Aggrecan degradation in human intervertebral disc and articular cartilage

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Aggrecan degradation in human intervertebral disc and articular cartilage has been studied by using anti-neoepitope antibodies specific for the N-terminal degradation products generated by cleavage within the interglobular domain at the metalloproteinase and aggrecanase sites. Immunoblot analysis of extracts of annulus fibrosus, nucleus pulposus and articular cartilage demonstrated age-related patterns in the abundance of both degradation products. In all three tissues the metalloproteinase-generated fragment was present at very low levels in young individuals but increased in abundance with age. In the disc tissues, the abundance of this degradation product levelled off in the juvenile; for cartilage this occurred in early adulthood. Despite these temporal differences, the levels attained in adults were comparable for the three tissues. In contrast, the aggrecanase-generated degradation product exhibited tissue-specific differences in the variation of its abundance with age. Whereas this degradation product increased with age in annulus fibrosus and articular cartilage and had levelled off by adulthood, in nucleus pulposus it was present in greatest abundance in young individuals and decreased to very low levels with age. Examination of discs exhibiting various degrees of degeneration did not reveal any differences in the levels of the metalloproteinase and aggrecanase-generated cleavage products that could not be accounted for by differences in age. In adults the product of aggrecanase action was much more abundant in articular cartilage than in either of the disc tissues, despite the age-related increase also observed for annulus fibrosus. Analysis of tissue extracts with an antibody recognizing the G1 domain of aggrecan identified two major degradation products whose abundance and size were correlated with the fragments detected by the anti-neoepitope antibodies. Taken together, these results indicate that cleavage at the metalloproteinase and aggrecanase sites are quantitatively important events in aggrecan catabolism in both articular cartilage and intervertebral disc *in vivo*. Moreover the two enzyme systems act independently and exhibit differences in the degree to which they contribute to aggrecan degradation in these tissues.

INTRODUCTION

Aggrecan is a large proteoglycan that forms macromolecular aggregates with hyaluronic acid, via an interaction that is stabilized by link protein. It is a functionally important component of both articular cartilage [1] and intervertebral disc [2]. Cloning and sequencing of the aggrecan cDNA and gene from different species have allowed an extensive characterization of the molecule at the structural level [3–5]. Aggrecan consists of a core protein with several distinct domains. At the N-terminus there are two structurally related globular domains, termed G1 and G2, separated by a short extended region known as the interglobular domain. Although the function of G2 has not been determined, G1 is known to mediate the interaction of aggrecan with hyaluronic acid and link protein, which serves to anchor aggrecan in the tissue. C-terminal to the G2 domain there is a long extended region consisting of two glycosaminoglycan-rich domains. The first is rich in keratan sulphate, whereas that which follows is rich in chondroitin sulphate. The high charge density imparted by the glycosaminoglycan chains results in a high degree of hydration for the proteoglycan aggregate, conferring on cartilage and intervertebral disc the ability to resist compression under load. At the C-terminus, aggrecan possesses a third globular domain, G3, consisting of epidermal growth factor-like, complement regulatory protein-like and lectin-like subdomains whose functions in aggrecan have yet to be elucidated. The G3 domain seems to be lost soon after synthesis and secretion in articular cartilage as a result of limited proteolytic cleavage of aggrecan near its C-terminus. The consequence of the loss of the G3 domain is not yet understood [1].

In articular cartilage, aggrecan has been demonstrated to undergo proteolytic cleavage at a number of different sites within the molecule. However, the two that have been most extensively characterized are the metalloproteinase and aggrecanase cleavage sites located within the interglobular domain, involving the Asn^{341}-Phe^{342} and Glu^{373}-Ala^{374} peptide bonds, respectively (Figure 1) [6–8]. These cleavage events are likely to be of significant functional consequence because they uncouple the G1 domain from the glycosaminoglycan-rich domains, which are then no longer anchored in the cartilage and hence might be lost. The small G1 domain is believed to remain anchored in the cartilage. The Asn^{341}-Phe^{342} site has been shown by studies *in vitro* to be cleaved by a number of matrix metalloproteinases (MMPs) (MMP-1, -2, -3, -7, -8, -9 and -13) [9–12]. In contrast, the proteinase mediating cleavage at the aggrecanase site has not been characterized. So far, only MMP-8, neutrophil collagenase, has been demonstrated to possess the ability to cleave this site *in vitro*, but this enzyme cleaves aggrecan preferentially at the metalloproteinase site [10]. There is also evidence that, in articular cartilage, aggrecan undergoes proteolysis at these interglobular domain sites *in vivo* [7,13]. Products of aggrecan cleavage at the metalloproteinase site have been demonstrated in normal and arthritic cartilage [13]. Other studies have shown that synovial fluids from normal individuals and patients with osteoarthritis, rheumatoid arthritis and acute knee injuries contain fragments generated by aggrecanase action [14]. Because the proteolytic degradation of aggrecan is considered to be an important event in the destruction and loss of function of articular cartilage, the results from studies *in vivo* suggest that metalloproteinases and

Abbreviations used: AGG-C antibody, antibody to aggrecanase-generated degradation product; MMP, matrix metalloproteinase; MMP-C antibody, antibody to metalloproteinase-generated degradation product.

