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Degradation of proteoglycan aggregate by a cartilage metalloproteinase

Evidence for the involvement of stromelysin in the generation of link protein heterogeneity *in situ*

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Cartilage proteoglycan aggregates were subjected to degradation by a metalloproteinase, capable of degrading proteoglycan, released from cartilage in culture. This proteinase was demonstrated to be immunologically identical with fibroblast stromelysin. An early release of hyaluronic acid-binding region and large glycosaminoglycan-attachment regions was observed. With increasing time the glycosaminoglycan-attachment regions were digested into smaller fragments and the hyaluronic acid-binding regions accumulated. The degradation of link proteins also occurred concomitantly with these events. Link proteins were converted into a component of similar size to that of the smallest native link protein component. *N*-Terminal sequence analysis of the three human link protein components indicated that they are all derived from the same protein core, which is closely homologous to that of the rat chondrosarcoma link protein. The two larger link proteins (M_r 48000 and 44000) contain the same *N*-terminal sequence, but they differ by the apparent presence of an *N*-linked oligosaccharide at residue 6 of the largest link protein component. The smallest link protein (M_r 41000), however, has an *N*-terminal sequence equivalent to that commencing at residue 17 in the larger link proteins. It was found that the cartilage metalloproteinase cleaves link proteins in human neonatal cartilage proteoglycan aggregates at the His-16–Ile-17 bond, the same position at which the smallest link protein component appears to be derived naturally from the two larger link protein components. These results suggest that stromelysin secreted by chondrocytes can account for the increased accumulation of hyaluronic acid-binding regions and much of the degradation of link protein observed during aging within human articular cartilage.

INTRODUCTION

Hyaline cartilage contains at least four distinct proteoglycans, the most abundant of which are the aggregating proteoglycans. A proteoglycan aggregate is composed of a central filament of hyaluronic acid to which numerous proteoglycan subunits are non-covalently attached (Hardingham & Muir, 1974). Each interaction between a proteoglycan subunit and the hyaluronic acid filament is further stabilized by a link protein (Hardingham, 1979). The proteoglycan subunits consist of a central protein core bearing many chondroitin sulphate and keratan sulphate chains and *O*-linked and *N*-linked oligosaccharides (Thonar & Sweet, 1979; Nilsson *et al.*, 1982). It has been established that the protein core of a proteoglycan subunit has several distinct regions (Doege *et al.*, 1987). These include a hyaluronic acid-binding region devoid of glycosaminoglycan chains, and regions rich in keratan sulphate and chondroitin sulphate (Paulsson *et al.*, 1987; Heinegård & Axelsson, 1977).

Link proteins are glycoproteins that exist in a number of forms. In human articular cartilage they can be separated into three components of M_r 48000, 44000 and 41000 (Roughley *et al.*, 1982). These are referred to in the present paper as LP1, LP2 and LP3 respectively. In mammalian cartilage, it has been suggested that the two larger protein link components may share the same protein core, but differ in their degree and/or type of oligosaccharide substitutions (Baker & Caterson, 1979; Mort *et al.*, 1985), and the smallest link protein component is thought to be derived from either of the two larger components by a proteolytic cleavage near the *N*-terminus (Le Glédic *et al.*, 1983).

The structure of proteoglycans is thought to be continuously modified during aging by proteolytic processes. This results in smaller proteoglycan subunits resulting from cleavage of the core protein (Buckwalter & Rosenberg, 1982; Paulsson *et al.*, 1987), as well as an accumulation of hyaluronic acid bearing free hyaluronic acid-binding regions (Roughley *et al.*, 1985). An increase in the concentration of the smallest link protein and further internal fragmentation of the link proteins also occur (Mort *et al.*, 1983).

Two distinct metalloproteinases capable of degrading proteoglycans have been extracted from human cartilage (Woessner & Selzer, 1984). In organ culture, however, only one of these proteinases appears to be secreted by the chondrocytes into the culture medium (Campbell *et al.*, 1986*a*). This metalloproteinase is able to degrade both the proteoglycan subunits and the link proteins (Campbell *et al.*, 1986*b*), and has properties similar to the

Abbreviations used: LP1, LP2 and LP3, link protein components of M_r 48000, 44000 and 41000 respectively.

