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Link protein as a monitor *in situ* of endogenous proteolysis in adult human articular cartilage

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The link protein components of proteoglycan aggregates in adult human articular cartilage show heterogeneity due to proteolysis. Cleavages near the *N*-terminus of the intact link proteins, before residues 17, 19 and 24, generate three proteins of slightly diminished size (LP3). Cleavages within the *N*-terminal disulphide-bonded loop, before residues 66 and 73 of the intact link proteins, generate proteins that yield smaller degradation products upon reduction (LP fragments). *In vitro*, modified link protein components of a similar size to LP3 can be generated by a variety of proteinases, but of the physiologically relevant enzymes only stromelysin, cathepsin B and cathepsin G have the ability to yield modified link proteins with *N*-termini identical with those observed *in situ*. None of the proteolytic agents tested was able to produce LP fragments with *N*-termini identical with those observed *in situ*, and the majority of proteinases were not able to cleave within the disulphide-bonded loops. Cathepsin L and hydroxyl radicals can cleave within the *N*-terminal disulphide-bonded loop, and have the potential of initially opening the loop to allow further proteolytic processing by other agents to generate the native cleavage sites.

INTRODUCTION

Proteoglycans and collagens are major structural macromolecules in the extracellular matrix of cartilage. While collagen fibrils give cartilage its tensile strength and maintain its shape, proteoglycan molecules endow the tissue with its osmotic properties, particularly its ability to resist compressive forces. In hyaline cartilage, most proteoglycan occurs in aggregates, which consist of a hyaluronate filament bearing many proteoglycan subunits (Hardingham & Muir, 1974). The non-covalent interaction between each proteoglycan subunit and the hyaluronate filament is stabilized by additional interaction with a link protein (Hardingham, 1979). In neonatal human articular cartilage, link proteins can be separated into three components of M_r 48000, 44000 and 41000, which are referred to as LP1, LP2 and LP3 respectively, with LP1 predominating (Roughley *et al.*, 1982). It has been shown that LP1 differs from LP2 by the presence of an extra *N*-linked oligosaccharide at residue 6 on LP1, and that LP3 is proteolytically derived from either LP1 or LP2 by the removal of the *N*-terminal 16 amino acid residues, probably via the action of stromelysin (Nguyen *et al.*, 1989). The recently elucidated primary structure of link protein reveals that the molecule contains three disulphide-bonded loops (Neame *et al.*, 1986; Dudhia & Hardingham, 1990). The first disulphide-bonded loop, nearest to the *N*-terminus, has an immunoglobulin-fold-like sequence and it is thought that this loop mediates the protein–protein interaction between link protein and a proteoglycan subunit (Périn *et al.*, 1987). The other two disulphide-bonded loops show a high degree of sequence similarity between themselves, and it appears that they are involved in the non-covalent interaction between link protein and the hyaluronate filament (Périn *et al.*, 1987). Proteolytic cleavage within the *N*-terminal disulphide-bonded loop is thought to give rise to a variety of link protein degradation products, termed LP fragments, that are apparent upon reduction of the molecules (Mort *et al.*, 1983).

The structure of the proteoglycan aggregate is not constant throughout life (Roughley & Mort, 1986), and some of the changes appear to be due to proteolytic degradation. Examples are the decrease in size of the proteoglycan subunits, the accumulation of isolated hyaluronate-binding regions derived from the proteoglycan subunits, the accumulation of LP3 at the expense of LP1 and LP2, and the accumulation of LP fragments (Buckwalter & Rosenberg, 1982; Mort *et al.*, 1983; Roughley *et al.*, 1985; Roberts *et al.*, 1989). The progressive depletion of proteoglycans in arthritic cartilage has also been attributed to the action of proteolytic agents. There is circumstantial evidence implicating a number of enzymes in this destructive process, including the cysteine proteinases cathepsins B and L (Bayliss & Ali, 1978; Barrett *et al.*, 1988), the aspartic proteinase cathepsin D (Poole & Mort, 1981), serine proteinases such as cathepsin G, polymorphonuclear-leucocyte elastase and plasmin (Sandy *et al.*, 1981; Mochan & Keler, 1984) and the neutral and acidic cartilage metalloproteinases (Woessner & Selzer, 1984; Azzo & Woessner, 1986). Recently it was shown that the neutral metalloproteinase from human articular cartilage is identical with stromelysin (Gunza-Smith *et al.*, 1989). These potentially destructive enzymes are primarily derived from the chondrocytes and cells within the surrounding tissues, such as synovial fibroblasts, macrophages and polymorphonuclear leucocytes. Proteinases released from the latter cell types are thought to play a dominant role in the degradation of cartilage under inflammatory conditions (Harris, 1989). However, the exact identity of proteolytic agents responsible for the degeneration of cartilage in aging and arthritis is still not certain. In the present study, the cleavage sites in link proteins generated *in vitro* by various proteolytic agents are compared with the cleavage sites found in link proteins isolated from normal, osteoarthritic and rheumatoid-arthritic adult cartilage, in order to identify the proteolytic agents involved in the degradation of cartilage during aging and arthritis.

