Age-related changes in the content of the C-terminal region of aggrecan in human articular cartilage

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The content of the C-terminal region of aggrecan was investigated in samples of articular cartilage from individuals ranging in age from newborn to 65 years. This region contains the globular G3 domain which is known to be removed from aggrecan in mature cartilage, probably by proteolytic cleavage, but the age-related changes in its abundance in human cartilage have not been described previously. The analysis was performed by immunosorbant assay using an antiserum (JD5) against recombinant protein expressed from a cDNA clone encoding the terminal 598 amino acid residues of human aggrecan, on crude extracts of cartilage without further purification of aggrecan. The results showed that the content of the C-terminal region decreased with age relative to the G1 domain content (correlation coefficient = 0.463). This represented a 92% fall in the content of this region of the molecule from newborn to 65 years of age. Furthermore, when the G1 content of the cartilage extracts was corrected to only include the G1 attached to aggrecan and to exclude the G1 fragments which accumulate as a by-product of normal aggrecan turnover (free G1), the age-related decrease in the C-terminal region remained very pronounced. Analysis by composite agarose/PAGE showed that the number of subpopulations of aggrecan resolved increased from one in newborn to three in adult cartilage. All of these reacted with an antiserum to the human G1 domain, but only the slowest migrating species reacted with the C-terminal region antiserum (JD5). Similar analysis by SDS/PAGE confirmed the presence of high-molecular-mass (200 kDa) proteins reactive with JD5, but no reactive fragments of lower electrophoretic mobility were detected. In contrast, when probed with the antiserum to the human G1 domain, the immunoblots showed protein species corresponding to the free G1 and G1-G2 fragments, which were present at high concentrations in adult cartilage. The results suggest that the loss of the C-terminal region is not directly part of the process of aggrecan turnover, but it is a slow independent matrix process that occurs more extensively with aging as turnover rates become slower. Young cartilage with the fastest turnover contains least molecules lacking the C-terminal region, whereas in old tissue with slow turnover few molecules retain this region. An increase in the cleavage of this region with age may also contribute to this change. The content of the C-terminal region may thus give a measure of the abundance of newly synthesized aggrecan.

INTRODUCTION

The predominant macromolecules in the extracellular matrix of articular cartilage are collagen type II and the large aggregating proteoglycan, aggrecan. Aggrecan consists of a multidomain protein core (reviewed in [1]) to which are attached many chondroitin sulphate (CS) and keratan sulphate (KS) chains. The complete human cDNA [2] and gene [3] sequences predict a protein core of molecular mass 250 kDa, which by rotary shadowing electron microscopy appears as three globular and two extended segments (for a recent review, see [4]). The N-terminal region contains two globular domains, G1 and G2, separated by an interglobular domain. At the C-terminus is a third globular domain, G3, which is joined to G2 by a long extended region that contains attachment sites for CS and KS chains. G1 and G2 are closely related in sequence but it is only G1 that is able to interact with hyaluronan and with link protein [5–8] in the formation of large aggregates. Such aggregates become immobilized within a rigid collagen network, and the high concentration of polyanion creates a hydration pressure which provides the functional basis of the biomechanical properties of articular cartilage.

Whereas the functions of the G1 domain and the KS-and CS-bearing region are known, that of the G3 domain remains unclear. The cDNA sequence has revealed it to be unrelated to the G1 and G2 domains, but it does contain elements with sequence similarities to three other protein families: (1) the complement B regulatory component [9]; (2) a family of cell-surface and extracellular carbohydrate recognition proteins, the Ca^{2+}-dependent mammalian type C lectins [10]; and (3) the epidermal growth factor (EGF)-like domains that have two motifs in tandem and can bind Ca^{2+} ions [11,12]. These EGF-like domains and the complement B component also exhibit alternative splicing in aggrecan [2,11,12]. The amino acid sequence of the G3 region is highly (> 90%) conserved amongst aggrecans from different species [13] and is about 65% similar to the corresponding C-terminal domain of other large aggregating proteoglycans such as versican from fibroblasts [14], and neurocan and brevican from brain tissue [15,16]. The same motifs are found in the selectins, which are cell-surface glycoproteins [17]. Such widespread conservation of these motifs suggests a specific role in carbohydrate recognition. The G3 domain of aggrecan has been expressed from cDNA and was shown to have low-affinity interactions with carbohydrates, in particular with fucose

Abbreviations used: ABTS, 2,2′-azinodi-(3-ethylbenzthiazoline sulphonate); anti-hG1, antiserum against human G1 domain; CS, chondroitin sulphate; EGF, epidermal growth factor; GST, glutathione S-transferase; IPTG, isopropyl thiogalactoside; KS, keratan sulphate.

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and galactose [10,18] and with hyaluronan [19], which may indicate binding to cellular or matrix ligands. One or more of the motifs of the G3 domain may have an important function during the intracellular synthesis and maturation of proteoglycan, as has been suggested from studies on a heritable chondrodystrophy of chicken, nanomelia. In this model the G3 domain fails to be synthesized during translation of the aggrecan mRNA [20,21], which leads to the degradation of the incomplete aggrecan core protein within the cytoplasm and an absence of proteoglycan in the matrix.

The G3 domain is known to be lost from aggrecan in the matrix, perhaps by a proteolytic mechanism [22–24]. Rotary shadowing electron microscopy shows the G3 domain to be present in the majority of proteoglycan monomers isolated from very young tissue, but is estimated to be present in only 10–50 % of molecules from older tissue [25]. There have been no detailed studies of the age-related changes in the G3 domain content of aggrecan from human cartilage, or any evidence for the mechanism leading to its removal from the protein core and its subsequent fate. The extensive heterogeneity of aggrecan in cartilage with increasing age is attributable to the size and number of CS (decreased) and KS (increased) chains, to the pattern of sulphation of the glycosaminoglycans [26,27] and to proteolytic activity on the core protein, particularly in the interglobular domain [28–30]. Catabolism of the C-terminal region of aggrecan may contribute significantly to this heterogeneity.

