Matrix metalloproteinases cleave at two distinct sites on human cartilage link protein

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The actions of human recombinant stromelysins-1 and -2, collagenase, gelatinases A and B and matrilysin on neonatal human proteoglycan aggregates were examined. With the exception of gelatinase B, aggrecan was degraded extensively by most metalloproteinases studied, whereas link protein showed only limited proteolysis. Sequencing studies of modified link protein components revealed that stromelysins-1 and -2, gelatinases A and B and collagenase cleaved specifically between His^{16} and Ile^{17}, and matrilysin, stromelysin-2 and gelatinase A cleaved between Leu^{25} and Leu^{26}. Cleavage at the former bond generated a link protein component with the same N-terminus as that isolated from newborn human cartilage. Based on previously determined *in situ* cleavage sites it is evident that matrix metalloproteinases are not solely responsible for the accumulation of link protein degradation products in adult human cartilage, indicating that additional proteolytic agents are involved in the normal catabolism of human cartilage matrix.

INTRODUCTION

Matrix metalloproteinases are thought to play an important role in the turnover of the extracellular matrix under normal and pathological conditions. Recent molecular cloning studies have revealed that there are at least nine members of the matrix metalloproteinase family, including interstitial collagenase, neutrophil collagenase, gelatinase A (72 kDa), gelatinase B (92 kDa), stromelysin-1, stromelysin-2, stromelysin-3, matrilysin (pump) and murine macrophage metalloelastase (Matrisian, 1992). The enzymes contain a Zn^{2+} ion in their active site and require Ca^{2+} ions for stability. They are secreted extracellularly as proenzymes which can be activated *in vitro* by various proteolytic agents, and the active species are inhibited by specific tissue inhibitors of metalloproteinases (TIMPs).

Link protein is a glycoprotein which stabilizes the non-covalent interaction between an aggrecan molecule (proteoglycan subunit) and hyaluronate (Hardingham, 1979) in proteoglycan aggregates. Human cartilage link protein can be separated by SDS/PAGE into three components of molecular mass 48, 44 and 41 kDa, referred to as LP-1, LP-2 and LP-3 respectively (Roughley et al., 1982). LP-1 and LP-2 are two glycosylated forms of the same intact protein core, while LP-3 is proteolytically derived from either LP-1 or LP-2 (Mort et al., 1983; Nguyen et al., 1989). In cartilage of newborns, LP-3 is a minor component, whose site of generation is compatible with the action of stromelysin-1 (Nguyen et al., 1989). With age, LP-3 accumulates at the expense of LP-1 and LP-2 (Mort et al., 1983). Moreover, LP-3 isolated from adult cartilage shows heterogeneity, consisting of at least three components. Two of the N-termini correspond to those generated *in vitro* by stromelysin-1 and cathepsin B or G (Nguyen et al., 1991). There is also an accumulation of fragmented link protein molecules in adult cartilage, resulting from cleavages within the N-terminal disulphide-bonded loop which is thought to mediate the interaction between link protein and aggrecan (Mort et al., 1983; Périn et al., 1987). The identity of the proteolytic agents responsible for this link protein fragmentation remains to be determined.

Degradative changes observed in aging and osteoarthritic cartilage are closely associated with an increase in neutral metalloproteinase activity (Martel-Pelletier et al., 1984; Martel-Pelletier and Pelletier, 1987; Dean et al., 1989). While such proteolytic activity is commonly associated with the action of stromelysin-1, it is possible that other members of the metalloproteinase family could participate. In the present study, the involvement of additional members of the matrix metalloproteinase family other than stromelysin-1 in normal cartilage catabolism is examined by comparing their *in vitro* cleavage sites with the *in situ* cleavage sites on human link proteins reported previously (Nguyen et al., 1991).

MATERIALS AND METHODS

Preparation of proteoglycan aggregates

Human femoral condylar cartilage was obtained from a newborn at autopsy within 20 h of death. Aggrecan and other soluble matrix constituents were extracted from cartilage slices in 4 M guanidinium chloride/0.1 M sodium acetate buffer, pH 6.0, containing proteinase inhibitors for 70 h at 4 °C. The filtered extract was subjected to CsCl density gradient centrifugation under associative conditions as described previously (Roughley et al., 1982). Proteoglycan aggregates were recovered from the bottom of the gradients.