MATERIALS AND METHODS

Source of proteinases and inhibitors

Polymorphonuclear-leucocyte elastase and cathepsin G, from human sputum, were purchased from Elastin Products (Pacific, MO, U.S.A.). Elastase and L-tosyl-lysylchloromethane-treated

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chymotrypsin, both from bovine pancreas, human plasma plasmin and all proteinase inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pepsin was obtained from Worthington Biomedical Corp. (Freehold, NJ, U.S.A.). Cathepsin D was isolated and purified from human liver as described previously (Mort *et al.*, 1981).

Preparation of proteoglycan aggregates

Human femoral condylar cartilage with no macroscopic evidence of cartilage degeneration was obtained from one newborn (4 months old) and three adults (33, 44 and 60 years old) at the time of autopsy and within 20 h of death. Surgical samples from three osteoarthritic patients (59, 64 and 65 years old) and two rheumatoid-arthritic patients (61 and 73 years old), undergoing total knee arthroplasty, were also obtained. Proteoglycan was 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, and the dialysed extract was subjected to CsCl-density-gradient centrifugation under associative conditions as described previously (Roughley *et al.*, 1982), with a starting density of 1.50 g/ml. Proteoglycan aggregates were recovered from the bottom of the gradient.

Isolation of link proteins

Link proteins were prepared from proteoglycan aggregates by CsCl-density-gradient centrifugation under dissociative conditions (Roughley *et al.*, 1982). For optimal separation between link proteins and hyaluronate molecules, a starting density of 1.35 g/ml was used. Link proteins were recovered from the top of the gradient.

Proteolytic degradation of proteoglycan aggregates

Neonatal proteoglycan aggregates (3 mg/ml), which contain minimal LP3, were digested by a variety of proteinases at an enzyme concentration of 10 μg/mg of proteoglycan. Incubation with human polymorphonuclear-leucocyte elastase, cathepsin G and bovine pancreatic elastase was in 0.1 M-Tris/HCl buffer, pH 8.5, for 4 h at 37 °C. The reactions were terminated by the addition of di-isopropyl phosphorofluoridate to a final concentration of 10 mM. Incubation with chymotrypsin for 4 h, and plasmin for 24 h, was in 0.1 M-Tris/HCl buffer, pH 7.5, at 37 °C. Chymotrypsin was inactivated by the addition of soya-bean trypsin inhibitor to a final concentration of 20 μg/mg of proteoglycan. Incubation with pepsin for 4 h, and cathepsin D for 24 h, was in 0.1 M-sodium acetate buffer, pH 5.0, at 37 °C. Pepsin was inactivated by the addition of pepstatin to a final concentration of 20 μg/mg of proteoglycan. The reaction between proteoglycan aggregate (3 mg/ml) and H_2O_2 (240 mM) was performed in 25 mM-sodium acetate buffer, pH 5.6, containing 80 mM-NaCl at 37 °C for 24 h, as described by Roberts *et al.* (1989). Modified link protein components still interacting with the hyaluronate were routinely separated from fragments of the proteoglycan subunits by CsCl-density-gradient centrifugation under associative conditions (Hascall & Heinegård, 1974). In the case of plasmin and H_2O_2 digestion, modified link proteins were purified by CsCl-density-gradient centrifugation under dissociative conditions (Roughley *et al.*, 1982), since these agents produced a less extensive degradation of the proteoglycan subunits, resulting in modified aggregates that still possessed a high density.

