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Activation of neutral metalloprotease in human osteoarthritic knee cartilage: evidence for degradation in the core protein of sulphated proteoglycan

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SUMMARY The neutral, metal dependent, proteoglycan degrading enzymes (NMPEs) in human osteoarthritic knee cartilage homogenates were activated by p-aminophenylmercuric acetate (APMA). The resultant effect on the structure of newly synthesised and already existing sulphated proteoglycan was measured. Newly synthesised and already existing proteoglycan aggregated to hyaluronic acid was reduced (p<0.01, p<0.05 respectively) when measured by chromatography on Sepharose CL-2B eluted with associative buffer. The APMA activated enzyme affected both the newly synthesised and already existing proteoglycan aggregate similarly (r=0.79, p<0.001). Treatment of cartilage homogenates with APMA and 1,10-phenanthroline (10 mM) showed that the amount of aggregated proteoglycan was at the control level. The hydrodynamic size of the proteoglycan monomer (A1D1) was also reduced by treatment of cartilage homogenates with APMA. Reaggregation experiments with fraction A1D1 and exogenous hyaluronic acid and link protein showed a similar defect in forming proteoglycan aggregates. These data showed that activation of the NMPEs altered the structure of proteoglycan in two ways. The most consistent change was a reduction in the ability of proteoglycan to form aggregates with hyaluronic acid. This was likely to have occurred via a cleavage of the core protein in or around the hyaluronic acid binding globular domain. A second alteration probably includes a limited proteolytic cleavage in the remainder of the core protein.

The destruction of articular cartilage is a hallmark of osteoarthritic pathology. In man, cartilage erosions are often slow but progressive. Gross pathological patterns implicate mechanical factors.1 2 Weight bearing cartilage is often preferentially compromised. This may account for the prevalence of medial compartment disease in osteoarthritis (OA) of the human knee.

One pathophysiological mechanism suggests degradation of interterritorial proteoglycan by neutral proteoglycan degrading enzymes (NMPEs). This enzyme class is metal dependent. NMPE activity is markedly inhibited by edetic acid and 1,10-phenanthroline.3-5 NMPE and collagenase concentrations are significantly raised in osteoarthritic human cartilage compared with macroscopically normal tissues.6 7 More recently it was reported that human osteoarthritic chondrocytes in culture synthesised more NMPEs.8 This suggested that authentic, stable alterations in the synthesis of this enzyme class occur sometime during the development of OA.

Most cartilage sulphated proteoglycans form large aggregates with hyaluronic acid.9 10 Aggregated proteoglycans are fixed in a network of type II collagen, minor collagen isotypes, and accessory non-collagenous matrix proteins.11 Whereas non-aggregated proteoglycan (even monomeric sub-
units) may diffuse out of the extracellular matrix, aggregated proteoglycans cannot. Thus maintenance of proteoglycans in the aggregated state is essential to enable cartilage to resist compressive mechanical forces and provide proper joint compliance.11

Any changes in the already existing proteoglycan aggregates as a result of mechanical transducing signals or increased NMPE levels, or both, would be expected to reduce the amount of tissue proteoglycan. Indeed, one of the earliest histopathological changes in osteoarthritic cartilage is a loss of metachromasia.12 If the chondrocytes were able to synthesise increased amounts of proteoglycan and replace the resorbed molecules, however, it is conceivable that cartilage integrity could be maintained.

Several recent studies from our laboratories13 14 (Shuckett et al, unpublished data) and from others15 16 have dispelled the previously held concept that chondrocytes of osteoarthritic cartilage fail to synthesise functional proteoglycans. Thus remaining alternatives must include the hypothesis that newly synthesised proteoglycan bearing a functional hyaluronic acid binding region (HABR) that allows for aggregation to hyaluronic acid is degraded by activated proteolytic enzymes before forming supramolecular structures. It has recently been shown that proteoglycan catabolism of mature rabbit cartilage maintained in serum involves proteolysis of the core protein and a separation of the hyaluronic acid bind region from the glycosaminoglycan-rich regions.16a This hypothesis was studied in knee cartilage derived from the lateral tibial plateau of patients undergoing total joint arthroplasty for debilitating OA. The changes in the structure of the already existing proteoglycans were compared with those of the newly synthesised proteoglycans radioisotopically labelled with 35SO4 by short term (24 hours), organ explant culture.17 The effect on proteoglycan structure of activating the endogenous latent cartilage NMPEs with p-aminophenylmercuric acetate (APMA) was assessed.

