Characterization of Aggrecan Retained and Lost from the Extracellular Matrix of Articular Cartilage

ININVOLVEMENT OF CARBOXYL-TERMINAL PROCESSING IN THE CATABOLISM OF AGGRECAN

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The catabolism of aggrecan in bovine articular cartilage explants is characterized by the release into the culture medium of high molecular weight aggrecan fragments, generated by the proteolytic cleavage of the core protein between residues Glu373 and Ala374 within the interglobular domain. In this study, the position of the carboxyl-terminus of these aggrecan fragments, as well as a major proteolytically shortened aggrecan core protein present in cartilage matrix, have been deduced by characterizing the peptides generated by the reaction of aggrecan core protein peptides with cyanogen bromide. It was shown that two out of three such peptide fragments having an amino terminus starting at Ala374 have their carboxyl terminus located within the chondroitin sulfate 1 domain. The third and largest aggrecan core protein peptide, with an amino terminus starting at Ala374, has a carboxyl terminus in a region of core protein between the chondroitin sulfate 1 domain and the chondroitin sulfate 2 domain. The carboxyl terminus of this peptide appeared to be the same as that of the proteolytically degraded aggrecan core protein, which is retained within the extracellular matrix of the tissue. Another two aggrecan fragments recovered from the medium of explant cultures with amino-terminal sequences in the chondroitin sulfate 2 domain at Ala1772 and Leu1872 were shown to have their carboxyl termini within the G3 globular domain. These results suggest that the catabolism of aggrecan between residues Glu373 and Ala374 in the interglobular domain by the putative proteinase, aggrecanase, may be dependent on prior proteolytic processing within the carboxyl-terminal region of the core protein.

Aggrecan, the large chondroitin sulfate proteoglycan of cartilage forms link protein-stabilized aggregates with hyaluronan and gives the tissue the ability to resist mechanical compression. The core protein of aggrecan is made up of three globular domains: G1, and G2, at the amino-terminal end of the molecule separated by an interglobular domain, and the G3 domain at the carboxy-terminal end, separated from the G2 domain by the keratan sulfate and chondroitin sulfate attachment regions.

Studies on the turnover of the total and 35S-labeled pools of aggrecan in explant bovine cartilage cultures have shown that catabolism of aggrecan involves proteolytic cleavage at alanine residue 374 within the interglobular domain of the core protein. Aggrecan fragments lacking the hyaluronan-binding domain are released into the culture medium (1). Two other cleavage sites have been identified within the chondroitin sulfate 2 domain, at Ala1772 and Leu1872. These three cleavage sites have been attributed to a specific proteinase, aggrecanase (1–3). The same degradation products of bovine aggrecan have been detected under different conditions of culture, which include tissue maintained in medium alone; medium supplemented with 20% calf serum; or medium containing interleukin 1α tumor necrosis factor or retinoic acid (1, 7, 8). Cleavage at Ala1774 in the interglobular domain of the aggrecan core protein has also been shown to occur in vivo (9), and we have evidence that cleavage at Ala1772 and Leu1872 in the chondroitin sulfate 2 domain also occurs in vivo.3 Related studies on the catabolism of newly synthesized 35S-labeled aggrecan in explant bovine cartilage cultures have shown that the core protein of aggrecan within the matrix decreases in size with time in culture (1, 10, 11). This occurs by the cleavage of the core protein in the carboxyl-terminal end of the core protein. Using explant cultures of articular cartilage and radiolabeling techniques, we have shown that there is a rapid processing of aggrecan core protein into a smaller polypeptide (1, 11). A major degraded aggrecan core protein with an amino-terminal sequence corresponding to that of bovine aggrecan and with the same apparent molecular weight as the degraded radiolabeled core protein, is also present in the total pool of aggrecan in the matrix (1).

The aim of this study was to determine the location of the carboxyl terminus of the major proteolytically degraded aggrecan core protein present in the matrix and those of aggrecan fragments lost to the medium of cartilage explant cultures in order to gain further understanding of the mechanism of proteolytic processing and catabolism of aggrecan core protein.