Analysis of link proteins

Link proteins were analysed by SDS/PAGE under reducing conditions with 10% (w/v) polyacrylamide gels (King & Laemmli, 1971), followed by electroblotting on to nitrocellulose membranes (Towbin *et al.*, 1979). The membranes were probed with monoclonal antibody to link protein, 9/30/8-A-4 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A.), which recognizes an epitope within the two *C*-terminal disulphide-bonded loops of link proteins isolated from many species (Caterson *et al.*, 1985; Neame *et al.*, 1986). Subsequently, the membranes were treated with ^{125}I-labelled Protein A, as described previously (Nguyen *et al.*, 1989), and link proteins were located by autoradiography with Kodak X-AR film.

Sequencing of link proteins

Link proteins were separated by SDS/PAGE, followed by electroblotting on to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA, U.S.A.) as described by Matsudaira (1987). Coomassie Brilliant Blue R250-stained link protein components were excised and analysed on an Applied Biosystems 470A gas-phase sequencer equipped with an on-line amino acid phenylthiohydantoin analyser.

RESULTS

N-Terminal sequencing of LP3 components generated *in vitro*

All the proteinases under investigation (polymorphonuclear-leucocyte elastase, cathepsin G, pancreatic elastase, chymotrypsin, plasmin, pepsin and cathepsin D) were able to degrade the link protein component of a neonatal proteoglycan aggregate to a product having a similar electrophoretic mobility to the native LP3. Only chymotrypsin, however, was capable of generating small LP fragments similar to those observed in adult human cartilage link protein preparations upon reduction. A similar observation for chymotrypsin has previously been reported for its action on the proteoglycan aggregates derived from the Swarm rat chondrosarcoma (Stevens & Hascall, 1986).

When the modified link protein components, generated *in vitro* by the action of various proteinases, were subjected to protein sequence analysis, all cleavage sites responsible for the production

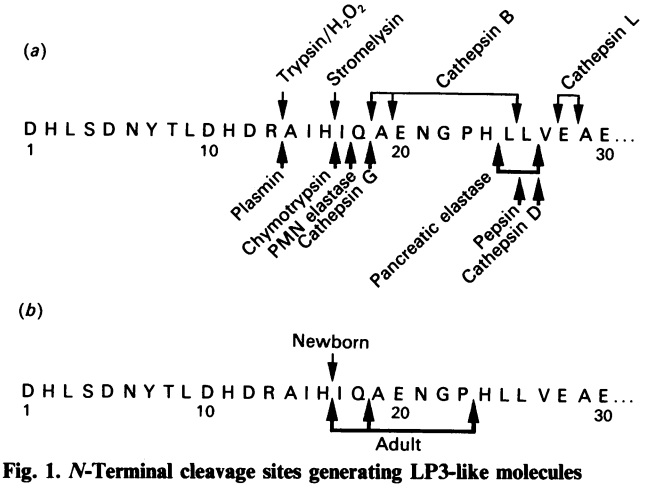

Fig. 1. *N*-Terminal cleavage sites generating LP3-like molecules

(*a*) Cleavage sites generated *in vitro* by the action of proteinases on link proteins in proteoglycan aggregates. Below, arrows indicate the cleavage sites for proteinases used in this study, and above, arrows indicate previously published cleavage sites for additional agents (Nguyen *et al.*, 1989, 1990; Roberts *et al.*, 1989). (*b*) Cleavage sites responsible for generating native LP3 in cartilage. Below, arrows indicate the sites at which cleavage has occurred to generate the modified *N*-terminal sequences in adult cartilage, and above, the arrow indicates the previously published site for LP3 generation in neonatal cartilage (Nguyen *et al.*, 1989). The sequence of the first 30 amino acid residues of intact human link protein deduced from cDNA clones (Dudhia & Hardingham, 1990) is indicated. Abbreviation: PMN elastase, polymorphonuclear-leucocyte elastase.