Materials and methods

Spatialmen selection

Lateral tibial plateau specimens (n=24) were obtained immediately after surgery. The mean age (SD) was 66-4 (5-7) years (range 53–81). The study comprised nine men and 15 women. The cartilage samples were obtained equally from right and left knees. One patient had a diagnosis of OA and chondrocalcinosis. The remaining patients were considered to have primary OA by radiological and clinical criteria. Cartilage samples were selected from the remaining tissue. This most often compr-
Neutral metalloprotease in osteoarthritic knee cartilage

scribed by Oegema et al was used.21 Proteoglycans were extracted for 24 hours at 4°C in 20 ml of 4 M guanidine-HCl, 50 mM sodium acetate buffer, pH 5.8, which contained several protease inhibitors (100 mM β-aminohexanoic acid, 10 mM disodium edetate, 5 mM phenylmethanesulphonyl fluoride, and 5 mM benzamide hydrochloride). The extract was centrifuged at 12 000 g for 30 minutes and the supernatant dialysed exhaustively at 4°C in Spectrapor membranes (2000 molecular weight cut off) against distilled water and freeze dried. The cartilage residue was washed with distilled water and incubated at 65°C for five hours with papain (1 µg/mg of original tissue wet weight) in sodium phosphate buffer, pH 6.5. The HexA and 35SO4 radioactivity levels were measured on the supernatant to determine the efficiency of the 4 M guanidine-HCl extraction.

CHARACTERISATION OF PROTEOGLYCANS

Ultracentrifugation analysis

The extracted freeze dried proteoglycans were first dissolved in 50 mM sodium acetate buffer, pH 5.8, containing 0.5 M guanidine-HCl. Density gradient ultracentrifugation, under associative conditions, was performed as previously described.22 Gradient fractions designated A1 to A4, based on their isopyknic densities, were obtained.

Aliquots of the A1 fraction were processed under dissociative conditions.22 Four equal fractions designated A1D1 (q=1.6 g/ml) to A1D4 (q=1.4 g/ml) by final isopyknic density were obtained.

Chromatographic analyses

Gel filtration chromatography was carried out along the lines of methods previously published.22 In brief, the A1 fraction was chromatographed on a Sepharose CL-2B column (0.4 cm×117 cm), equilibrated with 500 mM sodium acetate buffer, pH 7.0. Fractions of 0.5 ml were collected.

The A1D1 fraction was chromatographed under dissociative conditions (4 M guanidine-HCl, 500 mM sodium acetate, pH 5.8) on a Sepharose CL-2B column (0.8 cm×116 cm). Fractions of 1.0 ml were eluted. The glycosaminoglycan chain size was measured after reduction with NaBH4 and chromatography on Sepharose CL-6B.22 23

Proteoglycan monomer-hyaluronic acid interactions

The reaggregation procedure was carried out as previously described.14

Analytical study

The HexA measurement was determined by the carbazole method20 and was used as an index of endogenous proteoglycan content.

The newly synthesised proteoglycans were monitored by their 35SO4 radioactivity content. The radioactivity was measured in a Packard Tri-Carb model 3255 liquid scintillation spectrometer, which used an adjusted 14C/14CQ window for counting 35SO4. The counting efficiency was 75–80%.

STATISTICAL ANALYSES

Determinations were not made on all tissue samples. When the sample population exceeded 10 and the coefficient of variation indicated a lack of homogeneity between the groups the level of significance was obtained by a Wilcoxon rank sum test. Otherwise, a two tailed Student's t test was used. Linear regressions were calculated by the least squares method.

Results

NMPE ACTIVITY

Total, active NMPE activity was measured in 16 specimens. Treatment of cartilage extracts with APMA increased NMPE activity from 1.9% (SEM 0.24) (group 1) to 7.3% (0.6) (group 2). Thus a measurable, albeit small, constitutive activity was present in the osteoarthritic cartilage homogenates. The bulk of enzyme activity, however, was latent and could be activated by APMA. Treatment with 1,10-phenanthroline virtually eliminated the enzyme activity. These results were consistent with a previously published study.5

STRUCTURE OF PROTEOGLYCANS

Solubilisation

Freeze dried samples were reconstituted in 0.5 M guanidine-HCl, 0.1 M sodium acetate buffer before CsCl density gradient centrifugation. Only 3.3–3.5% of the HexA containing material and 1.7–2.0% of the 35SO4 labelled material remained insoluble. There were no significant differences between the groups.