EXPERIMENTAL PROCEDURES

Materials—H2SO4 (carrier-free) and AmphiLyte were purchased from Amersham (Buckinghamshire, UK); Dulbecco’s modified Eagle’s medium and newborn calf serum were from CSL (Melbourne, Victoria, Australia); retinoic acid, cyanogen bromide, and keratanase (endo-β-galactosidase from Pseudomonas sp.; EC 3.2.1.103) were from Sigma; and chondroitinase ABC (protease-free; from Proteus vulgaris; EC 4.2.2.4) was from ICN Biochemicals (Costa Mesa, CA). Monoclonal

2 Numbering of amino acid residues starts at the first residue of bovine aggrecan (4–6).

3 M. Z. Ilic, H. C. Robinson, and C. J. Handley, unpublished data.
antibodies 5/6/3-B-3 and 1/20/5-D-4 were kindly supplied by Prof. B. Caterson (School of Molecular and Medical Biosciences, University of Wales at Cardiff, UK). Horseradish peroxidase-conjugated antibody to mouse immunoglobulin (raised in sheep) was purchased from Silenus Laboratories (Hawthorn, Victoria, Australia), S-(cyclohexylamino)propanesulfonic acid, Calbiochem-Behring Corp. (Bedford, MA).

**Cartilage Cultures**—In experiments investigating the loss of \(^{35}\text{S}\)-labeled proteoglycans from cartilage explants and characterizing the catabolic products of radiolabeled aggrecan, articular cartilage (~4 g) from metacarpophalangeal joints of 2–3-year-old cattle was placed in organ culture in Dulbecco’s modified Eagle’s medium (1 g of tissue/10 ml of medium) containing 20% (v/v) newborn calf serum for 6 days. After 6 days in culture, the tissue was washed thoroughly with fresh sterile medium, and a proportion of the tissue (1 g) was immediately extracted for 48 h at 4 °C with 10 ml of 4M guanidinium chloride containing 20% (v/v) newborn calf serum for 6 days at 37 °C. At the end of the incubation period, the tissue was washed thoroughly with fresh sterile medium, and a proportion of the tissue (1 g) was immediately extracted for 48 h at 4 °C with 10 ml of 4 M guanidinium chloride in 0.05 M sodium acetate, pH 5.8, containing proteinase inhibitors (13). The remaining tissue was continued in culture in 0.5-g batches for a further 6 days in medium containing 1 μM retinoic acid (1). The medium was changed every 24 h and stored in the presence of proteinase inhibitors at −20 °C. At the end of the culture period, the tissue was extracted as above.

In experiments involving characterization of catabolic products of total levels of aggrecan, articular cartilage (~8 g) from two metacarpophalangeal joints of 6–8-year-old cattle was placed in organ culture in Dulbecco’s modified Eagle’s medium (1 g of tissue/10 ml of medium) containing 20% (v/v) newborn calf serum for 5 days. After 5 days in culture, the tissue was washed with medium alone and replaced in culture for a further 8 days in medium containing 1 μM retinoic acid. The medium was changed daily, and the spent medium was stored in the presence of proteinase inhibitors at −20 °C.

**Isolation and Purification of Aggrecan Core Proteins from Fetal Articular Cartilage** and Aggrecan Core Protein Fragments from Medium of Mature Articular Cartilage Explants—Fetal articular cartilage (~5 g) from the knee and metacarpophalangeal joints obtained immediately after dissection was extracted for 48 h at 4 °C with 50 ml of 4 M guanidinium chloride in 0.05 M sodium acetate, pH 5.8, containing proteinase inhibitors for 48 h. Aggrecan macromolecules were isolated by cesium chloride density gradient centrifugation and digested with chondroitinase ABC (0.5 units) in the presence of proteinase inhibitors (14). The deglycosylated aggrecan core proteins were purified by ion exchange chromatography on Q-Sepharose as described previously (1). Pooled medium (640 ml) from explant articular cartilage cultures from mature animals was applied in 320-ml lots to a column of Q-Sepharose as above.

