The action of human articular-cartilage metalloproteinase on proteoglycan and link protein

Similarities between products of degradation *in situ* and *in vitro*

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Interleukin 1 stimulation of human articular cartilage in organ culture produced the concomitant release of proteoglycan fragments and latent metalloproteinase. The released fragments ranged in size from that of almost intact proteoglycan subunits to the product of limiting digestion generated by the activated metalloproteinase. None of the fragments possessed the ability to interact with hyaluronic acid. Analysis of proteoglycan aggregate digested with the activated metalloproteinase showed that isolated hyaluronic acid-binding regions were produced from the proteoglycan subunits, and that the two higher- M_r link-protein components (M_r 48 000 and 44 000) were converted into the lowest- M_r component (M_r 41 000). Link protein extracted from cartilage under stimulation with interleukin 1 showed a similar conversion. These results suggest that interleukin 1 stimulates the release of latent metalloproteinase from chondrocytes and that a proportion of the enzyme is activated *in situ* in the cartilage matrix. The mode of action of the activated enzyme is compatible with a role in the changes in proteoglycan structure seen in aging.

INTRODUCTION

Articular cartilage can be thought of as a solution of proteoglycan entrapped in a collagenous network. The proteoglycan serves a major role in the retention of water within the tissue, and in so doing endows the cartilage with a resilience towards compressive forces (Maroudas *et al.*, 1985; Kempson *et al.*, 1976). The normal mode of existence of the proteoglycans within the cartilage is as aggregates, comprising a central filament of hyaluronic acid to which many proteoglycan subunits are attached via the hyaluronic acid-binding terminus of their core proteins (Hascall, 1977), with each interaction being stabilized by the presence of a link protein (Oegema *et al.*, 1977; Tang *et al.*, 1979). During aging the structure of the proteoglycan changes (Bayliss & Ali, 1978; Roughley & White, 1980). The proteoglycan subunits themselves show a decrease in size, primarily owing to variation in their glycosaminoglycan-attachment region. Some of the changes on aging can be ascribed to the action of proteinases within the extracellular matrix, resulting in retention of fragments containing the hyaluronic acid-binding region and loss of those comprising the glycosaminoglycan-attachment region (Roughley *et al.*, 1984, 1985). Further evidence for such proteolytic fragmentation can be seen in the modifications that occur to the link proteins with age (Mort *et al.*, 1983).

A family of monocyte-derived proteins, termed interleukin 1 (Dinarello, 1984), have the ability to induce proteinase secretion by chondrocytes, and hence promote degradation of the proteoglycan (Campbell *et al.*, 1986). These factors have been referred to as 'mononuclear cell factor' when assayed by stimulation of proteinase secretion from synovial cells and fibroblasts (Dayer *et al.*, 1977), or as 'catabolin' when assayed by cartilage

resorption (Saklatvala *et al.*, 1984). In bovine nasal cartilage it has been shown that the proteoglycan degradation induced by pig monocyte catabolin appears to occur preferentially at the hyaluronic acid-binding region of the proteoglycan subunits, resulting in release of apparently intact glycosaminoglycan-attachment regions, together with smaller fragments (Tyler, 1985). Such an action may be implicated in the depletion of proteoglycan associated with cartilage in arthritic joints, but it may also contribute to the normal age-related changes in proteoglycan structure.

We have shown previously (Campbell *et al.*, 1986) that explants of human articular cartilage, cultured in the presence of mononuclear-cell-conditioned medium as a source of interleukin 1, secrete a latent metalloproteinase, which when activated is capable of degrading proteoglycan. The purpose of the present work was to study the action of this proteinase on the proteoglycan components of human articular cartilage, both *in situ* and *in vitro*.