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metalloproteinase, stromelysin, secreted by human fibroblasts in culture (Chin *et al.*, 1985). In the present paper, the identity of the cartilage metalloproteinase and its mode of action on cartilage proteoglycan aggregates are described. In addition, *N*-terminal sequences of the three native human link proteins and the cleavage site of the metalloproteinase on the link proteins are presented.

EXPERIMENTAL

Materials

Acrylamide, methylenebisacrylamide, SDS and nitrocellulose sheets were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). ^{125}I-labelled Protein A and [^{35}S]methionine were obtained from Amersham (Oakville, Ontario, Canada). Rabbit anti-(mouse IgG1) antibody was from Sci-Can Diagnostics (Edmonton, Alberta, Canada). Immobilon (polyvinylidene difluoride) transfer membranes were obtained from Millipore (Bedford, MA, U.S.A.). Interleukin-1-rich medium, which was generously provided by Dr. Elaine E. Golds (Shriners Hospital, Montreal, Quebec, Canada), was prepared from human peripheral-blood mononuclear cells by stimulation with phytohaemagglutinin (Golds *et al.*, 1983). Recombinant human interleukin-1β was purchased from Cistron (Pine Brooke, NJ, U.S.A.).

Cartilage-derived metalloproteinase

Human articular cartilage from the femoral condyles of individuals having no clinical evidence of joint disease was obtained within 20 h of death. Cartilage pieces (1–2 mm^3) were cultured in serum-free medium (Handley & Lowther, 1977) with the presence of interleukin-1-rich medium at a concentration known to induce a maximal secretion of metalloproteinases (Campbell *et al.*, 1986*a*,*b*), or with 1 unit of recombinant human interleukin-1β/ml. The cultures were maintained for up to 14 days, with a change of fresh culture medium every 2 days. After 4 days of incubation in the presence of recombinant interleukin-1, some cartilage explants were incubated with [^{35}S]methionine (100 μCi/ml) for 24 h. These cultures were terminated and the medium was collected. The neutral metalloproteinase activity in harvested culture medium was monitored by a [^{3}H]casein-degradation assay (Campbell *et al.*, 1986*a*; Golds *et al.*, 1983). The metalloproteinase activity was concentrated by ultrafiltration on Amicon YM-5 membranes, and separated from endogenous proteoglycan fragments by gel filtration through Ultrogel AcA-44 (1.8 cm × 75 cm) as described by Campbell *et al.* (1986*a*), then reconcentrated. Similar enzyme preparations were obtained for cultures stimulated with either recombinant or natural interleukin-1. Analysis of the gel-filtration profile revealed only a single sharp symmetrical peak of proteoglycan-degrading activity, which was detectable only upon 4-aminophenylmercuric acetate activation. Futhermore, the activated enzyme was completely inhibited by EDTA (Campbell *et al.*, 1986*a*). These results suggest a single latent metalloproteinase.

Proteoglycan aggregates and link proteins

Human neonatal proteoglycan aggregates were prepared by extracting cartilage pieces with 4 M-guanidinium chloride/0.1 M-sodium acetate buffer, pH 6.0, containing proteinase inhibitors, at 4 °C for 70 h, and subjecting the dialysed extract to associative CsCl-density-gradient centrifugation (Roughley *et al.*, 1982). Proteoglycan was recovered from the bottom of the gradient, and on analysis by Sepharose CL-2B chromatography 70% of the proteoglycan was shown to be in the aggregate form. Link proteins were isolated from proteoglycan aggregates by dissociative CsCl-density-gradient centrifugation, followed by gel filtration through Sepharose CL-6B (Roughley *et al.*, 1982).