**SDS-Polyacrylamide Gel Electrophoresis**—Purified aggrecan core proteins isolated from the matrix of fetal calf articular cartilage and from the medium of explant cultures of cartilage and peptides generated from them by cyanogen bromide cleavage were subjected to electrophoresis under reducing conditions on SDS-4–10% gradient polyacrylamide slab gels (85 × 55 × 0.75 mm) (15). After electrophoresis, some gels were stained with silver (11), and the other gels were subjected to electroelution (250 mA; 4 °C; 1.5 h) using polyvinylidene difluoride membranes (Immobilon P; Ref. 16). The resultant membranes were blocked with 5% (w/v) skim milk in 0.8% sodium chloride buffered at pH 7.4 with 0.05 M sodium acetate, pH 5.8, containing proteinase inhibitors and digested with chondroitinase ABC (0.125 unit) and keratanase (1 unit) in the presence of proteinase inhibitors (14). The deglycosylated aggrecan core proteins were purified by ion exchange chromatography on Q-Sepharose as described previously (1). Three large cyanogen bromide peptides, which span most of the aggrecan core protein from Cys\(^{4278}\) in the G1 domain to Met\(^{2156}\) in the G3 domain, are predicted. The work described in this study required a tissue source rich in undegraded aggrecan. For this reason, aggrecan core proteins were isolated from fetal calf articular cartilage, since the level of the intact aggrecan core protein, containing the G3 domain, is higher in fetal tissue than in older animals. In calf cartilage, it makes up 50% of total aggrecan (19). In order to investigate the result of proteolytic processing of the intact core protein, articular cartilage from mature animals was used, since our previous work has shown that in such tissue there is a rapid proteolytic processing of the intact core protein (1). Several repeats of the disulfated disaccharides; Ref. 18), and the other gels were subjected to electroelution (250 mA; 4 °C; 1.5 h) using polyvinylidene difluoride membranes (Immobilon P; Ref. 16). The resultant membranes were blocked with 5% (w/v) skim milk in 0.8% sodium chloride buffered at pH 7.4 with 0.05 M sodium acetate, pH 5.8, containing proteinase inhibitors and digested with chondroitinase ABC (0.125 unit) and keratanase (1 unit) in the presence of proteinase inhibitors (14). The deglycosylated aggrecan core proteins were purified by ion exchange chromatography on Q-Sepharose as described previously (1).
Explant Cultures—Radiolabeled proteoglycan core proteins were isolated from the matrix of articular cartilage explants immediately after incubation with \([^{35}S]\)sulfate for 6 h. Samples of deglycosylated radiolabeled proteoglycan core proteins before and after reaction with cyanogen bromide were analyzed by SDS-PAGE followed by fluorography. Fig. 2, lane a, shows a single major radiolabeled aggrecan core protein band with \(M_r\) greater than 200,000 (peptide 1), which represents the intact core protein. Also present were minor radioactive bands of smaller molecular weight. After reaction of aggrecan core proteins with cyanogen bromide, four major radiolabeled peptides were generated (lane b): two intensely labeled peptides designated CNBr310 and CNBr120 as well as lower intensity bands designated CNBr430 and CNBr105.

Characterization of Cyanogen Bromide Peptides Derived from the Total Pool of Aggrecan Core Proteins Isolated from the Matrix of Fetal Calf Articular Cartilage—Deglycosylated aggrecan core proteins, isolated from the matrix of fetal articular cartilage were reacted with cyanogen bromide. Fig. 2 shows a silver-stained gel of aggrecan core proteins before (lane c) and after (lane d) reaction with cyanogen bromide. The most abundant aggrecan core protein present in the matrix of fetal cartilage was peptide 1. Present in smaller quantity was a diffuse band of smaller core proteins, which are consistent with aggrecan core proteins proteolytically shortened at the carboxyl-terminal end (20). The predominant band corresponded to the migration of peptide 2 observed in articular cartilage from mature animals (1, 11). Cyanogen bromide reaction with the same deglycosylated aggrecan core protein preparation from fetal calf cartilage generated four peptides (lane d) with the same electrophoretic mobility as those observed for radiolabeled cyanogen bromide peptides of aggrecan core proteins, CNBr430, CNBr310, CNBr120, and CNBr105. Fig. 2 also shows the immunodetection of the aggrecan core proteins (lanes e and f) and the cyanogen bromide peptides (lanes g and h) with monoclonal antibodies 1/20/5-D-4, which reacts to keratan sulfate oligosaccharides (lanes e and g) and 5/63-B-3, which reacts to terminal unsaturated chondroitin 6-sulfate disaccharides (lanes f and h). Aggrecan core protein bands were positive to keratan sulfate and to chondroitin sulfate epitopes.
Cyanogen bromide peptides CNBr430 and CNBr310 were positive to monoclonal antibodies 1/20/5-D-4 and 5/6/3-B-3, while peptide CNBr105 had only keratan sulfate epitopes, and peptide CNBr120 had only chondroitin sulfate epitopes.

Each of these cyanogen bromide peptides was subjected to amino-terminal amino acid sequencing, and the results are shown in Table I. Peptide CNBr310 had an amino-terminal sequence starting at Val residues 1 to Met residues 277 (45,000 Da) estimated from the molecular size of peptides CNBr210 and CNBr105 plus allowance for residues 1 to Met 277 and Met 2197 to the end (15,000 Da; Ref. 21). Cyanogen peptides CNBr105, CNBr310, and CNBr120 plus allowance for residues 1 to Met 277 and Met 2197 to the end (15,000 Da; Ref. 21).