METHODS

Materials

Human articular cartilage was obtained at the time of autopsy from the femoral condyles of individuals in whom there was no clinical evidence of joint disease. Tissue was taken within 20 h of death, and any areas showing macroscopic signs of degeneration were avoided. Interleukin 1 was kindly supplied by Dr. Elaine E. Golds (Shriners Hospital, Montreal), having been obtained from human peripheral-blood mononuclear-cell cultures by stimulation with phytohaemagglutinin as described previously (Golds *et al.*, 1983). Purification was by isoelectric focusing of the culture medium and subsequent

Abbreviation used: APMA, 4-aminophenylmercuric acetate.

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chromatography on Ultrogel AcA54. Adult human articular-cartilage proteoglycan subunits and neonatal proteoglycan aggregates were prepared as described previously (Roughley *et al.*, 1982).

Culture and stimulation of cartilage

Adult cartilage was maintained in organ culture as described previously (Campbell *et al.*, 1986; Handley & Lowther, 1977). After 2 days, culture was performed in the presence of interleukin 1, at a concentration shown previously to induce maximal stimulation of metalloproteinase secretion. Proteoglycan content was assayed by the dimethylmethylene blue dye-binding assay (ΔA_{535}) of Farndale *et al.* (1982), with bovine nasal-cartilage proteoglycan as a standard, and proteinase activity was monitored by the release of proteoglycan from polyacrylamide discs (Sandy *et al.*, 1981; Campbell *et al.*, 1986).

Proteolytic degradation of proteoglycan

Proteoglycan preparations were dissolved in 0.1 M-Tris/HCl, pH 7.0, containing 0.1 M-NaCl and 5 mM-CaCl₂. Digestion was initiated by adding partially purified cartilage metalloproteinase in the same buffer and making the resulting solution 1 mM in APMA. The metalloproteinase preparation was obtained from the culture medium of adult human cartilage stimulated with interleukin 1, and purified using Ultrogel AcA44 (Campbell *et al.*, 1986) to separate the enzyme (which is included) from endogenous proteoglycan fragments (which are eluted at the void volume). It was then dialysed into the incubation buffer before use. Samples were incubated at 37 °C for up to 48 h and, when required, enzyme activity was terminated by the addition of EDTA to 10 mM. In other experiments endogenous proteoglycan present in the culture medium was digested directly with endogenous proteinase by making the medium 1 mM in APMA and incubating at 37 °C.

Extraction of endogenous degradation products from cartilage

The proteoglycan content of the cartilage at the end of the culture period was isolated by extraction of the tissue pieces with 10 vol. of 4 M-guanidinium chloride/0.1 M-sodium acetate, pH 6.0, containing proteinase inhibitors (Roughley *et al.*, 1982). Extraction was performed at day 20 for tissue that had been cultured in the presence or absence of interleukin 1. The extracts were filtered and dialysed against 0.125 M-Tris/HCl, pH 6.8, containing 0.1 % SDS in preparation for analysis by SDS/polyacrylamide-gel electrophoresis.

Chromatography of proteoglycan

Proteoglycan and its proteolytic degradation products were examined by h.p.l.c. on Bio-Gel TSK50 and TSK60 columns (7.5 mm × 300 mm) in tandem (Roughley & Mort, 1985). When culture media were analysed, they were first dialysed against a one-tenth dilution of the h.p.l.c. buffer, freeze-dried, and then reconstituted with water to one-tenth the original volume. A Sepharose CL-2B column (1 cm × 110 cm) was used to assess the ability of the proteoglycan to interact with hyaluronic acid (Roughley *et al.*, 1984), after dialysis of the sample into the chromatography buffer. The column effluents were monitored for proteoglycan content as described above.