Preparation of metalloproteinases

Human recombinant prostromelysin-1 was expressed using a bovine papilloma-based vector with a mouse metallothionein I promoter in mouse mammary tumour (C127) cells, as described by Docherty and Murphy (1990). Human recombinant prostromelysin-2, procollagenase, progelatinase A, progelatinase B and promatrilysin were expressed without gene amplification

Abbreviations used: APMA, 4-aminophenylmercuric acid; LP-1, LP-2 and LP-3, link protein components of 48, 44 and 41 kDa respectively.

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in NSO mouse myeloma cells using a pEE12 vector containing glutamine synthetase as a selective marker (Bebbington et al., 1992). Prostromelysin-1 in serum-free culture medium of C127 cells was purified as described by Koklitis et al. (1991). Prostromelysin-2 and promatrilysin were purified as described by Murphy et al. (1991). The purification procedures for progelatinases and procollagenase have been described by Ward et al. (1991) and Murphy et al. (1992) respectively.

Enzyme activation

Proenzymes were activated under optimal conditions as described previously (Murphy et al., 1991) prior to reaction with the proteoglycan aggregates. Prostromelysin-1, prostromelysin-2, procollagenase, progelatinase B and promatrilysin were incubated with 2 mM 4-aminophenylmercuric acetate (APMA) at 37 °C for 1.5 h. Progelatinase A was activated with 2 mM APMA at 25 °C for 2 h.

Proteoglycan aggregate digestion

Proteoglycan aggregate (2 mg/ml) was digested with preactivated metalloproteinases at various enzyme concentrations (0.1, 1.0, 5.0, 10.0 and 25.0 μg of enzyme/mg of proteoglycan) in 0.1 M NaCl/10 mM $CaCl_2$/50 mM Tris/HCl buffer, pH 7.5, at 37 °C for 20 h. The reactions were terminated by the addition of EDTA to a final concentration of 10 mM. The proteoglycan aggregate was also digested with papain at 10 μg of enzyme/mg of proteoglycan in 5 mM dithiothreitol/5 mM EDTA/0.1 M sodium acetate buffer, pH 5.0, at 37 °C for 20 h. The digestion was terminated by the addition of iodoacetamide to a final concentration of 20 mM.

For sequencing studies, 10 mg of proteoglycan aggregate was digested with preactivated metalloproteinases at 5 μg of enzyme/mg of proteoglycan under the conditions described above. Modified link proteins in the reaction mixtures were purified by CsCl density gradient centrifugation under dissociative conditions (Roughley et al., 1982). Link proteins were recovered from the top of the gradients.

Analysis of aggrecan and link protein degradation products

Aggrecan fragments in the digestion mixtures were analysed using the 0.6% agarose/1.2% polyacrylamide slab-gel system, as described by Heinegard et al. (1985). Modified link proteins were analysed by SDS/PAGE under reducing conditions with 10% polyacrylamide gels (King and Laemmli, 1971), followed by electroblotting on to nitrocellulose membranes (Towbin et al., 1979). The membranes were probed with either monoclonal antibody 9/30/8A4 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A.) or CH3 (Hughes et al., 1992). Monoclonal antibody 8A4 recognizes an epitope within the C-terminal two disulphide-bonded loops of link proteins (Caterson et al., 1985; Neame et al., 1986), whereas antibody CH3 specifically recognizes a link protein component with Ile^{17} of the intact link protein as its N-terminus (Hughes et al., 1992). The blot probed with 8A4 was further incubated with ^{125}I-labelled Protein A, and link proteins were visualized by autoradiography using Kodak X-AR film, as described previously (Nguyen et al., 1989). The blot probed with antibody CH3 was incubated with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin second antibody. Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used for colour development as described previously (Hughes et al., 1992).

Sequencing of link proteins

Link proteins were separated by SDS/PAGE under reducing conditions, then electroblotted on to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA, U.S.A.), as described by Matsudaira (1987). The link proteins were visualized by brief staining with Coomassie Brilliant Blue R250, and the LP-3 components were excised and analysed on an Applied Biosystems 473A protein sequencer.