Digestion time course of proteoglycan aggregates

The concentrated metalloproteinase was dialysed into 0.1 M-NaCl/5 mM-$CaCl_2$/0.1 M-Tris/HCl buffer, pH 7.0. Neonatal proteoglycan aggregate was dissolved in the same buffer at 4.0 mg/ml. An equivalent volume of each solution (1.0 ml) was mixed together and incubated at 37 °C in the presence of 1 mM-4-aminophenylmercuric acetate. At intervals of 1 h, 2 h, 4 h, 8 h and 20 h, 0.25 ml of the digestion mixture was taken out and the reaction was terminated by adding EDTA to a final concentration of 10 mM. Papain digestion of proteoglycan aggregates was also carried out at 37 °C for 24 h. The reaction mixture contained 10 μg of enzyme/mg of proteoglycan in 5 mM-dithiothreitol/5 mM-EDTA/0.1 M-sodium acetate buffer, pH 5.0. The digestion was stopped by adding iodoacetamide to a final concentration of 20 mM.

Agarose/polyacrylamide-gel electrophoresis

Digested proteoglycan samples were dialysed into 1 mM-Na_2SO_4/40 mM-Tris/acetate buffer, pH 6.8, then analysed on the 0.6% agarose/1.2% polyacrylamide slab gel system described by Heinegård *et al.* (1985). To test the hyaluronate-binding ability of digested proteoglycan samples, they were incubated with 20% (w/w) hyaluronic acid at room temperature for 4 h before their analysis on agarose/polyacrylamide gels.

SDS/polyacrylamide-gel electrophoresis and immunoblotting

Digested proteoglycan samples were analysed under reducing conditions by the method of King & Laemmli (1971), with 10% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes by the method of Towbin *et al.* (1979). The transfer sheet was incubated in blocking solution [1% (w/v) bovine serum albumin in phosphate-buffered saline (10 mM-sodium/potassium phosphate buffer, pH 7.2, containing 0.145 M-NaCl and 0.05% NaN_3)] for 1 h at room temperature. The sheet was then incubated for 4 h at room temperature in the blocking solution containing monoclonal antibody, either 9/30/8-A-4 (to link protein) or 12/21/2-A-5 (to the hyaluronic acid-binding region). After several washes of the membrane with phosphate-buffered saline, it was incubated for 1 h at room temperature in blocking solution containing ^{125}I-labelled Protein A. After further washes of the nitrocellulose sheet with phosphate-buffered saline, link proteins and hyaluronic acid-binding regions were revealed by autoradiography with Kodak XAR film. To detect the hyaluronic acid-binding regions, an additional antibody step [rabbit anti-(mouse IgG1) antibody] was introduced before treatment with ^{125}I-labelled Protein A, because of the weak binding of Protein A to the 12/21/2-A-5 antibody.

[^{35}S]Methionine-labelled samples were similarly analysed by SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose membranes. Labelled

proteins were again revealed by autoradiography with X-ray film. The same transfer membrane was then treated with either sheep anti-(human collagenase) serum (Murphy *et al.*, 1986) or sheep anti-(human stromelysin) serum, followed by peroxidase-conjugated pig anti-(sheep IgG) antibody, and the peroxidase reaction was developed with 4-chloronaphthol as substrate. The anti-(human stromelysin) antibody was prepared by immunization with recombinant human fibroblast stromelysin (Murphy *et al.*, 1987).

Proteolytic cleavage of link proteins

To study the proteolytic cleavage position of the metalloproteinase on human link proteins, digested link proteins were prepared as follows. A 25 mg portion of proteoglycan aggregate dissolved in 10 ml of 0.1 M-NaCl/5 mM-$CaCl_2$/0.1 M-Tris/HCl buffer, pH 7.0, was incubated with metalloproteinase in the presence of 1 mM-4-aminophenylmercuric acetate, at 37 °C for 24 h. The digestion was stopped by adding EDTA to a final concentration of 10 mM, and digested link proteins remaining bound to hyaluronic acid were purified by associative CsCl-density-gradient centrifugation (Hascall & Heinegård, 1974). Rat link proteins digested by metalloproteinase were similarly prepared from Swarm rat chondrosarcoma proteoglycan aggregates, generously supplied by Dr. Clive Roberts (Shriners Hospital, Montreal, Quebec, Canada). Trypsin-digested human and rat link proteins were also prepared by digesting proteoglycan aggregates at an enzyme concentration of 10 μg/mg of proteoglycan aggregate in 0.1 M-Tris/HCl buffer, pH 7.5, at 37 °C for 4 h. Trypsin was then inactivated by the addition of soya-bean trypsin inhibitor (20 μg/mg of aggregate).