Associative CsCl density gradient

Table 1 shows the distribution of HexA and 35SO4. In all groups the amount of newly synthesised proteoglycan exceeded that of the macromolecules containing HexA in the most dense A1 fraction (p<0.002). In contrast, the already existing macromolecules containing HexA of middle density (A2 and A3) exceeded in amount that of the newly synthesised proteoglycans and reached statistical significance in A2 (p<0.002). No differences were seen in the least dense A4 fraction. These results also showed that no differences existed between the groups in the distribution of either HexA or 35SO4.
The extraction efficiency (89·8–92·4%), $^{35}$SO$_4$; 76·7–78·7%, containing HexA) did not differ between the groups and supported the conclusion that the CsCl density gradient results reflected the major component of extractable proteoglycans.

**Gel filtration**

Fig. 1 shows a representative chromatogram of the A1 fraction eluted from Sepharose CL-2B with associative buffer. In group 1 (trometamol (TRIS); constitutive enzyme activity) two peaks of $^{35}$SO$_4$ and HexA-containing material were resolved. The first peak eluted at the void volume and constituted the proteoglycan aggregate fraction. The second peak showed a broad polydisperse profile and is a non-aggregating proteoglycan subpopulation. APMA treated (Fig. 1; APMA) A1 fractions showed a reduction in the void volume peak and an increase in the amount of the retarded second peak. When the cartilage homogenate was activated and treated with 1,10-phenanthroline (Fig. 1; O–PHE+APMA) an A1 profile identical to that seen in group 1 A1 fractions was seen.

Fig. 2 shows the effect of activating latent NMPEs in individual samples on proteoglycan aggregates. A significant reduction in proteoglycan aggregates was

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**Table 1** Distribution of newly synthesised and endogenous hexuronic acid (HexA) proteoglycans in CsCl density associative gradient

<table>
<thead>
<tr>
<th>Group</th>
<th>Gradient fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A4 (top)</td>
</tr>
<tr>
<td>$^{35}$SO$_4$</td>
<td>5·9 (0·4)†</td>
</tr>
<tr>
<td>HexA</td>
<td>6·6 (0·6)</td>
</tr>
<tr>
<td>$^{35}$SO$_4$</td>
<td>5·9 (0·2)</td>
</tr>
<tr>
<td>HexA</td>
<td>7·9 (0·5)</td>
</tr>
<tr>
<td>$^{35}$SO$_4$</td>
<td>7·3 (0·5)</td>
</tr>
<tr>
<td>HexA</td>
<td>8·1 (0·8)</td>
</tr>
</tbody>
</table>

The freeze dried proteoglycans were solubilised in 0·5 M guanidine-HCl/0·1 M sodium acetate buffer, pH 5·8; containing protease inhibitors and subjected to CsCl density gradient ultracentrifugation.

• p<0·002 ($^{35}$SO$_4$ v HexA; n=19).

†Values are expressed as the mean (SEM) of the percentage of total radioactivity ($^{35}$SO$_4$) or HexA in each fraction.

Average final densities:

A1 1·7 g/ml; A2 1·64 g/ml; A3 1·57 g/ml; A4 1·50 g/ml.

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**Fig. 1** Sepharose CL-2B chromatography of CsCl density gradient fraction A1. An aliquot of fraction A1 was applied to the column and eluted with 0·5 M sodium acetate buffer, pH 7·0. Incorporated radioactivity and hexuronic acid (HexA) content (carbazole reactivity) were measured from the optical density at 530 nm. $V_o$ = elution position of bovine cartilage proteoglycan aggregate; $V_r$ = Na$_2$ $^{35}$SO$_4$. Group 1 received no treatment; group 2 was treated with p-aminophenylmercuric acetate (APMA); group 3 received APMA plus 1,10-phenanthroline.

**Fig. 2** The newly synthesised ($^{35}$SO$_4$) and endogenous (HexA) proteoglycan aggregate in fraction A1. A1 fractions were chromatographed on Sepharose CL-2B and eluted with associative buffer. The differences in $^{35}$SO$_4$ and HexA in the void volume (PG$_{agg}$) between the groups was tested for significance by Wilcoxon rank sum analysis. *p<0·01 (n=15), comparing group 2 with groups 1 or 3; **p<0·02 (n=15), comparing group 2 with groups 1 or 3.
found when group 2 was compared with groups 1 or 3 (p<0.01, $^{35}$SO$_4$; p<0.02, containing HexA). In addition, in all groups the amount of newly synthesised aggregates exceeded that of already existing proteoglycan aggregates, in keeping with previous studies of osteoarthritic cartilage extracted without prior incubation. These results also showed that only activated osteoarthritic cartilage NMPs reduced the amount of newly synthesised and endogenous proteoglycan aggregate. Of note, constitutive enzyme activity did not alter the gel filtration profile.