RESULTS

Characterization of aggrecan degradation products

Proteoglycan aggregates were digested with various concentrations of matrix metalloproteinases, and the aggrecan degradation products were analysed by agarose/polyacrylamide gel electrophoresis. Among the metalloproteinases tested, matrilysin most readily cleaved the aggrecan molecules, whereas gelatinase B had little degradative activity (Figure 1). At an enzyme concentration of 0.1 μg/mg of proteoglycan, matrilysin completely degraded the aggrecan into fragments of sizes only slightly larger than those of the single chondroitin sulphate chains generated by digestion with papain. At this low enzyme concentration, little aggrecan degradation was observed in the digestion mixtures generated by the other metalloproteinases, as indicated by the absence of a change in comparison with the electrophoretic profile of the untreated proteoglycan aggregate. At higher enzyme concentrations, collagenase and gelatinase A generated aggrecan fragments of smaller average size than those generated by stromelysin-1 or -2. The two stromelysins produced aggrecan fragments of a similar average size.

Characterization of link protein degradation products

Modified link proteins in the digestion mixtures were analysed by immunoblotting using monoclonal antibody 8A4 (Figure 2). Matrilysin completely converted all of the LP-1 and LP-2 into an LP-3 component, even at a concentration as low as 0.1 μg of enzyme/mg of proteoglycan. Collagenase, stromelysin-2 and gelatinase A were also capable of converting LP-1 and LP-2 into LP-3 components. However, a substantial amount of LP-1

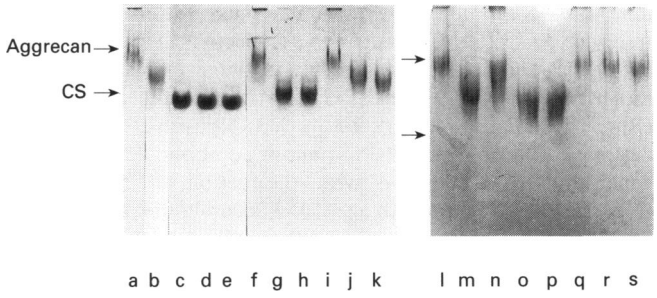

Figure 1 Degradation of aggrecan in proteoglycan aggregates with various concentrations of matrix metalloproteinases

Proteolysis was performed with human recombinant matrilysin (lanes c–e), collagenase (lanes f–h), stromelysin-2 (lanes i–k), gelatinase A (lanes n–p) and gelatinase B (lanes q–s) at increasing concentrations of enzymes (0.1, 5.0 and 10 μg/mg of proteoglycan respectively), and degradation products were analysed by agarose/polyacrylamide gel electrophoresis. Intact proteoglycan aggregate (lanes a and l) and proteoglycan degradation products produced by stromelysin-1 at 5 μg of enzyme/mg of proteoglycan (lanes b and m) were also analysed. The migration positions of intact aggrecan and chondroitin sulphate chains (CS) generated by the action of papain are indicated.

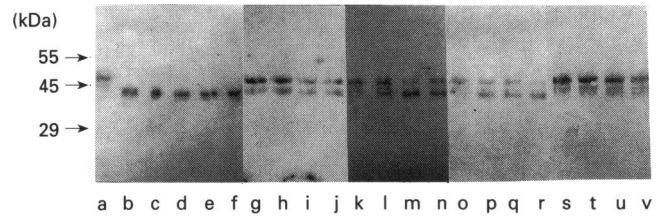

Figure 2 Degradation of link protein present in proteoglycan aggregates with various concentrations of matrix metalloproteinases

Link proteins present in intact proteoglycan aggregates (lane a) and link protein components generated by the actions of matrilysin (lanes c–f), collagenase (lanes g–j), stromelysin-2 (lanes k–n), gelatinase A (lanes o–r) and gelatinase B (lanes s–v) at increasing concentrations of enzyme (1.0, 5.0, 10.0 and 25 μg/mg of proteoglycan respectively) were analysed by SDS/PAGE, followed by immunoblotting using monoclonal antibody 8A4. Link protein modified by matrilysin at an enzyme concentration of 0.1 μg/mg of proteoglycan was also analysed (lane b).