Amino acid sequence analysis

Individual link proteins (100–500 pmol) were separated by SDS/polyacrylamide-gel electrophoresis, then electroblotted on to Immobilon membranes as described by Matsudaira (1987). Components identified by Coomassie Blue staining were excised and analysed directly with an Applied Biosystems model 470A sequencer equipped with an on-line model 120A phenylthiohydantoin analyser.

RESULTS

Degradation of proteoglycan aggregate

The action of the cartilage metalloproteinase on the proteoglycan aggregate was investigated by agarose/polyacrylamide-gel electrophoresis. This technique permits non-aggregated proteoglycans to be resolved within the gel but retains the aggregate at the gel surface. Not all proteoglycan in the aggregate preparation is actually bound to hyaluronic acid, and these free proteoglycan subunits are clearly visible (Fig. 1, lane A), though they can be induced to interact by the presence of additional hyaluronic acid. Some additional interaction may also occur if the aggregate preparation is incubated at 37 °C, this presumably being due to increased accessibility of binding sites over those that are exposed when samples are redissolved at room temperature. At early digestion times there was only minimal decrease in size of proteoglycan able to penetrate the gel, and much of the proteoglycan remains in the aggregated form (Fig. 1, lanes B and C). After 4 h of incubation there is no

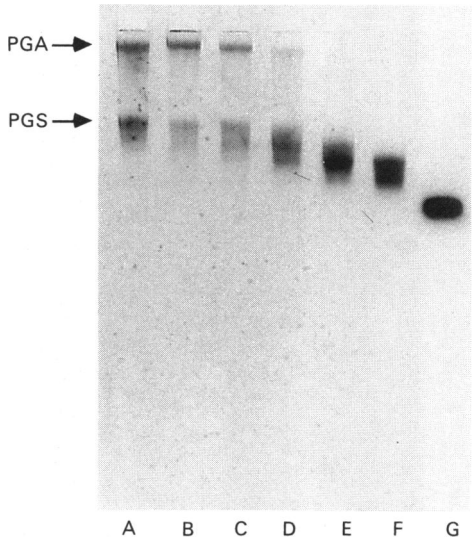

Fig. 1. Time course of degradation of human proteoglycan aggregates by activated cartilage metalloproteinase

Proteoglycan aggregate preparation (lane A) and 1 h (lane B), 2 h (lane C), 4 h (lane D), 8 h (lane E) and 20 h (lane F) digests were analysed by agarose/polyacrylamide-gel electrophoresis. Proteoglycan aggregates were also subjected to 24 h digestion by papain (lane G) and analysed on the same gel. The migration position of intact proteoglycan aggregates (PGA) and proteoglycan subunits (PGS) are indicated.

evidence for proteoglycan remaining in the aggregate form, and some of the proteoglycan is smaller than that observed for intact proteoglycan subunits. These large fragments were further degraded with time, and after an overnight incubation limit-degradation products were obtained as a single diffuse material, with a size larger than that of single chondroitin sulphate chains generated by papain digestion (Roughley, 1978).

When digestion mixtures at all time intervals were incubated with exogenous hyaluronic acid, then analysed by agarose/polyacrylamide-gel electrophoresis, no change in the mobility of the non-aggregated proteoglycan was observed, indicating a loss of hyaluronate-binding ability of all digested proteoglycan fragments. Furthermore, analysis of the samples by SDS/polyacrylamide-gel electrophoresis revealed a diffuse hyaluronic acid-binding region band that was first detected in the 2 h incubation mixture (Fig. 2, lane C). The intensity of this hyaluronic acid-binding region increased with longer incubation time (Fig. 2, lanes D–F), though there was no change in the size of this component with time. This is not to be expected if the proteolytic degradation were proceeding from the *C*-terminus of the proteoglycan subunits towards the hyaluronic acid-binding region. Thus it appears that release of the hyaluronic acid-binding region may represent one of the early cleavage sites for the activated metalloproteinase, and accounts for the accumulation of hyaluronic acid-binding regions at early digestion periods, along with the inability of large proteoglycan fragments to interact with exogenous hyaluronic acid.