The estimates of molecular mass of aggrecan core protein peptides and cyanogen bromide peptides depicted in Fig. 1, A and B. The approximate molecular size of peptide 2 was estimated by the addition of the molecular size of peptides CNBr210 and CNBr105 plus allowance for residues 1 to Met residues 277 (45,000 Da) estimated from the molecular size of the G1 domain (27.28). The approximate size of cyanogen bromide derived peptide CNBr310 was estimated from its migration on SDS-PAGE with respect to peptide 2 and peptide 3 derived from the medium of cartilage explant cultures with estimated mass of 280,000 Da. The approximate molecular size of peptide 1 was estimated from the sum of cyanogen peptides CNBr105, CNBr310, and CNBr120 plus allowance for residues 1 to Met residues 277 and Met residues 2197 to the end (15,000 Da; Ref. 21).

These results show that intact aggrecan core protein gave rise to four high molecular weight aggrecan core protein peptides after reaction with cyanogen bromide. This was indicated by the fact that the four peptides, derived from a preparation of aggrecan from fetal articular cartilage, match those from the radiolabeled pool of aggrecan rich in intact aggrecan core protein as it was isolated from mature articular cartilage immediately after incubation with [35S]sulfate. Three of these peptides, CNBr105, CNBr310, and CNBr120, had amino-terminal sequences originating in the G1 domain, keratan sulfate, and chondroitin sulfate 2 domain, respectively, as depicted in Fig. 1B. The peptide CNBr430, with the same amino-terminal sequence as peptide CNBr310, was present at a much lower level than peptide CNBr310. Peptide CNBr430 was therefore likely to be a product of incomplete reaction of the intact aggrecan core protein with cyanogen bromide at its carboxy-terminal end.

Cyanogen bromide peptides CNBr105, CNBr310, and CNBr120 combined account for the bulk of the aggrecan core protein, a total of 1920 amino acid residues out of 2308, spanning from Cys residues 278 in G1 to Met residues 2197 in G3. These results are consistent with previous studies where a keratan sulfate-containing fragment with amino-terminal sequence SSAGWLA-4 and two chondroitin sulfate-rich fragments, one of which had the amino-terminal sequence VTQV-, were isolated from the preparation of aggrecan from nasal cartilage treated with cyanogen bromide (21, 22). Peptide CNBr105 shows up as a weak band when detected by [35S]-fluorography of SDS-PAGE gels (Fig. 2, lane b) but as an intense band when detected by the silver stain or immunolocalization with monoclonal antibody 1/20/5-D-4 (Fig. 2, lanes d and g, respectively). The reasons for this difference are 2-fold; there is only a small number of keratan sulfate oligosaccharide stubs attached to this region of the aggrecan core protein and consequently a low level of incorporation of 35S, whereas the majority of aggrecan monomers in the total pool of aggrecan, even core proteins lacking parts of the carboxy-terminal domain, will generate this peptide following reaction with cyanogen bromide. Indeed, proteolytically shortened core proteins from the total pool of aggrecan, lacking much of the carboxy-terminal end of the molecule yet retaining an intact hyaluronan binding domain, can be expected to yield peptide CNBr105. Proteinic cleavage of the aggrecan core protein between Met residues 691 and Met residues 1792 would also be expected to generate cyanogen bromide peptides with a Mᵣ lower than that
Comparison of high molecular weight cyanogen bromide peptides of intact aggrecan core protein with cyanogen bromide peptides of proteolytically degraded aggrecan isolated from the matrix and medium of bovine articular cartilage was then used to estimate the location of the carboxyl termini of the core proteins of these degraded aggrecan macromolecules.

Characterization of the Major Proteolytically Processed Aggrecan Core Protein Present in the Extracellular Matrix of Cartilage—An experiment was set up to isolate $^{35}$S-labeled aggrecan that had undergone proteolytic processing in the cartilage-explant region. In this experiment, articular cartilage from mature animals was placed in explant cultures for 5 days, incubated with $^{35}$S-sulfate, washed well, and then returned to explant culture for a further 6 days in medium containing retinoic acid. During the course of this incubation, approximately 50% of the radiolabeled aggrecan molecules were lost from the tissue and represented chondrocyte-directed catabolism (data not shown). Radiolabeled aggrecan preparations were isolated from the matrix of articular cartilage explants immediately after incubation with $^{35}$S-sulfate and after a further period of 6 days in culture. Deglycosylated aggrecan core proteins and the peptides generated from them after reaction with cyanogen bromide were analyzed by SDS-PAGE followed by fluorography.