SDS/polyacrylamide-gel electrophoresis

Samples were analysed under reducing conditions by the method of King & Laemmli (1971), on 10% -acrylamide slab gels as described previously (Roughley *et al.*, 1982). After electrophoresis, protein was transferred to nitrocellulose membranes by the method of Towbin *et al.* (1979). Link proteins or hyaluronic acid-binding regions derived from the proteoglycan subunits were then identified by using monoclonal antibodies labelled with ¹²⁵I by the method of Greenwood *et al.* (1963), and subsequently detected by autoradiography, as described previously (Mort *et al.*, 1985). Monoclonal antibodies 9/30/8-A-4 (to link protein) and 12/21/2-A-5 (to the hyaluronic acid-binding region) (Cateron *et al.*, 1985) were kindly supplied by Dr. Bruce Cateron, University of West Virginia.

RESULTS

Analysis of proteoglycan in culture medium

Adult human cartilage was maintained in organ culture for 22 days, in either the presence or the absence of interleukin 1. At 2-day intervals the culture medium was changed and assayed for the presence of proteoglycan (Fig. 1a). In the absence of interleukin 1 there was an initial release of proteoglycan-containing fragments, which rapidly decreased to baseline values. In contrast, the amount of glycosaminoglycan released in the presence of interleukin 1 remained high throughout the culture period. This sustained proteoglycan release was accompanied by a sustained production of a latent proteinase, capable of degrading proteoglycan when activated (Fig. 1b).

The proteoglycan present in the culture medium at days 2, 10, 16 and 22 was examined by h.p.l.c. (Fig. 2). At day 2, when interleukin 1 was present in neither culture, most of the proteoglycan was of a large size (Fig. 2a), comparable with that obtained for proteoglycan subunits extracted from adult cartilage. When culture was continued in the absence of interleukin 1, there was no change in the proteoglycan profile on subsequent days, though the absolute amount of material present was considerably decreased. In contrast, the profile was altered for cultures in the presence of interleukin 1. On all the days examined the proteoglycan was eluted as a major peak with a more retarded shoulder (Figs. 2b–2d). The position of the major peak was slightly more included (and thus of smaller molecular size) than that of its day-2 counterpart or the extracted proteoglycan subunits. The shoulder was of a similar size to the degradation products obtained when the intact extracted proteoglycan was digested to completion with the isolated proteoglycan-degrading enzyme (results not shown).

The ability of the proteoglycan in the culture medium to interact with exogenous hyaluronic acid was assessed by chromatography through Sepharose CL-2B (results not shown). On day 2, when the culture medium contains proteoglycan that has diffused out of the cartilage on initiation of culture, 39% of the material can interact with hyaluronic acid. In contrast, on day 16, when the proteoglycan is released on stimulation by interleukin 1, only 7% has the ability to interact. These results can be compared with the intact extracted proteoglycan, which has a 63% ability to interact.

Fig. 1. Release of proteoglycan and metalloproteinase from articular cartilage under continuous stimulation by interleukin 1

Cartilage explants were cultured for 2 days in the absence of interleukin 1 to allow adjustment to culture conditions. Tissue was then cultured in the presence (●) or absence (○) of interleukin 1, with changes of medium every 2 days. Culture media were assayed for (a) proteoglycan content and (b) proteoglycan-degrading activity. One unit of activity represents the release of 1 µg of product/min.

To determine whether the endogenous proteoglycan present in the culture medium could be further degraded by the endogenous latent proteinase after activation, medium was incubated directly with 1 mM-APMA, and the proteoglycan analysed by h.p.l.c. (Fig. 3b). The limit degradation product was of the same size as that obtained with the purified enzyme. Furthermore, its elution position corresponded to that of the more retarded shoulder observed in the untreated medium (Fig. 3a). Incubation in the presence of EDTA completely abolished degradation (Fig. 3c). Thus the untreated medium appears to contain fragments ranging in size from almost intact proteoglycan subunits to limit-degradation products, though few appear able to interact with hyaluronic acid.