A linear regression analysis (Fig. 3) was carried out to assess whether the newly synthesised proteoglycan and already existing proteoglycan were affected to the same extent by the treatments. These results confirmed that the $^{35}$SO$_4$-containing and already existing proteoglycan aggregate were affected to the same extent.

**Dissociative CsCl gradient**

Fraction A1 was subjected to CsCl density gradient centrifugation to assess whether APMA treatment affected the density of proteoglycan subunits associated with either hyaluronic acid (void volume peak) or the retarded peak on Sepharose CL-2B. The distribution of $^{35}$SO$_4$ and HexA were unaffected by APMA. No differences existed between the groups in the percentage of high density A1D1 fraction (86-8-92.7% of the total) or in fractions of middle (A1D2, A1D3) or low density (A1D4).

**Fraction A1D1 hydrodynamic size**

The size of the high density proteoglycan monomer after treatment of cartilage homogenates with APMA was studied (Table 2). There was a statistically significant decrease in the hydrodynamic size of the A1D1 fraction of both the newly synthesised component (p<0.02) and the already existing proteoglycan (p<0.01) when group 2 results were compared with those for group 1. This increase in $n_{av}$ was not seen when APMA extracts were treated with 1,10-phenanthroline (group 3). APMA failed to alter the glycosaminoglycan chain size ($n_{av}$=0.58, $^{35}$SO$_4$; 0.62, HexA) (Fig. 4).

![Linear regression (least square) analysis. A strong positive correlation between the effect of each incubation condition on the amount of newly synthesised ($^{35}$SO$_4$) and already existing (containing HexA) proteoglycan aggregate is shown. UA=hexuronic acid.](http://ard.bmj.com/)

**Table 2** The hydrodynamic size of the proteoglycan monomer fraction of high density (A1D1) obtained by chromatography on Sepharose CL-2B

<table>
<thead>
<tr>
<th>Group</th>
<th>$n_{av}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 No treatment</td>
<td>$^{35}$SO$_4$ 0.26 (0.004)$^+$</td>
</tr>
<tr>
<td></td>
<td>HexA 0.32 (0.02)</td>
</tr>
<tr>
<td>2 APMA treated</td>
<td>$^{35}$SO$_4$ 0.36 (0.03)$^*$</td>
</tr>
<tr>
<td></td>
<td>HexA 0.39 (0.01)$^{**}$</td>
</tr>
<tr>
<td>3 1,10-Phenanthroline+APMA</td>
<td>$^{35}$SO$_4$ 0.25 (0.006)</td>
</tr>
<tr>
<td></td>
<td>HexA 0.36 (0.02)</td>
</tr>
</tbody>
</table>

Individual samples were chromatographed and the $n_{av}$ determined (see 'Materials and methods' for details). $^+$p<0.02, compared with $^{35}$SO$_4$ in group 1; $^{**}$p<0.01, compared with HexA in group 1.  
$^+$Values are mean (SEM), n=6.
Proteoglycan monomer from aggregate and non-aggregate subpopulations

The distribution of newly synthesised and HexA-containing proteoglycan monomer was studied on the individual Sepharose CL-2B peaks. A similar amount of high density proteoglycan was found in the peak I (void volume) D1 fraction when compared with the peak II (included peak) D1 fraction (data not shown). APMA treatment did not alter this. In preliminary studies APMA treatment appeared to reduce the average hydrodynamic size of the $^{35}$S0$_4$-containing as well as already existing proteoglycan monomer associated with the proteoglycan aggregate subpopulation.

Reaggregation of A1D1 fraction

Fig. 5 shows the results of reaggregation experiments using fraction A1D1 and exogenous umbilical cord hyaluronic acid in the presence of link protein. The data show that both the newly synthesised and HexA-containing A1D1 fractions showed similar aggregation to hyaluronic acid; that reaggregation of newly synthesised A1D1 was significantly diminished by treatment with APMA; (4/5 samples measuring reaggregation of endogenous A1D1 showed this); and that treatment with 1,10-phenanthroline + APMA.

Discussion

Proteoglycan monomers that form complexes with hyaluronic acid are stabilised by link protein.$^9$ These aggregates are essentially fixed within the extracellular matrix of articular cartilage. The present study showed that the constitutive NMPE activity in osteoarthritic cartilage was incapable of altering the amount of either newly synthesised or
endogenous proteoglycan aggregates. Activation of the mostly latent enzyme, however, reduced the amount of newly synthesised and already existing proteoglycan aggregate as well as the hydrodynamic size of the proteoglycan monomer itself.