Fig. 3 shows that deglycosylated aggrecan core proteins isolated from cartilage immediately after incubation with $^{35}$S revealed a major band of $^{35}$S label (peptide 1) (lane a). After 6 days in culture (lane b), the intensity of peptide 1 had diminished, and that of peptide 2 had increased to become the major $^{35}$S-labeled core protein peptide. The corresponding $^{35}$S-labeled peptides resulting from the reaction of the deglycosylated protein cores preparations with cyanogen bromide are shown in lanes c and d, respectively. In addition to bands of $^{35}$S label corresponding in position to peptides CNBr120, CNBr310, CNBr120, and CNBr105 (lane c), a new major peptide CNBr210 with an apparent $M_r$ of 210,000 was evident in lane d. Concomitant with this, there was a marked decrease in the intensity of the band corresponding to peptide CNBr120 after 6 days in culture.

Comparison of the cyanogen bromide peptides derived from radiolabeled aggrecan core proteins present in the matrix at different times of culture period of articular cartilage explant cultures demonstrated simultaneous appearance of peptide 2 and the cyanogen bromide peptide CNBr210. Peptide CNBr210 was therefore concluded to have been generated from peptide 2. This was supported by the densitometric measurements of the radiolabeled peptides where a marked decrease in the intensity of peptide 1 relative to peptide 2 with the time in culture was accompanied by a parallel decline in the intensity of cyanogen bromide peptides CNBr310 and CNBr120 (Fig. 4). The relative levels of peptide CNBr105 did not change significantly with the time in culture, which was to be expected, since it would derive equally from peptide 1 and peptide 2. It was predicted that peptide CNBr210 would have the same amino-terminus sequence as peptide CNBr310 if it arose as a result of proteolysis between Met$^{403}$ and Met$^{792}$. This would imply that the carboxyl terminus in peptide CNBr210 should be located at a site on the amino-terminal side of Met$^{792}$ within the chondroitin sulfate 2 domain as shown in Fig. 1B.

An estimate of the location of the carboxyl terminus of peptide 2 was based on the comparison of molecular weight of cyanogen bromide peptides CNBr310 and CNBr210. The difference in $M_r$ between the two cyanogen bromide peptides was estimated to be about 100,000 (Table I). Taking into account the substitution of aggrecan core protein with glycosaminoglycan stubs, remaining attached to the core protein after chondroitinase ABC and keratanase digestion, the most likely location of the carboxyl terminus of peptide 2 is in the extended nonglycosylated portion of aggrecan core protein between the chondroitin sulfate 1 domain and the chondroitin sulfate 2 domain of aggrecan, i.e. between Phe$^{1458}$ and Ile$^{1488}$. Other, less likely sites are the aggrecanase cleavage site between Glu$^{1771}$ and Ala$^{1772}$ observed in our previous studies (1) and another site between Glu$^{1666}$ and Gly$^{1667}$ reported in calf articular cartilage in culture (3). Because these sites lie, respectively, only 21 and 126 amino acid residues on the amino-terminus of Met$^{792}$, it would be expected that cleavage at these sites would generate cyanogen bromide peptides of considerably greater molecular size.

Comparison of Peptide Fragments Derived from the Radiolabeled and Total Pools of Aggrecan Isolated from the Medium of Cultured Articular Cartilage—Articular cartilage isolated from a mature animal was maintained in culture in medium con-
shown in after reaction with the monoclonal antibody, was subjected to with antibody 5/6/3-B-3 (Fig. 5 
the pooled medium were visualized by immunolocalization 
argin acid to the medium of cartilage cultures by 
peptides detected at the same time points.

Fig. 4. Densitometric measurement of [35S]sulfate-radiolabeled aggrecan core proteins and cyanogen bromide peptides. 
Aggrecan core proteins were isolated from the matrix of articular cartilage cultures of mature animal immediately after incubation with [35S]sulfate and 2 and 6 days after incubation with [35S]sulfate in culture maintained in medium containing 1 µM retinoic acid as shown in Fig. 3. A point representing matrix extracted on day 2 after radiolabeling has been added. A shows the percentage of peptides 1 and 2 in the matrix extracts as a function of time in culture after labeling with [35S]sulfate. B shows the percentage of different cyanogen bromide peptides detected at the same time points.

taining 1 µM retinoic acid for 6 days following incubation with [35S]sulfate. Deglycosylated aggrecan fragments isolated from the pooled medium were visualized by immunolocalization with antibody 5/6/3-B-3 (Fig. 5A, lane a). The same membrane, after reaction with the monoclonal antibody, was subjected to autoradiography; the radiolabeled peptide fragments are shown in lane b. Seven major peptides (peptides 1–7) with M_r between 100,000 and 600,000 were detected by the antibody and by autoradiography. This indicates that newly synthesized aggrecan is processed in the same manner as the aggrecan already present within the tissue. In previous studies (1) and in some of following experiments, we have detected an additional minor radiolabeled peptide (peptide 8) with M_r of 65,000.

