory activity against trypsin (Fig. 5). It was noted, however, that the trypsin inhibitory activity of \( \alpha_1 \) proteinase inhibitor when bound to HA was significantly less than when unbound using protein as a marker of inhibitor concentration (Fig. 5). The levels of the three acute phase proteins associated with HA when prepared by this in vitro procedure were comparable with those determined for HA of fluids derived from RA joints.

**EFFECTS OF ODFR ON HA-PROTEIN COMPLEXES**

Exposure of high molecular weight HA with low protein content (\(<1\)% to an ODFR flux generated by the Fe\(^{++}\) autoxidation system\(^{15,16}\) or PMA stimulated PMNL\(^{19}\) caused depolymerisation of HA, as shown by the inclusion of hexuronate positive material on the Sepharose CL-2B gel. Identical experiments conducted with equivalent amounts of HA derived from RA fluids in which the Vo fraction protein content was \(>7\)% of total were less degraded (Fig. 6).

In an attempt to identify which of the three acute phase proteins bound to HA in RA fluids was the most effective in protecting HA against ODFR degradation the respective in vitro prepared HA-protein complexes were exposed to an Fe\(^{++}\) autoxidation free radical flux. The results of these experiments are shown in Fig. 7, where it is evident that under the vigorous conditions used \( \alpha_1 \) proteinase inhibitor was ineffective at preventing degradation of HA, whereas haptoglobin and inter-\( \alpha \)-trypsin inhibitor showed some protection.

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**Fig. 4 Immunoprecipitation rings of (A) \( \alpha_1 \) proteinase inhibitor, (B) inter-\( \alpha \)-trypsin inhibitor, and (C) haptoglobin from four digested HA-protein complex samples (1,2,3,4) with specific antibodies on agarose plates obtained after 48 hours diffusion and stained with Coomassie Blue R. The diameters of the standards for \( \alpha_1 \) proteinase inhibitor (0.62–0.04 mg/ml), inter-\( \alpha \)-trypsin inhibitor (0.85–0.05 mg/ml), and haptoglobin (0.44–0.03 mg/ml) were compared with the diameters of the diffusion rings for the four unknowns (1–4).**
Discussion

Of the 201 HA preparations examined in the present study, those derived from joints of individuals with acute or chronic RA consistently showed the highest levels of bound protein. Furthermore, SDS-PAGE and immunodiffusion techniques clearly showed that the major proteins associated with HA from the inflamed joints were haptoglobin, inter-α-trypsin inhibitor, and α1 proteinase inhibitor. Like other acute phase proteins, these proteins arise as a physiological response to tissue injury or infection. All are markedly increased in serum and synovial fluid of patients with RA but are less abundant in the early phases than C reactive protein or serum amyloid A protein. The serum level of C reactive protein attained in RA is similar to that in rheumatic fever, where the protein concentration is high early in the acute phase of an infection but decreases before the inflammation subsides. C reactive protein can combine with the capsular polysaccharides of streptococcal cell walls, causing capsular swelling, thereby facilitating phagocytosis. We were surprised, therefore, to find that this protein did not form a strong complex with HA, which is structurally related to the bacterial membrane polysaccharides. The formation of stable complexes of HA with the acute phase proteins, α1 proteinase inhibitor, inter-α-trypsin inhibitor, and haptoglobin, appears, therefore, to be unique, probably arising from stereospecific charge-charge and hydrophilic interactions. In connection with this it is worth

Fig. 5 The in vitro binding between normal HA and acute phase proteins. (A) HA alone; (B) HA with haptoglobin; (C) HA with α1 proteinase inhibitor; (D) HA with inter-α-trypsin inhibitor. The incubated (4°C) mixtures of HA and the proteins were fractionated on Sepharose CL-2B column eluting with the same buffer as in Fig. 1. Fractions (0-6 ml) were monitored for hexuronic acid at 520 nm (—), protein at 280 nm (—•—•—), and trypsin inhibition at 405 nm (— --) as described. The bound protein was eluted with HA at the void volume, while the unbound material was eluted at the bed volume.
noting that once bound to HA, inter-α-trypsin inhibitor conserved its antiproteinase activity but that of α₁ proteinase inhibitor was substantially reduced, at least as determined by the assay conditions used here (Figs 5 and 7). Thus it is tempting to speculate that the conformation of α₁ proteinase was altered on interaction with HA. Alternatively, binding to HA may have masked the active site of the inhibitor.

Although it was possible to form complexes in vitro with non-degraded HA isolated from normal SF and the three acute phase proteins described above, we have not excluded the possibility that such complexes might be formed in vivo with HA modified structurally by its interaction with free radicals. Despite this uncertainty it appears that complex formation in vivo arises as a consequence of the high levels of the acute phase proteins present in SFs of RA joints, the concentration of these proteins being low in normal SFs.²⁰ ²¹ The identity of the smaller amounts of protein associated with osteoarthritic and gouty fluids was not established in the present study, but it is likely that they include one or all of the acute phase proteins found associated with HA in RA fluids. These proteins may have entered the osteoarthritic joint during previous inflammatory episodes.

Sandson, Hamerman, and Schwick showed more than 20 years ago that HA could form complexes with inter-α-trypsin inhibitor.²⁵ It was noted by this group that free inter-α-trypsin inhibitor was undetectable in normal SF but was bound to HA in SF from inflamed joints.²⁶ Brackertz, Hagmann, and Kueppers used immunoelectrophoresis to demonstrate the binding of α₁ proteinase inhibitor and a larger complex, possibly of this inhibitor and a proteinase, to HA of inflammatory SF.²¹ Our finding that HA also binds to haptoglobin appears therefore to be novel.