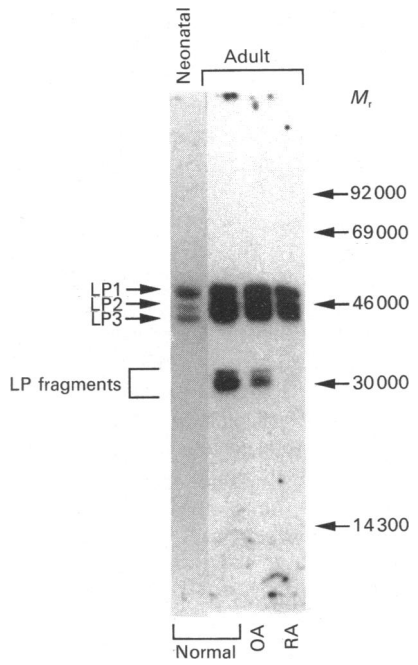

Fig. 2. Heterogeneity of link proteins isolated from normal and arthritic adult human articular cartilage

Link proteins isolated from normal, osteoarthritic (OA) and rheumatoid-arthritic (RA) adult cartilage were analysed by SDS/PAGE, followed by immunoblotting. For comparison, link proteins isolated from normal neonatal cartilage are also depicted. The migration positions of phosphorylase *b* (M_r 92000), BSA (M_r 69000), ovalbumin (M_r 46000), carbonic anhydrase (M_r 30000) and lysozyme (M_r 14300) are indicated.

of LP3-like molecules were found to be located within a 15-residue polypeptide segment commencing at residue 13 of the intact link protein (Fig. 1*a*). Furthermore, each proteinase, with the exception of pancreatic elastase, generated only a single LP3-like molecule, which possessed an *N*-terminus characteristic of the enzyme used for its generation. Pancreatic elastase yielded a mixture of two LP3-like molecules, one of which was analogous to that generated by cathepsin D.

N-Terminal sequencing of LP3 isolated from normal and arthritic adult cartilage

Link proteins extracted from adult human articular cartilage, obtained from eight different individuals, were analysed by SDS/PAGE, followed by immunoblotting with a monoclonal antibody to link protein. The pattern observed appeared to differ among the individuals, and representative samples are illustrated in Fig. 2. Link proteins extracted from normal adult cartilage obtained at autopsy, where no joint pathology was apparent, and those extracted from the cartilage remaining in osteoarthritic joints were characterized by a high abundance of LP3 and the presence of LP fragments, though the proportion of LP fragments appeared to be lower from the osteoarthritic samples. In contrast, link proteins isolated from the cartilage remaining in the rheumatoid-arthritic joints had LP1 as the most abundant component, and LP fragments were not detected.

When the LP3 components were subjected to protein sequence analysis, multiple *N*-termini were observed (Fig. 1*b*), corresponding to residues 17, 19 and 24 of the intact link proteins, and each sequence appeared to be present in similar abundance. An analogous result was obtained for all eight specimens examined, irrespective of the joint pathology. The sequence commencing at residue 17 is identical with that obtained when the intact link protein is treated *in vitro* with stromelysin or chymotrypsin, and the sequence commencing at residue 19 is identical with that obtained when the intact link protein is treated *in vitro* with cathepsin B or G (Fig. 1*a*). The remaining sequence commencing at residue 24 does not correspond to any of the LP3-like products generated by the proteinases used in this or previous studies (Fig. 1*a*).

N-Terminal sequencing of native LP fragments and LP fragments generated *in vitro*

Native LP fragments, derived from the adult cartilage link proteins by SDS/PAGE under reducing conditions, were also subjected to protein sequence analysis. Two dominant sequences were observed with *N*-termini commencing with tryptophan and tyrosine, corresponding to residues 66 and 73 respectively, in the intact link protein (Fig. 3). These *N*-terminal sequences are nine and 16 residues further towards the *C*-terminus of the link protein than the *N*-terminal sequence of a previously characterized LP fragment generated by the action of cathepsin L (Nguyen *et al.*, 1990).