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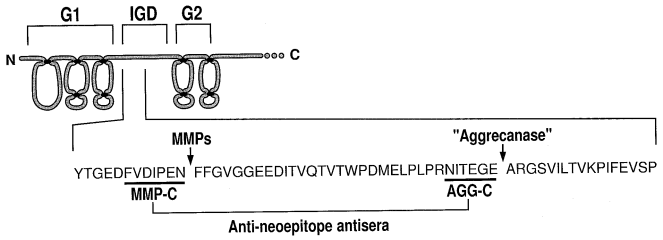

Figure 1 Sequences and locations within aggrecan of the peptides used to generate the anti-neoepitope antisera

The diagram depicts the structure of the N-terminus of the aggrecan core protein indicating the G1, G2 and interglobular domains. The amino acid sequence of part of the interglobular domain is shown below the diagram; also indicated are the sites cleaved by metalloproteinases (MMPs) and aggrecanase. The short lines underneath the sequence indicate the regions used in preparing the MMP-C and AGG-C anti-neoepitope antisera specific for the C-termini of the aggrecan N-terminal fragments generated on cleavage at these sites.

aggrecanase might be important in age-related tissue degeneration and in diseases such as arthritis.

In contrast with the numerous studies examining the cleavage of aggrecan at the metalloproteinase and aggrecanase sites in articular cartilage, relatively little is known about their contribution to aggrecan degradation in intervertebral disc [15]. Although these tissues are similar in that both are required to withstand compressive forces, they are quite distinct from a biochemical perspective. Thus aggrecan catabolism in the intervertebral disc might differ from that observed in articular cartilage. In this study, anti-neoepitope antibodies specific for the G1-containing fragments resulting from cleavage at the metalloproteinase and aggrecanase sites have been used to compare aggrecan degradation in the human intervertebral disc with that in articular cartilage.

MATERIALS AND METHODS

Source of tissues

Articular cartilage and intervertebral disc were obtained from human cadavers with no overt signs of connective tissue abnormalities, within 20 h of death. Intervertebral discs were from the L4–L5 spinal segment of 19 individuals covering an age range of 1.5–72 years and were dissected to collect samples of both annulus fibrosus and nucleus pulposus. The degree of disc degeneration was scored on a scale of 1 to 5 by the method of Thompson et al. [16], with higher scores indicative of more extensive degeneration. Articular cartilage was removed from the femoral condyles of 25 individuals, from newborn to 76 years of age. The tissues were then stored at −20 °C until extracted.

Preparation and glycosidase treatment of tissue extracts

Disc and cartilage tissue samples (100–200 mg) were chopped and extracted with 20 vol. (v/w) of 4 M guanidinium chloride/100 mM sodium acetate (pH 6.0) containing 1 mM disodium EDTA, 1 mM PMSF, 1 mM iodoacetamide and 10 μg/ml pepstatin A [17]. Extractions were performed with stirring in 4 ml Wheaton vials for 48 h at 4 °C. The extracts were clarified by centrifugation for 5 min in a microcentrifuge, dialysed for 48 h against two changes of 10 mM sodium acetate, pH 6.0, and stored at −20 °C. Before immunoblot analysis, tissue extracts were treated sequentially with chondroitinase ABC (ICN, Montreal, Quebec, Canada) and keratanase II (Seikagaku). Chondroitinase ABC was added to a concentration of 0.1 unit/ml and the incubation was performed at 37 °C for 4 h after which the enzyme was inactivated by heating at 100 °C for 10 min. Subsequently, keratanase II was added to a concentration of 10 m-units/ml and the incubations were left at 37 °C overnight. The incubations were then terminated by the addition of one volume of 2 × SDS/PAGE sample buffer [18].

SDS/PAGE and immunoblotting

Glycosidase-treated tissue extracts were heat-denatured at 100 °C for 5 min and subjected to electrophoresis under reducing conditions in 10 % (w/v) polyacrylamide gels [18]. After electrophoresis, resolved proteins were transferred to nitrocellulose membranes at 100 V for 2 h in a mini TransBlot cell (Bio-Rad, Mississauga, ON) utilizing the Towbin buffer system [19]. The membranes were then blocked overnight with 3 % BSA in Tris-buffered saline. Immunolocalization of aggrecan degradation products was performed with the antibodies and antisera described below and an alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibody (Sigma). Colour development was performed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [20].