Degradation of proteoglycan aggregate by metalloproteinase

The proteoglycan aggregate obtained from newborn human articular cartilage was subjected to degradation by the partially purified metalloproteinase preparation. Incubation was under conditions suitable for yielding limit-degradation products from intact proteoglycan subunit preparations, as assessed by the observation of

Fig. 2. Gel-filtration h.p.l.c. analysis of proteoglycan released into culture medium after stimulation of cartilage by interleukin 1: (a) day 2, (b) day 10, (c) day 16, (d) day 22

Proteoglycan subunits extracted from adult articular cartilage were eluted with a similar profile to that in (a). The column void volume (V_0) and total volume (V_t) are indicated, together with the elution position of chondroitin sulphate (V_{cs}).

no further change in size on h.p.l.c. Digestion resulted in the accumulation of isolated hyaluronic acid-binding regions, which were not present in the intact aggregate preparation (Fig. 4, lanes c and d). Furthermore, incubation with the metalloproteinase altered the pattern of the link-protein heterogeneity (Fig. 4, lanes a and b). Although the largest of the three link-protein components was most abundant in the starting material, the smallest predominated in the digested sample. In both cases, when aggregate was incubated with proteinase and EDTA, no hyaluronic acid-binding region was produced and no change in the link-protein pattern was seen. Such

Fig. 3. Degradation of released proteoglycan by released latent metalloproteinase after APMA activation

Medium from days 14 and 16 was incubated for 50 h in the absence or presence of 1 mM-APMA. Glycosaminoglycan-containing material was then analysed by gel-filtration h.p.l.c. (a) Medium incubated in the absence of APMA; (b) medium incubated with 1 mM-APMA; (c) medium incubated with 1 mM-APMA and 10 mM-EDTA. Medium before incubation gave the same profile as (a). The column void volume (V_0) and total volume (V_t) are indicated, together with the elution position of chondroitin sulphate (V_{cs}).

observations are comparable with those obtained previously, when intact proteoglycan aggregates were subjected to degradation by the bacterial proteinase clostripain (Roughley *et al.*, 1982), and suggest that most of the link proteins and the hyaluronic acid-binding regions of the proteoglycan subunits still retain function, and are resistant to degradation to peptides by the metalloproteinase when in the form of a proteoglycan aggregate.

Fig. 4. Effect of metalloproteinase on human proteoglycan aggregate

Neonatal human proteoglycan aggregate was incubated with APMA-activated metalloproteinase. Digests were analysed by SDS/polyacrylamide-gel electrophoresis followed by electroblotting. Link protein (a and b) and hyaluronic acid-binding region (c and d) were detected on the blots by using ^{125}I -labelled monoclonal antibodies. Lanes (a) and (c), undigested aggregate; lanes (b) and (d), aggregate incubated with metalloproteinase in the presence of APMA.

The effect of the endogenous proteinase *in situ* was compared with that of the isolated material *in vitro*, by studying the link protein extracted from the adult cartilage after culture for prolonged periods in the absence or presence of interleukin 1. In the absence of proteinase stimulation by interleukin 1, three link-protein components were observed (Fig. 5, lane a), of similar mobility and abundance to those present in extracts from cartilage that had not been subjected to culture (Mort *et al.*, 1983). However, in the presence of interleukin 1 the smallest of the three intact link proteins was considerably increased in abundance at the expense of its larger analogues (Fig. 5, lane b). Interestingly, the additional fragmentation of the link protein, observed in adult human cartilage (Mort *et al.*, 1983), was not affected by this procedure. Thus it would appear that, although the reported increase in the abundance of the smallest link protein with age could be accounted for by the action of

Fig. 5. Effect of culture in the presence of interleukin 1 on cartilage link-protein heterogeneity

Cartilage cultured for 18 days in the presence or absence of interleukin 1 was extracted with 4 M-guanidinium chloride, and the extracted link proteins were analysed by SDS/polyacrylamide-gel electrophoresis, followed by electroblotting and immunodetection. Lane (a), culture in the absence of interleukin 1; lane (b), culture in the presence of interleukin 1.

the metalloproteinase, the additional proteolytic fragmentation cannot. Furthermore, the observed fragmentation pattern on stimulation of proteinase production by interleukin 1 is compatible with the existence of link-protein-stabilized proteoglycan aggregates within the adult articular-cartilage matrix. If such aggregates were not present, one would not expect an accumulation of the smallest proteolytically derived link protein.