Concurrent with the release of the hyaluronic acid-binding region, and the degradation of the proteoglycan subunits to large fragments, link proteins were also being

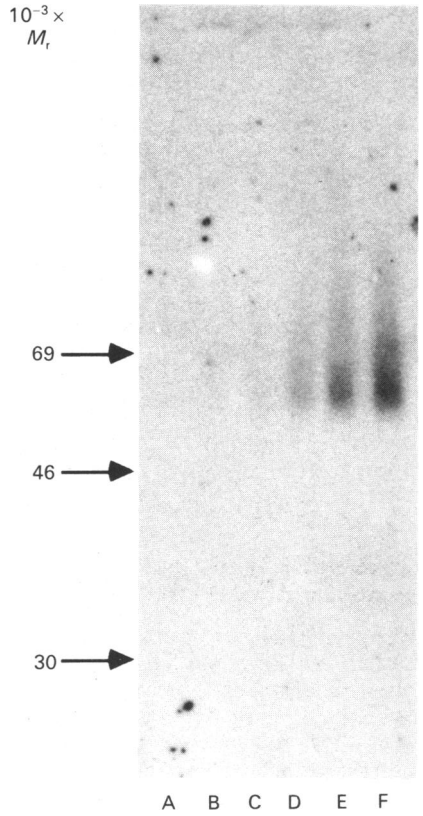

Fig. 2. Time course of hyaluronic acid-binding region release from proteoglycan aggregates incubated with the metalloproteinase

Intact proteoglycan aggregates (lane A) and 1 h (lane B), 2 h (lane C), 4 h (lane D), 8 h (lane E) and 20 h (lane F) digests were analysed by SDS/polyacrylamide-gel electrophoresis followed by immunoblotting.

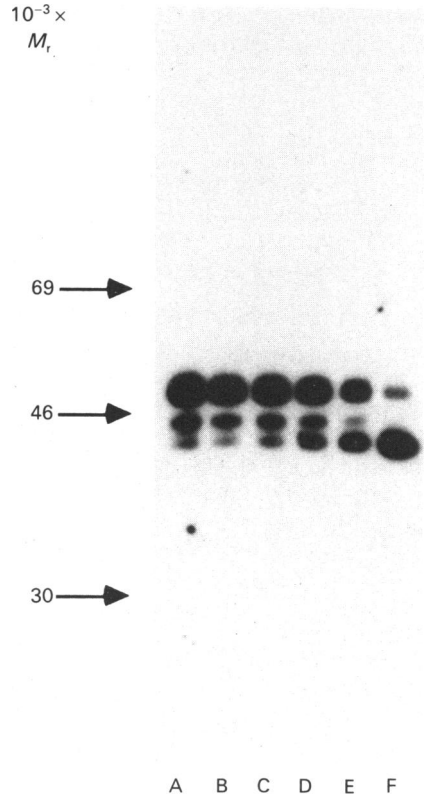

Fig. 3. Time course of link-protein modification in proteoglycan aggregates incubated with the metalloproteinase

Intact proteoglycan aggregate (lane A), and 1 h (lane B), 2 h (lane C), 4 h (lane D), 8 h (lane E) and 20 h (lane F) digests were analysed by SDS/polyacrylamide-gel electrophoresis followed by immunoblotting.

degraded by the cartilage metalloproteinase, as indicated by an increase in the abundance of a single protein band that apparently co-migrates with the native LP3 link component (Fig. 3, lane D). The abundance of this digested link protein component was found to be at the expense of LP1 and LP2 (Fig. 3, lanes E and F), and after an overnight incubation LP2 had completely disappeared while only a small amount of LP1 remained. At all digestion time intervals there was no evidence for the degradation of LP1 or LP2 into smaller intermediate components before their eventual conversion into the smallest link protein. The internal fragmentation of link proteins that is observed in adult cartilage was also not found in the present study. Since the modified link protein has the same mobility on SDS/polyacrylamide gel as that of native LP3, it appears that the cartilage metalloproteinase may be responsible for the native pattern of link-protein heterogeneity. To elucidate this potential role for the cartilage metalloproteinase, structural characterization of native link proteins and the degraded link protein component was performed.

