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 Sephacryl 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. If 46% of RA samples the level of protein was >4%, whereas only one fluid examined from osteoarthritis 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 29.7% 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.

Polymeric HA (HA) is the major macromolecular component of synovial fluid (SF) and accounts for 95% of its viscoelastic properties. These remarkable properties are dependent on the HA concentration and on its molecular weight. 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. It has been suggested that this arises from the HA and the most likely mechanism being free radicals, particularly those derived from oxygen (ODFR). These high energy species are generated in significant quantities by activated polymorphonuclear leukocytes (PMNL) and macrophages that mediate within the joints during inflammatory episodes.

The HA derived from SF of rheumatoid joints has also been reported to bind more proteins than HA in SF of normal joints. The identity and function of these HA bound proteins, however, have not been elucidated. 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-protease inhibitor (aPI) antiserum were supplied by Pharmacia (South Seas) Pty Ltd, Sydney, Australia. Hyaluronic acid (from Streptococcus sanguis) type XIV; TPCK treated bovine pancreas, N-α-carboxybenzoyl-L-arginine-α-naphthamide (CAAN), and porcine 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co, St Louis, MO, USA. Before use the α1-protease 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.1 All other chemicals used were of anatryptic 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 disease. The pathological fluids were classified by one of us (PMB) as gouty, RA, or osteoarthritic using standard clinical/laboratory criteria. The pathological and cellular content of all fluids was removed by centrifugation at 1500 r.p.m 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 precalculated Sepharose CL-2B column (2 x 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 haemorrhage by the a-butyryl-hydrolase method of Blumenkrantz and Asboe-Hansen.7 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 haemorrhagic positive fractions were pooled, concentrated by Amicon Diaflo, and the retentate was reanalysed for hexuronic and protein. The protein bound to HA was calculated on a weight basis by the formula.

\[
\text{protein (g)} = \frac{\text{absorbance at 280 nm}}{\text{molar extinction coefficient of protein}}
\]

Characterisation of Proteins Bound to HA

The pooled concentrated HA fractions eluting from the void volume of the Sepharose CL-2B columns were subjected to Streptomyces hyaluronidase digestion (25 turbidity reducing units/ml) 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 Ouchterlony's method.4 The amount of individual proteins associated with HA was assessed by radial immunodiffusion as described previously.14

Generation of ODHR

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

Ferrous ion autooxidation

A 1-0 ml reaction mixture was used consisting of (a) HA 0.5 mg/ml, (b) ferrous sulphate 30 μM, (c) edetic acid 30 μM (i.e., in a 1:1 ratio with ferrous ion). Under these conditions, and in the presence of atmospheric oxygen, autooxidation of ferrous ions generates hydroxyl radical, the species reported to be effective in depolymerisation of HA.2,3,15 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 haemorrhage by the Blumenkrantz and Asboe-Hansen method.6

PMA-stimulated polymorphonuclear leucocytes

FMNL from the blood of healthy volunteers were separated by centrifugation over Ficoll-Hypaque as described by Hutton et al.16 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
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separations showing greater than 90% ejection were used.

To a 1.0 ml reaction mixture consisting of PMA
250 µg), HA (5-50 mg/ml), sericidase (300 units/ml)
and FCS (100 µmM) was added 0-5-2 x 10^9 PMN.
The reaction mixture was inactivated at 37°C for one
hour and then the cells sedimented by centrifugation
for 10 minutes at 1500 g. The supernatant was then
typed directly to a Sepharose CL-2B column,
which was eluted and monitored as described above.

NITRIC PEROXIDE PROTEINS TO HA FROM
NORMAL SYNOVIAL FLUIDS

Hypo-tic HA (0.4 mg/ml), isolated as the void
volume (Vo) fraction from normal SF obtained at
the time of synovectomy by Sepharose CL-2B chromato-
graphy, was incubated for 16 hours at 4°C with an
志 amount of each of the three ace phase
proteins identified as binding to HA in the RA SF.
At the end of the incubation, the proteins were immediately applied to a Sepharose CL-2B column
and fractionated as described above. All the
fractions were monitored for hemase, protein,
and trypsin inhibitory activity in the case of α1-
proteinase inhibitor and α2-antitrypsin inhibitor.
Trypsin inhibitory activity was determined in the
following manner with 10 µl of each fraction:
digoxigenate were incubated with 100 ng trypsin
and 1 mM N-A-carboxybenzenesulfonyl-p-nitroanilide.
H2 in 0-15 M NaCl for 16 hours at 37°C and the
absorbance read at 405 nm.