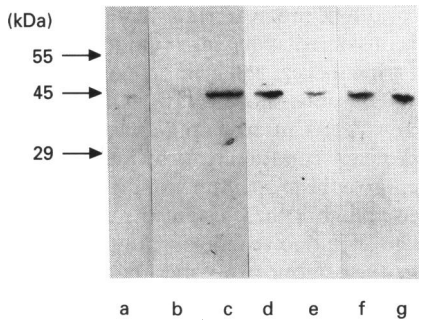

Figure 4 CH3 epitope in link proteins modified by matrix metalloproteinases

Link proteins present in intact proteoglycan aggregates (lane a) and those modified by matrilysin (lane b), collagenase (lane c), stromelysin-1 (lane d), stromelysin-2 (lane e), gelatinase A (lane f) and gelatinase B (lane g) were analysed by SDS/PAGE, followed by immunoblotting using monoclonal antibody CH3.

remained, even at concentrations of 10 μg of enzyme/mg of proteoglycan. Link protein was quite resistant to degradation by gelatinase B, as indicated by the lack of change in the relative abundance of the various link protein components even at a concentration of 25 μg of enzyme/mg of proteoglycan. There was no evidence that any member of the matrix metalloproteinase family is capable of generating fragmented link protein molecules analogous to those observed in normal adult cartilage (Mort et al., 1983).

N-terminal sequence analysis of modified link proteins

LP-3 components generated *in vitro* by the action of human recombinant stromelysin-1, collagenase and gelatinase B yielded a single sequence having Ile^{17} of the intact link protein as its N-terminal residue (Figure 3). A single sequence starting at Leu^{26} of the intact link protein was obtained for the link protein components modified by matrilysin. Stromelysin-2 and gelatinase A produced link protein components containing two sequences with Ile^{17} and Leu^{26} as their respective N-termini. The yield of the sequence commencing with Ile^{17} accounted for about 50% and 70% of the modified link proteins generated by stromelysin-2 and gelatinase A respectively.

Analysis of the CH3 epitope in modified link proteins

Proteoglycan aggregate samples which were digested under the same conditions used for sequencing analysis were also analysed by immunoblotting using monoclonal antibody CH3 (Figure 4). There was minimal CH3 epitope in link proteins present in undigested proteoglycan aggregates and a complete absence of this epitope in link proteins modified by matrilysin cleavage. However, link proteins generated in the digestion using stromelysin-1, collagenase, gelatinase A or gelatinase B yielded a strong CH3-reactive band, with a relatively weaker band for those produced by stromelysin-2. These results are compatible with the sequencing data. Moreover, they clearly indicated that most matrix metalloproteinases yielded *in vitro* a link protein component having the same N-terminus as that of an LP-3 component present in human cartilage (Nguyen et al., 1991).

DISCUSSION

The mechanism for the turnover of the extracellular matrix in connective tissues under normal and pathological conditions is complex and probably involves many proteolytic agents. In the present study, the potential role of several members of the family of matrix metalloproteinases in cartilage degradation was investigated.

Among the enzymes tested, matrilysin most extensively degraded the components of a neonatal human proteoglycan aggregate, whereas this appeared to be a very poor substrate for gelatinase B. This result is compatible with activity data previously reported using a proteoglycan bead assay (Murphy et al., 1991). It is interesting that collagenase has the ability to degrade

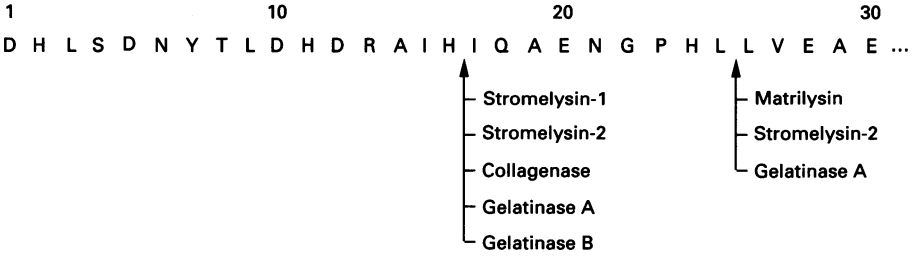

Figure 3 Cleavage sites generated by the action of matrix metalloproteinases on link proteins present in proteoglycan aggregates

The N-terminal sequence of intact human link protein deduced from cDNA clones (Dudhia and Hardingham, 1990) is indicated, together with the cleavage sites generated *in vitro* by matrilysin, collagenase, stromelysin-1, stromelysin-2, gelatinase A and gelatinase B.