DISCUSSION

In this work we have shown that the proteoglycan degradation products that diffuse from cartilage in culture, upon stimulation of proteinase production by interleukin 1, have a range of sizes. Most are of large size, with many being comparable with the intact proteoglycan subunits present in the cartilage matrix before incubation. Others are of smaller size, with some representing the

limit degradation product possible with the proteinase under consideration. As only a small proportion of the degradation products interact with hyaluronic acid, it appears probable that much of the proteoglycan is released from the tissue by a single cleavage near the hyaluronic acid-binding region, and then further degraded upon prolonged incubation. There is certainly no evidence of predominantly small fragments in the culture medium, which would be expected if degradation were proceeding from the glycosaminoglycan-attachment termini of the proteoglycan subunits within the tissue. The hyaluronic acid-binding region has been postulated to be a preferred site of action for many proteinases *in vitro* (Roughley, 1977), and preliminary studies indicate that this is also true for the metalloproteinase under investigation. As to whether this is actually the initial site of cleavage is open to question, and one must bear in mind that the pathway of degradation seen in dilute solutions *in vitro* need not be identical with that followed *in situ*.

In general the action of the activated metalloproteinase *in vitro* on both proteoglycan subunits and link proteins is the same as that seen *in situ* for cartilage under stimulation by interleukin 1. This suggests that the concomitant release of both proteinase and proteoglycan upon stimulation by interleukin 1 represents a cause-and-effect relationship. As most of the proteinase in the culture medium is present in an inactive latent form, one needs to postulate that active enzyme is present within the cartilage matrix. It is possible that a proportion of the secreted enzyme is activated endogenously upon release and does not escape from the tissue in an active form, either because of interaction with its substrates or through subsequent inhibition to yield a form that can no longer be activated, such as through interaction with the tissue inhibitor of metalloproteinases ('TIMP'), which is known to be present in the cartilage matrix (Reynolds *et al.*, 1981). The activatable enzyme, which is present in the culture medium, can then be thought of as an overflow which was not subjected to the endogenous activation process. Partial activation of the endogenous latent enzyme observed on initiation of culture, even in the absence of interleukin 1 (Fig. 1b), presumably accounts for the lower percentage aggregation of the proteoglycan released during this time, compared with the extracted material.

It has been previously reported that the structure of the proteoglycan in human articular cartilage changes during the aging process. Some of these changes are compatible with a proteolytic mechanism: (a) the decrease in proteoglycan subunit size with age, resulting in an abundance of isolated hyaluronic acid-binding regions in the mature adult (Roughley *et al.*, 1985), and (b) the increased abundance of smaller link proteins with age, occurring concurrently with further fragmentation of the molecules in highly disulphide-bonded regions (Mort *et al.*, 1983). It is apparent that the proteinase produced by the chondrocytes under stimulation by interleukin 1 can perform many of these cleavages. It is certainly capable of producing hyaluronic acid-binding regions and smaller link proteins from proteoglycan aggregates, both of which would be expected to accumulate within the matrix, owing to their inability to diffuse freely if they remain in association with the hyaluronic acid filaments. In contrast, the additional proteolytic fragmentation of the link proteins does not appear to be due to the

chondrocyte proteinase. The origin of these cleavages is at present unknown, though it is possible that they result from other mechanisms, such as free-radical damage, as has been postulated for many changes in macromolecular structure observed during aging (Herman, 1984). It is also possible that free radicals may contribute generally to proteolytic degradation in the proteoglycans with aging, although the present data are indirectly more supportive of the action of proteinases.

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