Results

ACTION ON SYNOVIAL FLUIDS BY
SEPHAROSE CL-2B CHROMATOGRAPHY

Fractionation of all cell-free SF samples by Sepharose
CL-2B chromatography separated the high
志 amount of each of the three active phase
proteins identified as binding to HA in the RA SF.
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Fig. 1. Sepharose CL-2B chromatogram of (A) normal and (B) RA synovial fluids eluted (10 ml) with 10 mM
phosphate, 0.15 M NaCl, 7-2 g/l at 4°C. Fractions (7.5 ml)
were monitored for hemase activity at 520 nm (1-1.2)
and for protein at 750 nm (2-2.2) as described in Materials
and Methods. Note, for the RA synovial fluids, the protein
(bound) firmly associated with the heparosan acid
containing fractions that eluted the void volume (Vo)
of the column.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein content in percentage of RA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>b-1 1-2 2-3 3-4 4-5 5-7</td>
</tr>
<tr>
<td>Normal</td>
<td>15 0 0 0 0 0 0</td>
</tr>
<tr>
<td>Osteoarthritic</td>
<td>24 2 2 1 0 0 0</td>
</tr>
<tr>
<td>Casualty</td>
<td>9 1 0 0 0 0 0</td>
</tr>
<tr>
<td>Rheumatoid</td>
<td>59 6 7 8 3 2 5</td>
</tr>
</tbody>
</table>

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

IDENTIFICATION OF PROTEINS BOUND
TO HA

The RA of concentrated (Aquase II) pooled Vo
fractions from the Sepharose CL-2B chromatog-

4

B
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vo fraction protein content was >75% of total was 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 irrespective in vitro prepared HA-protein complexes were exposed to an Fe²⁺ autodigestion free radioligand. The results of these experiments are shown in Fig. 7, where it is evident that under the rigorous conditions used, protease inhibitor was ineffective in preventing degradation of HA, whereas hyaluronidase and inter-acrylase inhibitor showed some protection.

In vitro complex formation between acute-phase proteins and HA isolated from normal joint SF

In these experiments HA in the vo fraction from chondrocytes 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 fraction was determined by UV-absorption at 280 nm and by the trypan blue exclusion assay for protease inhibitor and inter-acrylase inhibitor. Binding of three acute phase proteins to protect HA was demonstrated by the appearance of protein-positive material eluting at vo and by the inhibitory activity against trypan blue. It was noted, however, that the trypan blue inhibitory activity of vo protease inhibitor when bound to HA was significantly less than when bound using protein A in a manner of inhibition of conformation (Fig. 5). The levels of the three acute phase proteins associated with HA when prepared by the 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 protease content (vo) to an ODFR flux generated by the Fe²⁺ autodigestion system or PMA-stimulated PMA was shown to form a complex of HA, as shown by the exclusion of high molecular weight material in the Sephadex CL-2B gel. Identical experiments conducted with equivalent amounts of HA derived from RA fluids in which the
Discussion

Of the 201 HA preparations examined in this 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 protein associated with HA from the inflamed joints were haptoglobin, interα-trypsin inhibitor, and α1-proteinase inhibitor. Like other acute phase proteins, these proteins arise in 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 of serum amyloid A protein. The serum level of C reactive protein attains high level 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 complex of HA with the acute phase proteins, α1-proteinase inhibitor, interα-trypsin inhibitor, and haptoglobin, appears, therefore, to be unique, probably arising from steric repulsion charge-charge and hydrophilic interactions. In connection with this, it is worth...
noting that one bound to HA, inter-α-trypsin inhibitor
conserved its antiproteolytic activity but
that of α1 proteinase inhibitor was substan-
tially reduced, at least as determined by the assay
conditions and here (Figs 1 and 7). Then it is tempting
to speculate that the configuration of α1 proteinase
inhibitor altered its interaction with HA. Alternatively,
binding to HA may have masked the active site of
the inhibitor.