A consistent feature of the chromatographic profiles of HA from the inflamed SF investigated in our study was the exclusion of most of the material from the Sepharose CL-2B column (see Fig. 1B). This indicated that most HA in SF of inflamed joints had a molecular weight greater than 1×10⁶ daltons. A recent report by Dahl et al., who used high porosity gels, showed that the molecular weight of HA declines, on average, from 7-0×10⁶ in normal SF to about 4-8×10⁶ in SF from patients with RA.⁴ This study also indicated that at least 30% of the HA present in the RA SF was of high molecular weight. This group suggested that although depolymerisation of HA occurs within the inflamed joint, the dilution of SF by the plasma dialysate was largely responsible for the frequently observed decline in viscosity. This conclusion may have to be modified in the light of present studies as the high molecular weight proteins that bind to HA in inflamed joints could also alter the hydrodynamic size and rheological properties of the HA preparations examined by gel permeation

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Fig. 7 Separose CL-2B chromatograms of HA-protein complexes prepared in vitro with (A) HA alone, (B) HA with haptoglobin, (C) HA with α₁ proteinase inhibitor, (D) HA with inter-α-trypsin inhibitor and then exposed to 50 μM ferrousedetic acid at 25°C for 15 minutes. Fractions were monitored for hexuronic acid (---), protein at 280 nm (--•--•--), and trypsin inhibitor (--) as previously described.
chromatography. As the high porosity gels employed by Dahl et al are, as yet, unavailable commercially, our gel filtration experiments were limited to the exclusion properties provided by the Sepharose CL-2B agarose gels. As discussed above, these gels are incapable of resolving HA molecular species with apparent Mr in excess of 1.0×10⁶.

It has been suggested that depolymerisation of HA could result from attack by oxy radicals produced by phagocytic cells or by enzymatic processes.⁵ ²⁷ Although hyaluronidase, β-glucuronidase, and β-N-acetylglucosaminidase of lysosomal origin have been shown to be present in synovial fluid of RA joints,₂⁸ they are only capable of degrading HA in an acidic environment. This requirement probably excludes their effectiveness outside the cell. The more likely mechanism, therefore, is via ODFR, which have been shown by several groups to cause rapid depolymerisation of HA in vitro.₆ ¹₉ ₂₉-₃₁

Studies with ionising radiation have indicated that the hydroxyl free radicals can attack hyaluronate at either the glycosidic bond or at the C5 hydrogen of the pyranose ring.₃₂ If such susceptible groups on the polysaccharide chain were screened by bound protein, then degradation by ODFR might be abrogated, or at least modified. Our results indicate that haptoglobin and inter-α-trypsin inhibitor could qualify for such a role as HA complexes formed in vitro with these proteins (Fig. 7) were less degraded by an ODFR flux (produced by Fe⁺⁺ autoxidation) than protein free HA. On the other hand, α₁ proteinase inhibitor was not effective in this regard, which was a surprising result in view of the known affinity of the methionyl sulphur present in this protein for activated oxygen.₃₃ As the trypsin inhibitory activity of α₁ proteinase inhibitor when bound to HA was substantially reduced, however, it is likely that the conformation of this protein was quite different from that present in the unbound form, and the methionyl sulphur may be unavailable for oxidation. It is also possible that the small amount (relative to the other proteins) of α₁ proteinase inhibitor bound to HA was insufficient to ‘neutralise’ the intensity of the level of free radical flux used in our experiments.

When combined with haemoglobin, haptoglobin has been shown to possess peroxidase activity.₃⁴ If such complexes were formed in SF they could contribute to the protection of HA from hydrogen peroxide, which is known to be generated by activated PMNL from hydroxyl free radicals.₃ This latter mechanism of protection has been suggested for synovial proteins which were not bound to HA. Motohashi and Mori studied the degradation of HA by ascorbic acid using high performance gel permeation chromatography.₃⁵ Ascorbate was oxidised to hydrogen peroxide by molecular oxygen in the presence of trace amounts of metal ions such as Cu⁺⁺ or Fe⁺⁺. With physiological concentrations of ascorbate, HA was degraded in vitro to less than a third of its original hydrodynamic size. Caeruloplasmin and apotransferrin inhibited this degradation, but holotransferrin and albumin were less effective. It was concluded that the mechanism of inhibition of HA depolymerisation of caeruloplasmin was probably due to its ferroxidase activity, i.e., its ability to oxidase Fe⁺⁺ to Fe⁺⁺⁺. When the availability of Fe⁺⁺⁺ was reduced the oxidation of ascorbate to ascobic acid was suppressed. Apotransferrin can also bind Fe⁺⁺⁺ and could likewise influence the role of Fe⁺⁺⁺ in the oxidation processes.

From the present studies we concluded that certain acute phase proteins diffusing into the joints as a consequence of synovial inflammation may, in addition to some resident proteins, serve a useful role in protecting HA from degradation by ODFR and hydrogen peroxide. Furthermore, as these electron rich species are also known to degrade basement membranes and components of the cartilage matrix⁶ ³⁷ and suppress macromolecular biosynthesis by cartilage and synovial cells,³⁸-⁴¹ their attenuation by acute phase proteins could represent an important mechanism for the preservation of tissue integrity during inflammatory cell invasion.

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