Preparation of antisera

Monoclonal anti-neoepitope antibodies have recently been developed to permit the specific detection of the cleavage products of proteolytic action [21,22]. However, it has since been shown that polyclonal antisera with similar specificity can be prepared [23,24]. Thus rabbit antisera specific for the C-termini generated on the cleavage of the aggrecan interglobular domain at the metalloproteinase (…FVDIPEN) and aggrecanase (…NITEGE) sites were prepared, by using the peptides CGGFVDIPEN and CGGNITEGE respectively (Figure 1). The cysteine residues were added to allow coupling of the peptides to ovalbumin, and the glycine residues were included as spacers. In addition to these antisera, a general anti-G1 antiserum was raised, using a mixture of peptides spanning different regions of the aggrecan G1 domain (TPEQLGAAYEDGFHQCDAGW-LAD, KFTFQEAANECRRLGARLATTG, RVLLGTSLTIP-CYFIDPMHPVTTAPS, SNDSGVYRCEVMHGIEDSEATL-EVVVK, ICYTGEDFVDIPENFFGVGGEEDITV, DRAQR-ACLQNSAIIATPEQL and KARPNCGGNLLGVRTV). These peptides were coupled to ovalbumin through their internal cysteine residues. The peptides were synthesized on an Applied Biosystems model 431 peptide synthesizer, purified by HPLC and coupled to ovalbumin with *N*-hydroxysuccinimidyl bromoacetate [25]. Individual conjugates or, for the G1 peptides, a mixture of conjugates were injected into rabbits for the production of polyclonal antisera [26]. When required, antibodies were purified by affinity chromatography by using the immobilized immunizing peptides or peptide mixture [27]. For convenience the antisera and purified antibody preparations specific for the metalloproteinase- and aggrecanase-generated degradation products will be referred to as MMP-C and AGG-C antibodies respectively. The characterization of the MMP-C antibody has previously been described (E. R. Lee, L. Lamplugh, C. P. Leblond, S. Mordier, M.-C. Magny and J. S. Mort, unpublished work). The AGG-C antibody has been characterized by competitive ELISA, which showed it to be highly specific for the C-terminus of the immunizing peptide. Truncated and extended peptides were required in an approx. 100-fold molar excess to compete with the immunizing peptide for antibody binding (E. R. Lee, L. Lamplugh, C. P. Leblond, M.-C. Magny, J. S. Mort and K. Chan, unpublished work).

RESULTS

Variation of aggrecan degradation product levels with age

Aggrecan degradation products resulting from cleavage at the metalloproteinase and aggrecanase sites have already been described in articular cartilage [7,13], but intervertebral disc has not been studied. To determine whether such aggrecan degradation products are present in intervertebral disc and whether they exhibit age-related patterns, extracts of annulus fibrosus and nucleus pulposus from individuals covering a wide age range (1.5–72 years old) were analysed. Analysis of annulus fibrosus extracts with the MMP-C antibody revealed a single major immunoreactive protein species migrating with an apparent molecular mass of 55 kDa (Figure 2A), a size compatible with cleavage at the metalloproteinase site [23,28]. This degradation product was undetectable or present in very low quantities in the youngest individuals studied, but increased in abundance with age before levelling off during juvenile development. Analysis with the AGG-C antibody similarly identified a single major immunoreactive species in extracts of annulus fibrosus, but this migrated with a relative molecular mass of approx. 70 kDa (Figure 2B). This degradation product, whose size was consistent with proteolysis at the aggrecanase site [24], also showed an age-related increase in abundance with age, but this seemed to level off only by late adulthood. Thus the increase in the abundance of the metalloproteinase-generated degradation product seems to precede that of the aggrecanase-generated product. When extracts of articular cartilage from individuals of different ages (newborn to 76 years old) were also analysed with the MMP-C and AGG-C antibodies, immunoreactive degradation products of 55 and 70 kDa were likewise observed. Both degradation products exhibited age-related increases in abundance that seemed to level off by early adulthood (results not shown). Thus the results indicate that, in articular cartilage and annulus fibrosus, both degradation products exhibit increased abundance with age, relative to levels observed in the young juvenile. However, whereas metalloproteinase-mediated aggrecan cleavage precedes that mediated by aggrecanase in annulus fibrosus, the two enzyme systems exhibit similar temporal patterns in cartilage.