We have previously shown that the peptide bands with electrophoretic mobilities of peptides 1 and 2 (Table I) isolated from the matrix of bovine articular cartilage and from the medium of explant articular cartilage cultures have the same amino terminus as the intact bovine aggrecan (VEVS; Ref. 23). These species are probably lost to the medium of cartilage cultures by passive means (1). Other workers have reported the appearance of similar size aggrecan core proteins with the amino-terminal sequence of bovine aggrecan in the medium of explant cartilage cultures (2, 24). We have previously characterized peptides 3 (M_r 280,000), 4 (M_r 200,000), and 5 (M_r 170,000) and have shown that these peptides have the same amino-terminal sequence ARGS- (1) located within the interglobular domain of aggrecan core protein starting at Ala^{17456}. Furthermore, we have shown that peptides 6 (M_r 130,000) and 7 (M_r 100,000) have amino termini AGEG- and LGQR-, respectively, which correspond to cleavage at residues Ala^{1772} and Leu^{1872} located within the chondroitin sulfate 2 domain. An uncharacterized proteinase, aggrecanase, has been implicated in the cleavage at these three sites based on the similarity in amino acid sequence at the three cleavage sites. This work demonstrates that peptides 3–7 must originate from aggrecan core proteins with the molecular weight of peptide 2 and above, which are the major radiolabeled aggrecan core proteins observed in the matrix.

Characterization of Cyanogen Bromide Peptides Formed from Peptide Fragments Derived from Aggrecan Core Protein Isolated from the Medium of Explant Cultures—Articular cartilage isolated from mature animals was maintained in culture in medium containing 1 µM retinoic acid for 8 days as described previously. Fig. 5B shows deglycosylated aggrecan fragments derived from aggrecan core protein isolated and purified from the pooled medium, visualized with silver staining before (lane a), and after (lane b) reaction with cyanogen bromide. Four peptide bands designated CNBr430, CNBr310, CNBr210, and CNBr120 were detected, which were also shown to be generated from the aggrecan core proteins present in the matrix of articular cartilage. Three other peptide bands were designated CNBr130, CNBr100, and CNBr70.

Fig. 5B also shows the corresponding immunodetection pattern of peptides fragments derived from aggrecan core protein (lanes c and d) and after reaction with cyanogen bromide (lanes e and f) with monoclonal antibodies 1/20/5-D-4 (lanes c and e) and 5/6/3-B-3 (lanes d and f). Peptides 1–5 contained keratan sulfate epitopes (detected with monoclonal antibody 1/20/5-D-4), whereas peptides 1–7 contained chondroitin sulfate epitopes (detected with monoclonal antibody 5/6/3-B-3). Cyanogen bromide peptides CNBr430, CNBr310, and CNBr210 contained both keratan and chondroitin sulfate epitopes. Although peptides CNBr130 and CNBr120 migrated close together, it was evident that peptide CNBr130 reacted positively for keratan sulfate and chondroitin sulfate epitopes, whereas peptide CNBr120 contained only chondroitin sulfate epitopes. Peptide CNBr100 was strongly positive for keratan sulfate and also showed weak staining for chondroitin sulfate. Peptide CNBr70 contained only keratan sulfate epitopes.

The cyanogen bromide peptides were also subjected to amino-terminal amino acid sequencing, and the results are shown in Table II. Peptides CNBr210 and CNBr130 had the amino-terminal sequence commencing at Val^{1092} within the keratan sulfate domain. Peptide CNBr120 had an amino-terminal sequence commencing at Ser^{1793} in the chondroitin sulfate 2 domain. The band assigned to peptide CNBr100 contained three peptides with amino-terminal sequences commencing at Val^{1092} within the keratan sulfate domain, Cys^{280} within the G1 globular domain and Leu^{1872} within the chondroitin sulfate 2 domain. Peptide CNBr70 had an amino-terminal sequence commencing at Ala^{374} in the interglobular domain.