The fact that activation of the NMPEs did not alter glycosaminoglycan chain size, or result in a marked redistribution of proteoglycan when analysed by density gradient centrifugation, indicated that core protein proteolysis was rather limited. Noteworthy was the finding that the newly synthesised and the already existing proteoglycan population were affected similarly. Thus these results support the conclusion that synthesis of proteoglycans occurs in a normal fashion in osteoarthritic cartilage, and substantiates the contention that activated NMPE cleaves the core protein before acquiring high affinity binding for hyaluronic acid.

The formation of proteoglycan aggregates has been thought to reduce proteolytic cleavage of the core protein. Recent studies, however, have shown the presence of the HABR associated with hyaluronic acid in the absence of the chondroitin sulphate attachment region, and this together with the present data suggests that proteolytic cleavage may occur distal to the HABR itself, releasing the bulk of core protein. Thus the present data support at least two possible interpretations. The activated NMPE may cleave the core protein before the formation of stable proteoglycan aggregates (see above). This is probably the case with the newly synthesised proteoglycans. It is equally possible that proteolytic cleavage occurs in proteoglycans already aggregated to hyaluronic acid.

The NMPE is favoured as the major contributor to the disaggregation and further cleavage of proteoglycans in the interterritorial region. Acid metalloproteases may also participate in the pericellular region as well as in the interterritorial matrix as at least some of the acid enzyme activity (40%) is retained near neutral pH. Activation of acid metalloproteoglycanase also caused a distinct reduction in the size of proteoglycan containing HexA. Several forms of the NMPE are synthesised by cultured chondrocytes and are also found in cartilage. The various forms of this NMPE class may act at different cleavage sites on the core protein. We are uncertain at present whether the failure of the constitutive enzyme (group 1) to alter proteoglycan structure resulted from an insufficient amount of enzyme or the failure of enzyme to degrade specific domains of the core protein.

The amount of NMPE is markedly increased in osteoarthritic cartilage compared with normal cartilage or with macroscopically normal specimens removed from patients of the age (about 50 years) of the present sample. Recent studies compared NMPE concentrations in macroscopically fibrillated cartilage as a function of age and the depth from the articular surface. These studies showed that superficial cartilage contained higher concentrations of total enzyme than found in the deeper regions. Moreover, increased active enzyme was associated with fibrillation. As it is likely that a significant proportion of the superficial cartilage is eroded in OA the total NMPE concentrations in the present study (7.3 (SEM 0.6)% ) compare favourably with those measured in cartilages taken at necropsy from patients older than 50 years.

Reaggregation experiments in which purified A1D1 was incubated with hyaluronic acid helped resolve one contentious component of this study. In the specimens that received no addition and in those treated with 1,10-phenanthroline the amount of reaggregated proteoglycan (Fig. 5) slightly exceeded the value reached by equilibrium dialysis. The reduced proteoglycan aggregate formation seen in the AMPA treated samples (Fig. 2) could have occurred if insufficient hyaluronic acid were present in the original homogenate. The amount of proteoglycan aggregates formed by reaggregation and by dialysis were similar, however. Any differences in proteoglycan aggregation obtained in this study and in the previous one may reflect additional post-maturational changes in the HABR (i.e., additional 42 hours). Thus the most attractive explanation to account for these results is that some proteoglycan monomers do not possess the capacity to form aggregates. Presumably a missing HABR or a non-functional HABR accounts for this.

The pathophysiological consequences of reductions in the size of the proteoglycan monomer have yet to be established. Based on the calculations of Ohno et al., the newly synthesised proteoglycan monomer A1D1 from group 1 (Table 2) would be in the order of 1.43×10⁶ M₂ or 36 nm. The already existing proteoglycan monomer size (Kₘ=0.32) would be slightly smaller (1.17×10⁶ M₂) and similar to that reported in other studies for adult human cartilage. Treatment with APMA reduced the size of the newly synthesised proteoglycan monomer to 88-88×10⁶ M₂ or 32-5 nm, and the already existing monomer to an even smaller size.

These present findings suggested that activators of the latent metalloenzymes are either inhibited in situ or are not synthesised even in cartilage of patients with severe OA. Putative activators of NMPE in vivo (plasmin?) would appear to be intimately involved in the alteration of the newly synthesised and endogenous proteoglycans. In this regard, marked synovial involvement occurring...
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early in experimental canine OA and presumably occurring at some time in the continuum of human OA resulted in complete absence of proteoglycan aggregates and apparent multiple cleavages of the core protein. The present results suggest that therapeutic intervention designed to inhibit NMPE activators may significantly limit the progressive aspect of osteoarthritic cartilage destruction.

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