Extracts of nucleus pulposus were also found to contain the 55 and 70 kDa aggrecan degradation products as the major immunoreactive species, but these exhibited age-related variations in abundance that were opposite to each other (Figure 3). The degradation product generated by metalloproteinase action increased in abundance with age and levelled off during juvenile development (Figure 3A), as shown for annulus fibrosus. The abundance of this product seemed to exhibit a slight decrease in the mature adults studied. In contrast, the fragment produced by cleavage at the aggrecanase site was present in greatest abundance in the youngest individuals and decreased with age to low levels by middle to late adulthood (Figure 3B). These results indicate that in nucleus pulposus, unlike in annulus fibrosus and cartilage, proteolysis mediated by aggrecanase occurs earlier in life than that mediated by metalloproteinase.

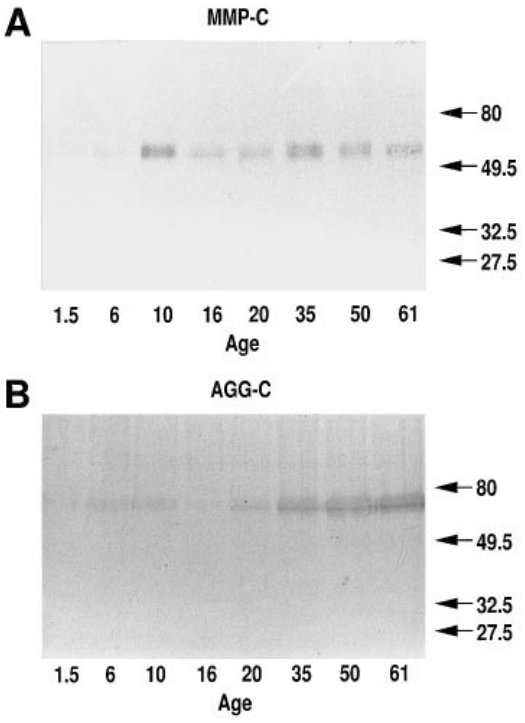

Figure 2 Variation with age of aggrecan degradation products generated by metalloproteinase and aggrecanase action in annulus fibrosus

Extracts of annulus fibrosus (5 μl for MMP-C blot and 20 μl for AGG-C blot) from 17 individuals of different ages (1.5–72 years of age) were analysed by immunoblotting with the MMP-C and AGG-C anti-neoepitope antibodies. The analysis of a representative group covering a range of 1.5–61 years of age is shown. The arrows at the right indicate the migration positions of molecular mass markers (in kDa).

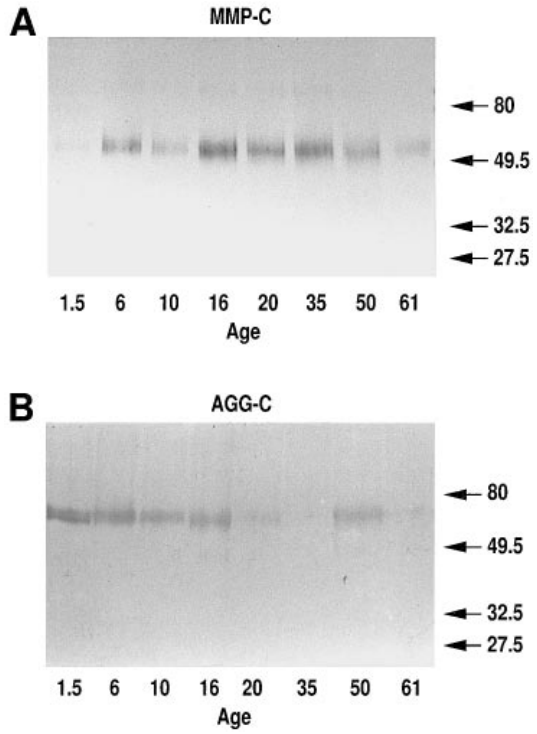

Figure 3 Variation with age of aggrecan degradation products generated by metalloproteinase and aggrecanase action in nucleus pulposus

Extracts of nucleus pulposus (5 μl for MMP-C blot and 20 μl for AGG-C blot) from 17 individuals of different ages (1.5–72 years of age) were analysed by immunoblotting with the MMP-C and AGG-C anti-neoepitope antibodies. The analysis of a representative group covering a range of 1.5–61 years of age is shown. The arrows at the right indicate the migration positions of molecular mass markers (in kDa).

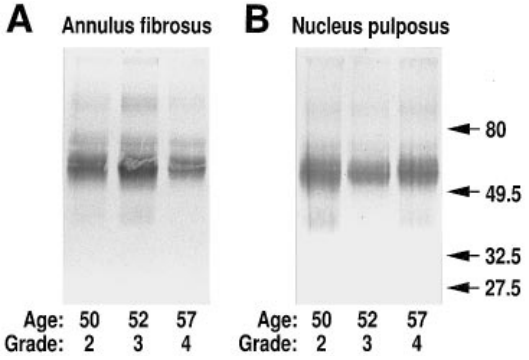

Figure 4 Analysis of metalloproteinase-generated degradation product with disc degeneration

Extracts (5 μl) of annulus fibrosus (**A**) and nucleus pulposus (**B**) were analysed with the use of the MMP-C anti-neoepitope antibody. The samples were chosen to cover various grades of degeneration in the sixth decade of life. The arrows at the right indicate the migration positions of molecular mass markers (in kDa).