both aggrecan and link proteins, since mammalian collagenases are commonly considered as having a unique action in cleaving the α-chains of triple-helical collagens at specific sites. However, Hughes et al. (1991) have shown that recombinant collagenase can also degrade aggrecan from pig laryngeal cartilage. The generation of proteoglycan fragments by these proteinases could be very detrimental to the proper functioning of cartilage, because of the presumed ability of these fragments to diffuse readily out of the tissue. This would lower the overall fixed negative charge and thus change the osmotic properties that enable the cartilage to resist the effects of compressive loading.

Sequencing studies, together with the immunoblotting analysis using monoclonal antibody CH3, revealed that matrix metalloproteinases cleave link protein at two distinct peptide bonds, His^{16}–Ile^{17} and Leu^{25}–Leu^{26}. Stromelysin-1, collagenase and gelatinase B yielded a product due to cleavage at the bond between His^{16} and Ile^{17}, whereas matrilysin gave a product resulting from cleavage between Leu^{25} and Leu^{26}. This change in peptide bond specificity is apparently not due to the lack of a hemopexin-like C-terminal domain in matrilysin, the only truncated member of the matrix metalloproteinase family, since stromelysin-2 and gelatinase A can hydrolyse both bonds. Moreover, previous studies have shown that the removal of the C-terminal domain has little effect on the activation, proteolytic activity and substrate specificity of stromelysin-1 (Okada et al., 1986; Koklitis et al., 1991; Murphy et al., 1992).

Cleavage at the bond between His^{16} and Ile^{17} in intact link protein would yield a modified link protein component with the same N-terminus as that of native LP-3 isolated from neonatal human cartilage (Nguyen et al., 1989). This component also corresponds to one of several LP-3 components extracted from normal adult cartilage (Nguyen et al., 1991). Previously it was observed that stromelysin-1 purified from the culture medium of cartilage explants stimulated with interleukin-1β was the only physiologically relevant enzyme capable of cleaving link proteins at this site *in vitro* (Nguyen et al., 1991). However, the results of the present study indicate that most matrix metalloproteinases can hydrolyse this bond *in vitro*. It is apparent, though, that some of the proteinases would also generate cleavage at the Leu^{25}–Leu^{26} bond, and such a site of action has not been observed in the generation of LP-3 components in human cartilage. Thus the most probable metalloproteinases acting in the cartilage matrix are stromelysin-1 and collagenase. It has been shown recently that prostromelysin is expressed to a greater extent in human cartilage than is procollagenase (Nguyen et al., 1992). In addition, proteoglycan is a preferred substrate for stromelysin (Murphy et al., 1991), and hence it is likely that stromelysin plays a dominant role in cartilage proteoglycan metabolism relative to other metalloproteinases.

It is evident from this study that none of the matrix metalloproteinases is able to cleave within the first N-terminal disulphide-bonded loop of link protein to generate the fragmented link protein molecules observed in human adult cartilage (Mort et al., 1983). This implies that other proteolytic agents are involved in the catabolic processes occurring within the cartilage matrix. The occurrence of multiple forms of LP-3 in adult cartilage would also support such a conclusion (Nguyen et al., 1991). Recently, stromelysin-1, gelatinases and matrilysin have been shown to cleave the interglobular domain of human and porcine cartilage aggrecan at the bond between Asn^{341} and Phe^{342} (Fosang et al., 1991, 1992; Flannery et al., 1992). The product of cleavage at this site corresponds to the C-terminus of a minor portion of the G_1 domain (hyaluronic-acid binding region of aggrecan) that accumulates in adult human cartilage (Flannery et al., 1992). However, the major degradation product of aggrecan present in the human cartilage matrix (Flannery et al., 1992), in human synovial fluid collected at all phases of osteoarthritis (Sandy et al., 1992), and in culture media of bovine cartilage explants under control conditions and on stimulation by interleukin-1β appears to be derived from cleavage at the bond between Glu^{373} and Ala^{374}. The identity of the proteolytic agent responsible for the hydrolysis of this bond is still unknown, and it is possible that the same proteolytic agent might be responsible for some link protein cleavage in the adult.

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