The likely origin of cyanogen bromide peptides associated with peptides 3–7, which appear in the medium of explant cultures, is indicated in Fig. 1B. Cyanogen bromide cleavage between Met^{693} and Val^{692} in peptide 3, 4, or 5 would be expected to generate peptide CNBr70, spanning from Ala^{374} in the interglobular domain of aggrecan to Met^{693} in the keratan sulfate domain. This peptide contains keratan sulfate and no chondroitin sulfate epitopes (Fig. 5, lane e) and was clearly a major derivative of the total pool of aggrecan fragments ap-
Amino-terminal sequences of CNBr peptides derived from aggrecan core protein fragments present in the medium

| Peptide  | Approximate mass | NH2-terminal sequence | Amount sequencing |
|----------|------------------|-----------------------|-------------------|
| CNBr210  | 210              | VXQVPVVGVA            | 9                 |
| CNBr130  | 130              | VXQVPVP                | 1.5               |
| CNBr120  | 120              | XGQGOXRD              | 4                 |
| CNBr100  | 100              | VTQVPVP                | 1.5               |
| CNBr105  | 105              | XSQWLAADR             | 3                 |
| CNBr70   | 70               | ARSGVILXAK            | 15                |

Fig. 5. A, comparison of radiolabeled and total peptide fragments derived from aggrecan core protein recovered from the medium of articular cartilage explants of mature cattle cultured in medium containing 1 μM retinoic acid. SDS-PAGE electrophoresis on a 4–20% gradient gel of peptide fragments isolated from the pooled medium of articular cartilage explants of mature cattle cultured for 6 days following incubation with [35S]sulfate. Lane a, immunolocalization with monoclonal antibody 5/63-B-3; lane b, autoradiography of the same membrane. B, aggrecan peptide fragments isolated from the medium of cartilage cultures. Purified peptide fragments, before and after digestion with cyanogen bromide, were separated by electrophoresis on a 4–10% gradient polyacrylamide gel and were visualized with silver staining (lanes a and b) and by immunolocalization with monoclonal antibodies (lanes c–f). Peptide fragments are shown before (lanes a, c, d, and g) and after reaction with cyanogen bromide (lanes b, e, and f), immunolocalization with antibody 1/20/5-D-4 (lanes c and e), and with antibody 5/63-B-3 (lanes d and f).

The results also suggest that cyanogen bromide treatment of peptide 6 yielded peptide CNBr120, which was rich in chondroitin sulfate epitopes (Fig. 5B, lanes b and f). Cyanogen bromide cleavage of peptide 6 would be expected to yield an additional peptide containing 21 amino acid residues spanning from Ala1772 to Met1792 (Fig. 1B), but this peptide could not be detected. Reaction of peptide 7 with cyanogen bromide would be expected to yield a peptide commencing at Leu1872 within the chondroitin sulfate 2 domain, which would migrate close to the CNBr100 band; such a peptide was detected (Table II and Fig. 5B, lanes b and f). Peptides 6 and 7 appear to extend into the G3 domain, and the precise location of their carboxyl termini has still to be determined.

Involvement of Carboxyl-terminal Processing in the Catabolism of Aggrecan—Analysis of the radiolabeled pool of aggrecan within the matrix of cartilage explants with time in culture indicated that peptide 2 was generated by extensive carboxy-terminal proteolytic processing of the core protein, which resulted in the loss of the chondroitin sulfate domain 2. These proteolytic processes involved cleavage of the core protein at Ala1772 or Leu1872, resulting in the appearance of peptides 6 and 7 in the culture medium (Fig. 1C). Additional proteolytic processing of the core protein also occurred between chondroitin sulfate 1 and chondroitin sulfate 2 domains, as indicated in Fig. 1C. This can be concluded because a single cleavage of intact aggrecan core protein at a site between the chondroitin sulfate 1 domain and the chondroitin sulfate 2 domain would generate a large (>200-kDa) aggrecan core protein fragment after deglycosylation with chondroitinase ABC and keratanase in the medium with a novel amino-terminal sequence. Such a peptide fragment, which would be intensely 35S-labeled, might be expected to occur in high total levels and to migrate between peptides 3 and 4. No such peptide fragment was observed, yet high levels of much smaller peptide fragments, peptides 6 and 7, were detected in the medium of explant cartilage cultures.