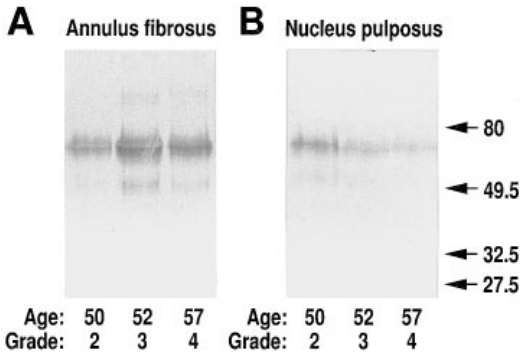

Figure 5 Analysis of aggrecanase-generated degradation product with disc degeneration

Extracts (20 μl) of annulus fibrosus (**A**) and nucleus pulposus (**B**) were analysed with the use of the AGG-C anti-neoepitope antibody. The samples were chosen to cover various grades of degeneration in the sixth decade of life. The arrows at the right indicate the migration positions of molecular mass markers (in kDa).

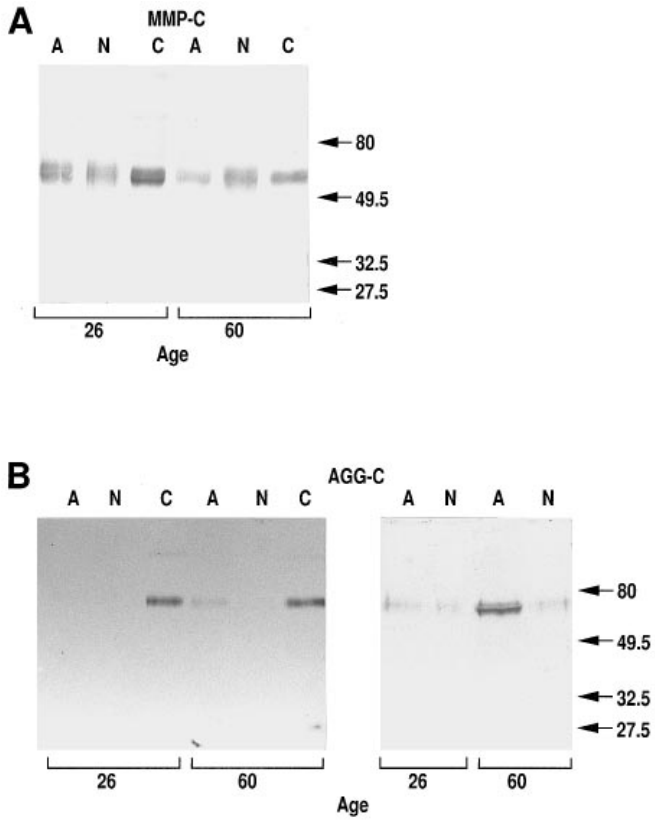

Figure 6 Comparison of aggrecan degradation products generated by metalloproteinase and aggrecanase action in adult intervertebral disc and articular cartilage

Extracts of annulus fibrosus (A), nucleus pulposus (N) and articular cartilage (C) from two adults, aged 26 and 60 years, were analysed by immunoblotting with the MMP-C (**A**) and AGG-C antibodies (**B**). For the MMP-C blot 5 μl of tissue extract was used, whereas for the AGG-C blots two amounts of extract (left panel, 5 μl; right panel, 20 μl) were used owing to the much lower abundance of the aggrecanase-generated degradation product in disc than in cartilage. The arrows at the right indicate the migration positions of molecular mass markers (in kDa).

Analysis of metalloproteinase and aggrecanase cleavage products with disc degeneration

It was also of interest to examine whether the aggrecan degradation products generated by metalloproteinase and aggrecanase action varied in abundance with disc degeneration. The discs analysed were selected to cover a wide range of degeneration while minimizing age differences within the sixth decade of life. Analysis with the MMP-C neoepitope antibody did not reveal any obvious differences in the abundance of the metalloproteinase-generated degradation product (Figure 4). Analysis with the AGG-C antibody likewise did not identify any differences in extracts of annulus fibrosus (Figure 5A). Extracts of nucleus pulposus exhibited an abundance of the aggrecanase-generated degradation product which decreased with increasing degeneration (Figure 5B). However, this decrease probably corresponds to the trend in abundance observed with aging shown above (Figure 3B). Thus disc degeneration itself does not seem to be associated with an increased abundance of the aggrecan degradation products resulting from metalloproteinase and aggrecanase action above that attributable to the normal aging process.