N-Terminal sequencing of native and degraded human link proteins

N-Terminal sequences of human LP1, LP2 and LP3 were determined after their separation by SDS/polyacrylamide-gel electrophoresis. It was found that the two larger link protein components (LP1 and LP2) have the same *N*-terminus and similar sequences for the first 20 amino acid residues (Fig. 4). The only exception is that LP1 differed from LP2 by giving a blank cycle on sequencing instead of an asparagine at residue 6. The Asn-Tyr-Thr sequence for residues 6–8 in LP2 represents a potential glycosylation site for *N*-linked oligosaccharides, and presumably such an oligosaccharide is present in LP1. LP3 appears to share the same protein core as the two larger link protein components, but its *N*-terminal end is shortened by the loss of a peptide of 16 residues (Fig. 4).

The cleavage sites of trypsin and the metalloproteinase on human and rat link proteins were deduced from the partial *N*-terminal sequences of digested link proteins. It was found that human and rat link proteins have different trypsin-susceptible sites (Fig. 4). In rat link proteins trypsin cleaves on the carboxy side of Arg-24, whereas in the human analogues the cleavage site is nearer the *N*-terminal end at Arg-13. In the human link protein the metalloproteinase cleaves at the His-16–Ile-17 bond, whereas the cleavage site in the rat link protein is at the Ala-19–Glu-20 bond (Fig. 4). The cleavage site of the metalloproteinase on human link proteins is at exactly the same position as expected if LP3 had been generated *in situ* by the action of this proteinase at the expense of LP1 and LP2.

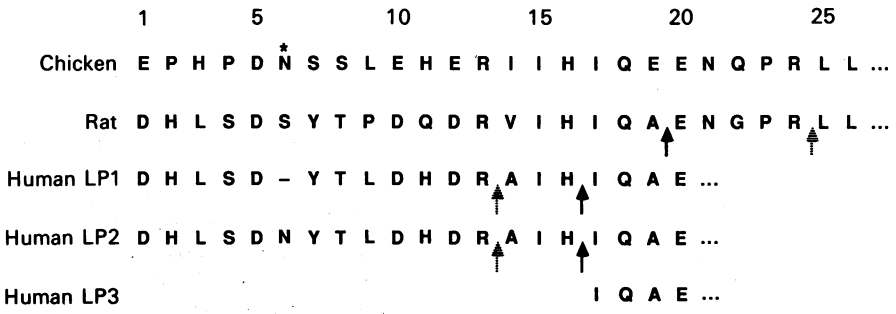

Fig. 4. Partial *N*-terminal sequences of human link proteins

N-Terminal sequences of human link proteins (LP1, LP2 and LP3) are depicted together with those reported for rat chondrosarcoma link protein (Neame *et al.*, 1986) and inferred from cDNA sequencing for chicken cartilage link protein (Deák *et al.*, 1986). The symbols used are: *, potential asparagine-linked glycosylation site; –, a blank cycle on sequencing; solid arrow, site of metalloproteinase cleavage; dashed arrow, site of trypsin cleavage.

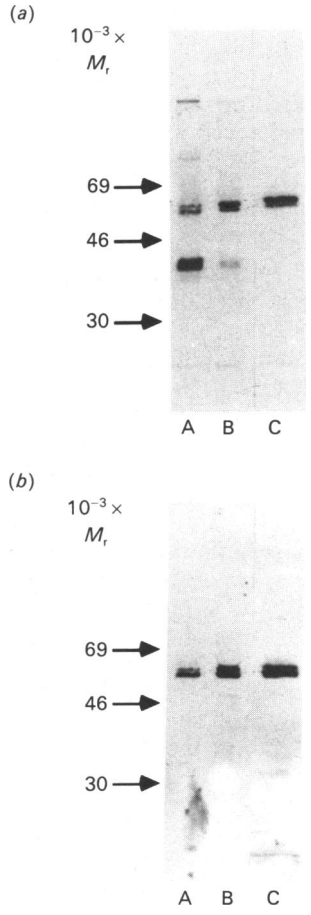

Fig. 5. Immunological characterization of the cartilage metalloproteinase

Protein secreted by human articular cartilage cultured in the presence of [^{35}S]methionine was analysed by SDS/polyacrylamide-gel electrophoresis, then transferred to nitrocellulose and investigated by (*a*) direct autoradiography and (*b*) immunoperoxidase localization with antibody specific for stromelysin. Concentrated medium from cartilage cultured in the absence (lane A) or in the presence of interleukin-1 (lane B), and pooled fractions from the peak showing latent metalloproteinase activity obtained after fractionation of the culture medium through Ultrogel AcA-44 (lane C), were used for analysis.