The catabolism of aggrecan within the extracellular matrix was shown to be accompanied by the appearance in the culture medium of glycosylated forms of peptides 3–5 with amino termini commencing at Ala1772 in the interglobular domain. These three peptides were smaller than peptide 2, and their carboxyl termini were mapped to either the end of the chondroitin sulfate 1 domain in the case of peptide 3 or to the middle of the chondroitin sulfate 1 domain in the case of peptides 4 and 5 (Fig. 1C).
It should be pointed out that no peptide produced by direct cleavage at Ala\(^{374}\) within the interglobular domain of aggrecan core protein and extending into the G3 globular domain was detected in this study. Such a peptide would be expected to migrate with a molecular size between peptides 1 and 2 and might be expected to occur in the medium of explant articular cartilage cultures at a level that would exceed that of peptides 6 and 7 combined (Fig. 5B, lane a). That no such peptide was detected indicated that a single initial cleavage within the interglobular domain of aggrecan core protein cannot be considered a primary event in the proteolytic catabolism of aggrecan core protein in articular cartilage. This is consistent with a model of aggrecan degradation in which cleavage within the interglobular domain occurs only after processing of the core protein within the carboxyl-terminal region.

Indeed, the cleavage within the interglobular domain of peptide 2 appears to give rise to peptide 3. This is shown in Fig. 3, which shows the radiolabeled aggrecan fragments appearing in the medium of explant cultures with time. There were significantly higher levels of \(^{35}S\) label associated with peptides 2 and 3 in the medium later in the culture period during days 3–6 (Fig. 3, lane f) compared with the first 2 days after incubation with \(^{35}S\) sulfate (Fig. 3, lane e). The analysis of corresponding cyanogen bromide peptides showed elevated levels of cyanogen bromide peptide CNBr210 for the culture period during days 3–6 (Fig. 3, lane h). This indicates that the band assigned to peptide CNBr210 originated from cyanogen bromide cleavage of peptide 2 or 3. Because of the limitation in resolution of SDS-PAGE, it is impossible to determine whether the carboxyl termini of peptides 2 and 3 are the same or are located close to each other.

Analysis of peptide fragments appearing in the medium during the first 2 days in culture after incubation with \(^{35}S\) sulfate (Fig. 3, lane e) showed that peptides 4 and 5 were present at considerably higher levels than peptide 3. The fact that peptides 4 and 5 were present in the medium together with peptides 6 and 7 is consistent with carboxyl-terminal processing of peptide 1 as described above. The paucity of peptides 2 and 3 during this period of culture suggested that the steps involved in carboxyl-terminal processing of aggrecan core protein are closely coupled and rapid. Furthermore, the data shown in Fig. 3 lanes e–f support the concept that there were multiple cleavage sites within the chondroitin sulfate 1 domain, since no intensely radiolabeled peptide fragments with amino-terminal sequences located within the chondroitin sulfate 1 domain could be detected. The molecular size of such a peptide fragment formed by a single cleavage of peptide 2 within the chondroitin sulfate 1 domain might be predicted to lie between 80,000 and 110,000 Da, corresponding to the difference in size between peptide CNBr210 and peptide CNBr130 or between peptide CNBr210 and peptide CNBr100.

Such extensive proteolytic processing along the aggrecan core protein either within the region between chondroitin sulfate 1 domain and the chondroitin sulfate 2 domain to generate peptide 2 or within chondroitin sulfate 1 domain to generate peptides 4 and 5 could involve aggrecanase, but involvement of other proteinases cannot be ruled out.

The pattern of proteolytic processing described above also occurs in aggrecan macromolecules present in bovine cartilage of animals up to 8 years old and under different conditions of culture where the rate of catabolism of aggrecan is stimulated. The analysis of aggrecan fragments present in the synovial fluid of these animals suggests that this process also occurs in vivo. In human cartilage, a similar pattern of proteolytic processing occurs for radiolabeled aggrecan formed in explant cultures of articular cartilage, in which peptides 1 and 2 make up the main species present (11). In mature human cartilage, however, a range of size of aggrecan in the extracellular matrix is observed, and the majority of core proteins are smaller than peptide 2. This suggests that as a result of aging, aggrecan core proteins in the total pool of aggrecan undergo more extensive carboxyl-terminal processing than observed in bovine cartilage. This results in the presence in culture medium and synovial fluid of core protein fragments with amino-terminal sequence starting at Ala\(^{374}\) with a range of molecular sizes smaller than that of peptide 3 (9, 11).

This work suggests that the generation of peptide 2 by extracellular proteolytic cleavage of the intact aggrecan core protein represents a key intermediate in the proteolysis of aggrecan, which ultimately leads to its loss from the tissue. This process thus represents an essential step in the catabolism in normal and diseased cartilage as well as in skeletal development and in aging.

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