Comparison of aggrecan degradation products in adult intervertebral disc and articular cartilage

The age studies demonstrated that the aggrecan degradation products increased in abundance between the newborn and adult in disc and cartilage, with the exception of the aggrecanase-generated fragment in nucleus pulposus. However, this did not determine how the levels attained in adults compared between tissues for each degradation product. Thus, to compare their relative levels directly in adult disc and cartilage, tissue extracts from two individuals (26 and 60 years old) were analysed with the MMP-C and AGG-C antibodies. Immunoblotting with the MMP-C antibody revealed that the 55 kDa metalloproteinase-generated degradation product did not differ significantly in abundance between the three tissues (Figure 6A). In contrast, the aggrecanase-generated degradation product was present in much greater abundance in articular cartilage from both adults, with barely detectable levels in annulus fibrosus from the mature adult (Figure 6B, left panel). Analysis of larger quantities of annulus

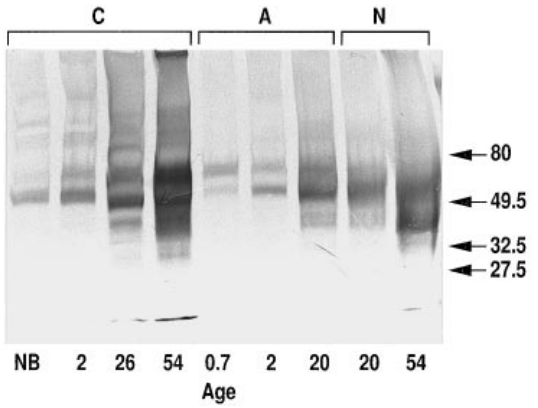

Figure 7 Analysis of G1-containing aggrecan degradation products in intervertebral disc and articular cartilage.

Extracts (20 μl) of articular cartilage (C), annulus fibrosus (A) and nucleus pulposus (N) from individuals of different ages were analysed with the use of an anti-G1 antibody to detect all aggrecan degradation products containing the G1 domain. The arrows at the right indicate the migration positions of molecular mass markers (in kDa). Abbreviation: NB, newborn.

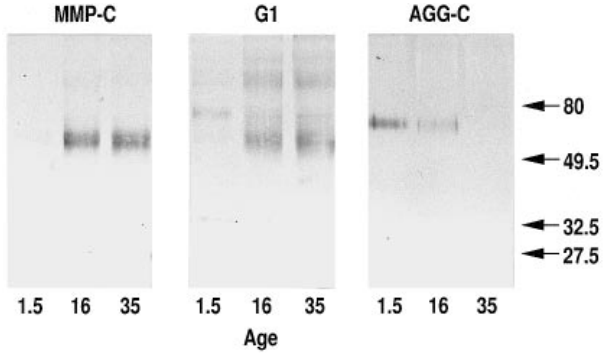

Figure 8 Comparison of the relative abundance of the total G1-containing aggrecan degradation products with those specifically generated by cleavage at the metalloproteinase and aggrecanase sites

Extracts of nucleus pulposus (1.5 μl for blots with MMP-C and anti-G1 antibodies; 10 μl for blots with AGG-C antibody) from three individuals were analysed by immunoblotting with the anti-neoepitope and anti-G1 antibodies. The extracts from these individuals were chosen because they exhibited marked differences in the relative abundances of the MMP-C and AGG-C neoepitopes. The arrows at the right indicate the migration positions of molecular mass markers (in kDa).

fibrosus and nucleus pulposus extracts showed that the 70 kDa degradation product was present in comparable amounts in the young adult, but was more abundant in annulus fibrosus in the mature adult (Figure 6B, right panel), consistent with the age-related patterns described above. The results indicate that whereas the degree of metalloproteinase action is similar in intervertebral disc and cartilage, aggrecanase-mediated proteolysis seems to be much more predominant in cartilage than in disc.

Contribution of metalloproteinase and aggrecanase site cleavage to aggrecan degradation

Even though the metalloproteinase and aggrecanase sites have been the most extensively characterized aggrecan cleavage sites, their contribution to total aggrecan degradation in cartilage and intervertebral disc *in vivo* has not been determined. Thus, to examine the importance of cleavage at these sites, tissue extracts were analysed by immunoblotting with an anti-G1 antibody to detect all degradation products containing this domain. Analysis of annulus fibrosus, nucleus pulposus and articular cartilage demonstrated that there were two major G1-containing degradation products that were consistently present irrespective of age and tissue source, albeit with variations in their relative abundance (Figure 7). These migrated with apparent molecular masses of approx. 55 and 70 kDa, sizes similar to those of the degradation products detected with the MMP-C and AGG-C antibodies respectively. It was also evident that a more extensive degradation of aggrecan was occurring with increasing age, which was manifested by the appearance of components that migrated more rapidly than the 55 kDa degradation product, but no components intermediate in size between the major 55 and 70 kDa species were apparent. This indicated that further proteolytic cleavages were occurring within the G1 domain rather than in the interglobular domain.