Immunological characterization of the secreted cartilage metalloproteinase

Proteins synthesized and secreted by human cartilage explants cultured in the presence of [^{35}S]methionine were analysed by SDS/polyacrylamide-gel electrophoresis, followed by electroblotting on to nitrocellulose and autoradiography. Several labelled bands were found in the control medium (Fig. 5*a*, lane A). However, under interleukin-1 stimulation, the intensity of a doublet band in a M_r range 55000–60000 was significantly increased, while the intensity of other secreted proteins was diminished (Fig. 5*a*, lane B). The elevated secretion of these two proteins coincided with an increase in latent casein-degrading activity in the culture medium. Furthermore, on gel-filtration analysis of the medium from interleukin-1-stimulated cultures a single peak of latent casein-degrading activity was observed, which on autoradiography showed an identical doublet pattern (Fig. 5*a*, lane C).

The identity of these two bands was established by immunoblotting the same nitrocellulose membrane used for autoradiography with either anti-(human stromelysin) serum or anti-(human collagenase) serum. It was found that the radioactive doublet band corresponded exactly to a doublet band observed on immunoblots probed with anti-stromelysin serum (Fig. 5*b*). These two bands co-migrated with the doublet bands observed with human fibroblast stromelysin (results not shown). These data, together with those reported earlier on the characterization of this proteinase (Campbell *et al.*, 1986*a*), substantiate the conclusion that only one proteinase, stromelysin, is present in the preparations used in this work. There was no evidence for the secretion of collagenase by human cartilage explants into the culture medium, even under the stimulation of interleukin-1, as indicated by the lack of detectable collagenase bands on immunoblots.

DISCUSSION

In this investigation we have observed that cleavage adjacent to the hyaluronic acid-binding region of the proteoglycan subunits may represent an early proteolytic event when human articular-cartilage proteoglycan aggregates are subjected to degradation by a cartilage

metalloproteinase, now identified as stromelysin. Although release of the hyaluronic acid-binding region need not occur before cleavage within the glycosaminoglycan-attachment region of the proteoglycan subunits, the results indicate that *in vitro* stromelysin does not act by cleaving from the *C*-terminus of the subunits towards the hyaluronic acid-binding region. Thus cleavage adjacent to the hyaluronic acid-binding region appears to be one of the preferred sites of degradation. This may be even more pronounced in the cartilage, where the high proteoglycan concentrations may render the glycosaminoglycan-attachment region less accessible to proteinases owing to decreased hydration of the glycosaminoglycan chains. The degradation of the link proteins also appears to be an early proteolytic event, and may occur concomitantly with the release of the hyaluronic acid-binding regions. Given sufficient time, all link proteins were degraded to a single protein band that co-migrated with the smallest native component. Thus the action of cartilage stromelysin on the proteoglycan aggregates is compatible with the accumulation of hyaluronic acid-binding regions and modified link proteins observed in normal adult human cartilage (Roughley *et al.*, 1985; Mort *et al.*, 1983).