Treatment of the proteoglycan aggregate *in vitro* with H_2O_2 has also been shown to generate two major products of similar size to the native LP fragments (Roberts *et al.*, 1987). On protein sequencing, the larger LP fragment (M_r 32000) generated by the action of H_2O_2 failed to yield a sequence, whereas the smaller LP fragment (M_r 29000) had lysine, corresponding to position 87 in the intact link protein, as its *N*-terminus (Fig. 3). This cleavage site is *C*-terminal to those that give rise to the native LP fragments. Interestingly, the His-Lys sequence that surrounds this cleavage site also occurs at residues 60 and 61, which if cleaved would generate an LP fragment of comparable size with the M_r 32000 species. Potentially, radical action could result in a blocked *N*-terminus at this site, so accounting for the inability to sequence this molecule. The only proteinase, apart from cathepsin L, observed to cleave within a disulphide-bonded loop is chymotrypsin. The action of this enzyme was found to be between residues 55 and 56 of the intact link protein, a site just one residue before that at which cathepsin L acts.

DISCUSSION

One potential approach for gaining an insight into the identity of the endogenous proteolytic agents responsible for cartilage degradation is the use of a constituent matrix protein as a

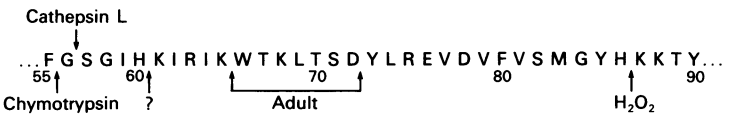

Fig. 3. Internal cleavage sites generating LP fragments within adult cartilage link proteins

The partial sequence (residues 55–90) in the first disulphide-bonded loop of human link protein is depicted, based on data inferred from cDNA clones (Dudhia & Hardingham, 1990). Below, arrows indicate the cleavage sites responsible for generating the native LP fragments, and the observed and potential (?) cleavage sites generated by H_2O_2 and chymotrypsin. Above, the arrow indicates the previously published cleavage site for cathepsin L (Nguyen *et al.*, 1990).

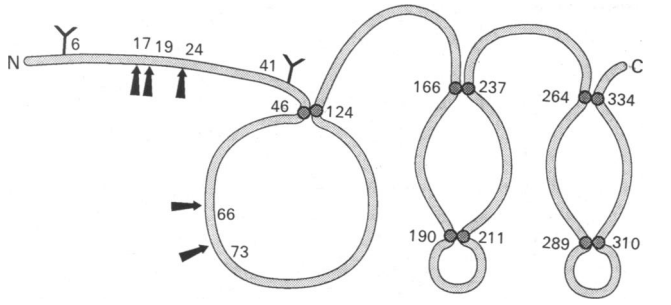

Fig. 4. Schematic representation of an intact human link protein, drawn to scale based on cDNA sequencing data (Dudhia & Hardingham, 1990)

Arrows indicate the sites where endogenous proteolytic modification occurs in adult cartilage. The number adjacent to each arrow indicates the amino acid residue in the intact link protein that becomes a free *N*-terminus in the modified link protein. The *N*-terminus (N), *C*-terminus (C), *N*-linked glycosylation sites (Y) and the position of disulphide bonds (●●) are also indicated.

monitor *in situ*. Ideally, the selected protein should show a limited susceptibility to degradation, and after proteolytic modification it should be retained within the matrix. Furthermore, each proteolytic agent should act at distinct cleavage sites, so that characteristic products are produced. Link protein, when part of a proteoglycan aggregate, exhibits such a limited susceptibility to proteolysis, and the results of this and previous papers (Nguyen *et al.*, 1989, 1990; Roberts *et al.*, 1989) indicate that different proteolytic agents do indeed produce characteristic cleavage sites. Furthermore, similar degradation products have been shown to accumulate within the cartilage matrix with age (Mort *et al.*, 1983), suggesting that the degradation products are retained within the matrix over many years of life. Thus link protein would appear to satisfy all the criteria necessary to justify its use as a monitor *in situ* to identify proteolytic agents involved in the endogenous degradation of cartilage extracellular matrix throughout life.