To determine whether the abundance of the major G1-containing degradation products could be correlated with that of the fragments detected with the MMP-C and AGG-C antibodies, extracts of nucleus pulposus from three individuals were analysed with all three antibodies. The fragment detected with the MMP-C antibody increased with age (Figure 8, left panel), and the change in intensity correlated with that exhibited by the 55 kDa product detected with the anti-G1 antibody (Figure 8, middle panel, lower band). The degradation product detected by the AGG-C antibody decreased with age in nucleus pulposus (Figure 8, right panel), and the change in its abundance was similar to that of the 70 kDa fragment detected with the anti-G1 antibody (Figure 8, middle panel, upper band). To establish further the identity of the major degradation products observed with the anti-G1 antibody, their electrophoretic mobilities were compared directly with those of the fragments detected by the MMP-C and AGG-C antibodies. Blots of individual nucleus pulposus extracts

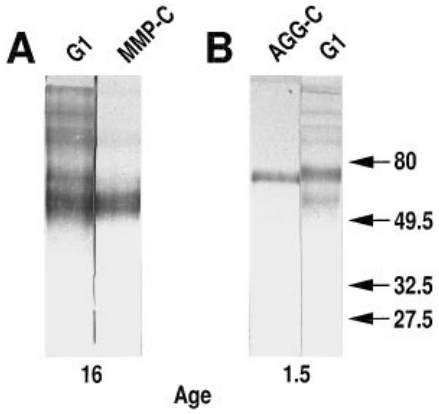

Figure 9 Comparison of the electrophoretic migration of the major G1-containing aggrecan degradation products with those generated by specific cleavage at the metalloproteinase and aggrecanase sites

Extracts of nucleus pulposus from 16-year-old (**A**) and 1.5-year-old (**B**) individuals were analysed by immunoblotting with the anti-neoepitope and anti-G1 antibodies. For each analysis an individual sample of extract (5 μl for **A**, 10 μl for **B**) was loaded into a double-width lane, subjected to electrophoresis and subsequently transferred to nitrocellulose. Thereafter each membrane was cut longitudinally along the middle of the sample lane and the halves were blotted with MMP-C and anti-G1 (**A**) or with AGG-C and anti-G1 (**B**). The arrows at the right indicate the migration positions of molecular mass markers (in kDa).

were cut into halves and analysed with the anti-G1 antibody and either the MMP-C or AGG-C antibody. The comparison demonstrated that the 55 and 70 kDa degradation products detected with the anti-G1 antibody co-migrated with those observed with the anti-MMP-C and anti-AGG-C antibodies respectively (Figure 9). Taken together, these results indicate that the 55 and 70 kDa degradation products possessing termini resulting from cleavage at the metalloproteinase and aggrecanase sites constitute major N-terminal degradation products of aggrecan present in intervertebral disc and articular cartilage *in vivo*.

DISCUSSION

In this study, anti-neoepitope antibodies have been used to assess the role of metalloproteinases and aggrecanase in the degradation of aggrecan in human intervertebral disc and articular cartilage. The profiles of proteinase action during aging in annulus fibrosus and articular cartilage are similar [7,13] in that the degradation products arising from both aggrecanase and metalloproteinase action increase between the juvenile and the adult. However, the tissues differ in the order in which the products of these enzyme systems appear and reach a plateau with age. In contrast with annulus fibrosus and articular cartilage, nucleus pulposus exhibits age-related patterns of metalloproteinase and aggrecanase action that are opposite to each other. The tissue-specific differences in the temporal order of metalloproteinase and aggrecanase action and their opposite age-related trends in nucleus pulposus indicate that the two enzyme systems act independently, and suggest that they might have distinct functions in matrix turnover and degradation in disc and cartilage. In particular, the early appearance of aggrecanase action in nucleus pulposus might be indicative of an important role for this enzyme in the development and/or growth of this tissue. It is interesting to note that a distinguishing feature of the nucleus pulposus of the young juvenile, in comparison with annulus fibrosus, is the presence of notochordal cells that are present at birth but gradually disappear by approx. 10 years of age [15]. It might be postulated that notochordal cells are more sensitive to catabolic stimuli than other cells present in the nucleus pulposus and the resident cells of the annulus fibrosus.