Structural differences among the three components of human link proteins were also established in this study on the basis of their partial *N*-terminal sequences. All human link proteins appear to share the same protein core, but they differ in the degree of glycosylation and the protein core length. LP1, the largest link protein, has the same *N*-terminal sequence as LP2, but appears to possess an extra *N*-linked oligosaccharide as residue 6. Differences in oligosaccharide substitution have also been suggested to account for link-protein heterogeneity in the bovine system (Baker & Caterson, 1979; Le Glédic *et al.*, 1983). Both LP1 and LP2 have a protein core longer than that of LP3 by a peptide of 16 amino acid residues. This shortening of the core protein is likely the result of a proteolytic cleavage at the His-16–Ile-17 bond. In previous studies (Le Glédic *et al.*, 1983; Mort *et al.*, 1985) it was suggested that the smallest link protein, LP3, was derived from the larger link protein components by a proteolytic cleavage within the glycosylated *N*-terminal, but the natural cleavage position has never previously been identified. It was also found in the present work that the cartilage metalloproteinase cleaved human link proteins at the His-16–Ile-17 bond, the proteolytic cleavage site identical with that accounting for the generation *in situ* of the smallest human link protein from the two larger components. Thus cartilage stromelysin is a prime candidate responsible for the link-protein heterogeneity and the accumulation of the smallest link protein at the expense of the two larger components observed during aging (Mort *et al.*, 1985).

In comparison with the complete amino acid sequence of rat chondrosarcoma link protein reported by Neame *et al.* (1986), the *N*-terminal sequence of the human analogues shows some dissimilarities. In addition to an extra *N*-linked oligosaccharide glycosylation site at residue 6, resulting from the substitution of an asparagine for a serine residue, there are three other substitutions (residues, 9, 11 and 14) among the first 20 amino acid residues (Fig. 4). One of these substitutions involves a charged residue (histidine instead of glutamine at position 11 in the human link protein). This, along with the extra carbohydrate moiety at residue 6, may contribute to the variation in proteolytically susceptible sites within the *N*-terminal region that was observed between the two species. Because of the general similarity in the *N*-terminal sequences of rat and human link proteins, and evidence from chemical deglycosylation that human LP3 is glycosylated (Mort *et al.*, 1985), it is likely that all human link proteins also contain a glycosylation site homologous to that at residue 41 of rat link protein. Therefore, in the largest human link component, there are probably two *N*-linked oligosaccharides, one at residue 6, the other at the second site, whereas both LP2 and LP3 contain an oligosaccharide only at the second site. A complete amino acid sequence of chicken cartilage link protein, deduced from cDNA clones (Deák *et al.*, 1986), also contains two potential glycosylation sites at identical positions, but its protein core in the *N*-terminal 12 amino acid residues is quite distinct from the human and rat sequences (Fig. 4).

Immunoblotting analysis provides direct evidence that the cartilage metalloproteinase is identical with stromelysin, a latent metalloproteinase previously characterized from fibroblasts (Chin *et al.*, 1985) and secreted by a variety of connective-tissue cells. Stromelysin secretion by rabbit articular-cartilage chondrocytes is enhanced by the presence of interleukin-1 (Murphy *et al.*, 1986), and as reported in the present work by human articular cartilage. However, in the chondrocyte system a co-ordinate secretion of stromelysin and collagenase was observed, whereas in the human cartilage cultures only stromelysin could be detected in the culture medium. This does not necessarily imply that co-ordinate expression of the two enzymes does not occur in the human system, but more probably reflects binding of collagenase to the cartilage matrix rather than release into the culture medium. The human cartilage stromelysin shows two electrophoretic bands of mobilities identical with those observed for human fibroblast stromelysin, where the two forms are known to represent different degrees of glycosylation of the enzyme (Wilhelm *et al.*, 1987). However, the intensity of the two components does vary between the two tissues, indicating a variation in the proportion of oligosaccharide substitution (results not shown).

On the basis of evidence presented in this paper, stromelysin secreted by human articular-cartilage chondrocytes (Campbell *et al.*, 1986*a*) is a good candidate to account for many of the degradative changes in the structure of human proteoglycan aggregates observed during aging (Roughley & Mort, 1986), and as such it may represent the normal mechanism responsible for physiological turnover. The mechanisms regulating the synthesis and activation of this metalloproteinase as well as its inhibitors are therefore undoubtedly important in maintaining the structure of the extracellular matrix and the proper functioning of cartilage. Under normal conditions, it is unclear what factors control the level of enzyme activity, though interleukin-1 could be involved, as its production by chondrocytes has been demonstrated (Olivierre *et al.*, 1986). There is, however, more compelling evidence to associate interleukin-1 with the degradation of cartilage macromolecules associated with arthritic conditions (Pujol & Loyau, 1987).

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