In studies *in vitro* all proteolytic agents tested in this and previous work were able to cleave the link protein within the *N*-terminal region, to yield a modified link protein (LP3) whose new *N*-terminal residue corresponds to an amino acid situated between residues 14 and 29 in the intact link protein (Fig. 1*a*). When native adult LP3 was examined, three *N*-terminal sequences were apparent, all falling within the same region (Fig. 1*b*), suggestive of a proteolytic origin. The only physiologically relevant proteolytic agents able to produce identical degradation products are stromelysin, cathepsin B and cathepsin G. This is different from the situation previously observed in neonatal human articular cartilage, where only a single *N*-terminus was observed for LP3, compatible with the action of stromelysin (Nguyen *et al.*, 1989).

In addition to their increased proportion of LP3, adult cartilage link proteins also show proteolytic fragmentation within one of their disulphide-bonded loops (Mort *et al.*, 1983). The size of these LP fragments and their ability to interact with the monoclonal antibody 8-A-4 have led to the suggestion that they are derived by cleavage within the first (*N*-terminal) disulphide-bonded loop. Sequence analysis of the native LP fragments confirms this observation (Fig. 4). Only two agents of physiological relevance, cathepsin L and hydroxyl radicals, are able to cleave within this region, but neither can reproduce the natural cleavage sites. Nevertheless, these proteolytic agents could still be involved in the generation of native LP fragments by initially cleaving the first disulphide-bonded loop at sites before the native cleavage sites, so altering its conformation and allowing additional proteolytic processing to take place by other agents.

The participation of stromelysin in the normal turnover of cartilage matrix in both the newborn and adults is not unexpected, since chondrocytes constitutively synthesize and secrete prostromelysin (Nguyen *et al.*, 1989), which when activated in the matrix can function over a wide pH range (Campbell *et al.*, 1986). The extracellular activity of cathepsins B and L is less clear, as these lysosomal cysteine proteinases are unstable and rapidly denatured under neutral physiological pH. More stable high-M_r forms of both cathepsins B and L have been reported in several organ and cell culture systems (Mort & Recklies, 1986; Kominami *et al.*, 1988), though a physiological role for these forms is still uncertain. Cathepsin G is found in the azurophil granules of polymorphonuclear leucocytes, and its action on cartilage matrix components could be the result of its release from these cells, possibly during subclinical episodes of inflammation, which may occur throughout life. Surprisingly, there was no evidence for the activity of polymorphonuclear-leucocyte elastase, another major azurophil-granule proteinase, though this could be due to the higher abundance of the natural elastase inhibitor (α_1-proteinase inhibitor) than the natural cathepsin G inhibitor (α_1-antichymotrypsin) (Ohlsson, 1978). Also, during such subclinical inflammatory episodes, hydroxyl radicals, which are generated from reactive oxygen metabolites ($O_2^{\cdot-}$, H_2O_2) released by activated inflammatory cells, could find access to cartilage matrix components. In addition, it has recently been shown that articular-cartilage chondrocytes are able to produce and secrete H_2O_2 (Tiku *et al.*, 1990).

With age, there is an accumulation of the proteolytically modified link protein components (Mort *et al.*, 1983) and such modified link proteins could potentially have an adverse effect on the proper functioning of cartilage. Although the *N*-terminal cleavages described in this study are unlikely to affect link protein function, those occurring within the first disulphide-bonded loop, which has been shown to mediate the protein–protein interaction between link protein and proteoglycan subunit (Périn *et al.*, 1987), could potentially alter the degree of proteoglycan aggregate stability. In contrast, there was no evidence for proteolytic modification occurring within the tandemly repeated disulphide-bonded loops associated with the interaction between link protein and hyaluronate, or in the regions that join the different loops. Thus interaction of the modified link proteins with hyaluronate should not be impaired, and this may account for their observed accumulation with age.

In the limited number of specimens examined, the link protein heterogeneity pattern appears different between cartilage from normal and arthritic joints, with the cartilage from the arthritic joints consistently showing a decreased proportion of proteolytically modified link proteins, possibly indicative of an ongoing hypertrophic repair process in the cartilage remaining in these joints. Irrespective of these differences the *N*-terminus present in the modified link proteins was the same in all cases. This would indicate that as a general rule the proteolytic processes taking place in the arthritic cartilage need not necessarily differ from those taking place in normal cartilage, though the rate at which degradation is proceeding would be expected to vary. In addition, one cannot exclude the possibility that unique agents may have been acting at the focal sites of degeneration, and not throughout the cartilage in the arthritic joint.

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