The direct comparison of adult annulus fibrosus, nucleus pulposus and cartilage demonstrated comparable levels of the metalloproteinase-generated degradation product in adult tissues. This indicates that the contribution of metalloproteinases to aggrecan degradation is similar in disc and cartilage, despite their temporal differences. In contrast, the aggrecanase degradation product is present in much greater abundance in adult cartilage compared with either of the disc tissues, despite the age-related increase in abundance also shown for annulus fibrosus. Thus aggrecanase action seems to have a much more important role in articular cartilage than in intervertebral disc.

It should be noted that there are two factors that could affect the interpretation of the observed neoepitope levels. First, the correlation between the extent of proteinase action and the abundance of the detected neoepitopes might be altered if the neoepitopes generated by metalloproteinase and aggrecanase action are removed from the initial degradation products or are lost from the tissues before tissue sampling. This could be due to further proteolytic degradation, thereby resulting in the release of the neoepitope-bearing C-terminus or in abrogation of hyaluronate binding via cleavage within the G1 domain. Alternatively, the degradation of hyaluronate might cause a release of bound G1 regions and their loss from the tissue by diffusion. Because our data indicate that metalloproteinases and aggrecanase are major contributors to aggrecan degradation in these tissues, one effect of further proteolysis could be the loss of some aggrecanase-generated neoepitope as a result of subsequent cleavage at the metalloproteinase site. In addition it is not known how long metalloproteinase- and aggrecanase-generated degradation products remain in the tissues bound to hyaluronate and unaltered by further proteolysis. If the degradation products do indeed survive for many years, they would accumulate and their observed abundance would be an indicator of total proteolytic action occurring before tissue sampling. If, however, they are lost from the tissues at appreciable rates, their abundance would rather be a reflection of their rate of turnover during a shorter time frame before tissue sampling.

The second factor that could affect the interpretation of these data is that glycosylation near the aggrecanase site might interfere with its cleavage by aggrecanase. The sequence surrounding this site, N^{368}ITEGE/ARG, possesses potential sites for N- and O-linked keratan sulphate chain attachment. Barry et al. [29] have demonstrated that aggrecan from steer, but not calf, articular cartilage is substituted at residue(s) Asn^{368} and/or Thr^{370}. Although it has not been shown, it is possible that aggrecan from adult human cartilage and disc could exhibit a similar age-related change in glycosylation; if the resulting keratan sulphate chains were of large enough size, this might interfere with aggrecanase action in the adult owing to steric hindrance. This factor might to some extent contribute to the apparent decrease in aggrecanase-mediated degradation observed with age in nucleus pulposus, which has previously been shown to contain keratan sulphate with greater chain lengths than in articular cartilage [2].

Although the present results illustrate the major contribution of metalloproteinases and aggrecanase to total aggrecan degradation in disc and cartilage *in vivo*, they do not preclude the possibility that other agents also contribute to aggrecan degradation in these tissues. In fact, in some adults, immunoblot analysis with the anti-G1 antibody did reveal minor degradation products whose molecular sizes were indicative of additional proteolysis within the G1 domain, although the agents responsible for the generation of these degradation products are not known. It is interesting to note that the decrease in abundance of the metalloproteinase-generated fragment exhibited by nucleus pulposus late in adulthood coincides with the appearance of such low-molecular-mass degradation products. Although studies *in vitro* have shown that many proteinases of potential physiological relevance are able to cleave the aggrecan interglobular domain between the sites of action of metalloproteinases and aggrecanase [12], there was no evidence of degradation products intermediate in size between the major 55 and 70 kDa fragments, suggesting that such proteinases do not have a major role in aggrecan degradation in these tissues. In addition to proteinases, other agents such as free radicals [30], which might be generated in significant amounts as a result of the avascular nature of cartilage and disc, could contribute to aggrecan degradation, although evidence for their involvement remains to be established.

Taken together, the results presented here demonstrate the importance of both metalloproteinases and aggrecanase in the degradation of aggrecan in human intervertebral disc and articular cartilage *in vivo*. The functional importance of aggrecan in conferring on these tissues their characteristic mechanical properties suggests that its proteolytic degradation during aging might result in altered tissue function. This might occur as a result of both aggrecan depletion from the tissues as well as by the accumulation of degradation products that might compete with newly synthesized molecules for sites within the matrix. These events might contribute to altered functional properties of the tissues and a predisposition to age-related degeneration.

We thank Marie-Claude Magny for technical assistance, Elisa de Miguel for synthesizing the peptides, and Jane Wishart for the photography and artwork. This work was supported by the Arthritis Society of Canada and the Shriners of North America.

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Received 17 January 1997/10 April 1997; accepted 18 April 1997