Although it was possible to form complexes in vivo with denatured 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 50% of RA joints, the concentration of those proteins being low in normal sera. 15, 20 The identity of the smaller amounts of protein associated with osteoarthritic and poorly defined 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.

Sandborn, Hesmon, and Schwaig showed more
than 20 years ago that HA could form complexes
with inter-α-trypsin inhibitor. 24 It was noted by the
Fig. 7 Sepharose CL-2B chromatography of HA-protein
complexes prepared in vitro
with (A) HA alone, (B) HA and hyaluronic
acid (C) HA with α1 proteinase
inhibitor, (D) HA with
inter-α-trypsin inhibitor and then
exposed to 50 μM ferromastic acid at 25°C for 15 minutes.

Fractions were assayed for hexosaminidase activity
(- - - -), total protein at 280 nm (• - • - • -),
and trypsin inhibition (○ - ○ - ○ - ○ - ○).

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than 20 years ago that HA could form complexes
with inter-α-trypsin inhibitor. 24 It was noted by the
group that free inter-α-trypsin inhibitor was unde-
tectable in normal SF but was bound to HA in SF
from inflamed joints. 25 Brecken, Hagman, and
Kuipers used immunochemical methods to demon-
strate the binding of α1 proteinase inhibitor and a
larger complex, possibly of this inhibitor and a proteinase, to HA of inflammatory SF. 26 Our
finding that HA also binds to hyaluronic acid appears to be novel.

A consistent feature of the chromatographic profiles of HA from the inflamed SF investigated in our study was the exclusion of IgG 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 10^6 daltons. A recent report by Dahl et al, who used high density gels, showed that the molecular weight of HA declin
on average, from 7.0 x 10^6 in normal SF to about 4.0 x 10^6 in SF from patients with RA. 22 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 deposition of HA occurs within the inflamed joint, the dilution of SF by the plasma diuresis 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 perm size
chromatography. As the high porosity gels employed by Doshi et al. are, as yet, unavailable commercially, our gel filtration experiments were limited to the elution properties provided by the Sepharose CL-2B agarose gels. As discussed above, these gels are incapable of resolving HA monoclonal species with apparent Mr in excess of 1 x 10^6.

It has been suggested that decomplexation of HA could result from attack by oxy radicals produced by phagocyte cells or by enzymatic processes.13,14 Although hyaluronidase, β-glucuronidase, and β-N-acetylglucosaminidase of lysosomal origin have been shown to be present in the peritoneal fluid of RA joints,20 they are only capable of degrading HA in an acidic environment. This limitation probably excludes their effectiveness outside the cell. The more likely mechanism, therefore, is via O2 or H2O2, which have been shown by several groups to cause rapid depolymerization of HA in vitro.1,20,21

Studies with ionizing radiation have indicated that the hydrophilic free radical can attack hyaluronate at either the glycosidic bond or at the CS hydrogen of the γ-interaction ring.22 If such susceptible groups on the polysaccharide chain were screened by bound protein, then degradation by O2 or H2O2 might be abrogated, or at least modified. Our results indicate that hyaluronate and inter-alpha-trypsin inhibitor could qualify for such a role as HA complexes formed in vitro with these proteins (Fig. 7) were less degraded by an O2 or H2O2 flux (produced by Fe3+ activation) than protein free HA. On the other hand, α2 proteinase inhibitor was not effective in this regard, which was a surprising result in view of the known affinity of the serine protease for this protein.23-25 O2 or H2O2 as a trypsin inhibitory activity of α2 proteinase inhibitor when bound to HA was substantially reduced, however, it is likely that the combination of this protein was not different from that present in the unbound form, and that small polypeptide may be unavailable for oxidation. It is also possible that the small amount of free protease (i.e. the other proteins) of α2 proteinase inhibitor bound to HA is insufficient to ‘neutralize’ the intensity of the level of free radical flux used in our experiments.

When combined with hyaluronate, hyaluronate has been shown to possess peroxidase activity.12,13 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 PMN, from hydrogen free radicals. 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 perma-
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