It was the purpose of the present study to determine the age-related changes in the content of the C-terminal region of aggrecan from human articular cartilage, and to investigate the presence of peptides corresponding to this region released from aggrecan within the cartilage matrix. An ELISA was developed using a polyclonal antiserum to the C-terminal region of aggrecan and was used to determine the concentration of this region in extracts of human cartilage. The antiserum was also used to identify proteoglycan subpopulations carrying the C-terminal region and to detect protein fragments derived from its cleavage.

EXPERIMENTAL

Materials

All reagents were of analytical grade from BDH Chemicals (Poole, Dorset, U.K.). Guanidinium chloride, 6-aminohexanoic acid, benzamidine HCl, PMSF, soya-bean trypsin inhibitor, anti-(rabbit IgG) antibody–peroxidase conjugate and 2,2′-azinodi-(3-ethylbenzthiazoline sulphonate) (ABTS) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Chondroitinase ABC and keratanase I were from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Reagents for enhanced chemiluminescence (ECL) assay and Hyperfilm-MP were obtained from Amersham International (Little Chalfont, Bucks., U.K.).

cDNA sequences of the C-terminal region of aggrecan

A human cartilage cDNA phage library in λgt11 [31] was screened with a 1.2 kbp insert from clone λP3, which contained sequences of the pig G3 domain [32]. Two clones, λH4 and λC4, from the human library were isolated and sequenced; these contained inserts of 1590 bp and 1923 bp respectively and had identical sequence in the overlap region. Both clones started at the same nucleotide position within the CS attachment region, but clone λC4 extended beyond the translation reading frame of the mRNA and contained 123 bp of the 3′ untranslated region.

Construction of expression plasmids for G3 fusion protein

All DNA manipulations and bacteriological media and procedures were essentially as described [33]. The coding region of the insert in λC4 was amplified by PCR in 50 μl volumes using Ampli*Taq* DNA polymerase (Perkin Elmer, Warrington, U.K.), in buffer conditions as listed by the supplier and containing 1 nmol each of the synthetic oligonucleotides 5′-GCGGATCCAGTGGCCTACCAAGTGGC (forward) and 5′-GCGAATTCGTGGGCTGTGCTGGGGCG (reverse) acting as primers corresponding to the 5′- and 3′-ends respectively of the cDNA. PCR amplification conditions were: 92 °C, 1 min (denaturation); 46 °C, 1 min (annealing); and 72 °C, 1.5 min (extension) for 30 cycles, followed finally by an extension at 72 °C for 10 min. The primers were designed to incorporate a *Bam*H1 or an *Eco*R1 restriction site at each end of the amplified product in order to facilitate unidirectional and in-frame cloning into the pGEX-2T plasmid (Pharmacia Biotech, St. Albans, U.K.). The amplified DNA was ligated into the *Bam*H1--*Eco*R1 site of pGEX-2T, enabling the synthesis of the CS-G3 insert in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST), under control of the isopropyl thiogalactoside (IPTG)-inducible *tac* promoter. A unique thrombin cleavage site aids the release of the CS-G3 protein from the conjugate. Plasmid constructs were transformed into *E. coli* DH5a competent cells (Life Technologies, Paisley, Scotland, U.K.) and ampicillin-resistant recombinants were selected on L-agar plates. Plasmid DNA was prepared from several colonies and inserts were analysed by predicted size and restriction mapping. DNA from one clone, pGEX1C4, was sequenced across the *Bam*H1 site to ensure that the coding sequence of the insert was in-frame with the GST sequence.

Cultures of pGEX1C4 induced with 1 mM IPTG were grown as described [33] in order to prepare fractions of supernatants and insoluble inclusion bodies from cell extracts. Briefly, cell pellets were resuspended in 10 vol. of STE buffer (0.01 M Tris/HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 0.5 mM PMSF) and lysozyme was added to a concentration of 10 μg/ml. After 30 min at room temperature, cell lysis was achieved by a rapid freeze–thaw of the cell suspension. The released DNA and insoluble debris was pelleted by centrifugation at 100000 ***g***. Supernatants were analysed by SDS/PAGE with non-induced cultures as controls. Supernatants from induced cultures containing approx. 5 mg of total protein were combined with an equal volume of Freund's complete adjuvant and used to raise a polyclonal antiserum in New Zealand White rabbits. Rabbits were boosted after 14 days with approx. 1 mg of total protein in incomplete adjuvant. The antiserum against recombinant CS-G3 from one rabbit, JD5, was used in this study.

Preparation of articular cartilage extracts

Articular cartilage from individuals ranging from 5 years to 65 years of age was obtained from mid-thigh and hind-quarter amputations at the time of surgery and was macroscopically normal. Sections of cartilage (20 μm) were extracted with 4.0 M guanidinium chloride, 0.05 M sodium acetate buffer, pH 6.8, at 4 °C for 24 h. Insoluble debris was removed by filtration. Supernatants containing the protein preparations were stored at −20 °C in small aliquots. Prior to assay, samples were dialysed extensively into 0.05 M sodium acetate, pH 6.8. Aliquots of samples containing 80 μg of uronic acid were treated with chondroitinase ABC (0.02 unit) overnight at 37 °C, and used directly in the ELISA. All extraction and dialysis buffers in the cartilage preparative procedures contained proteinase inhibitors

(0.01 M disodium EDTA, 0.01 M 6-aminohexanoic acid, 5 mM benzamidine HCl, 0.5 mM PMSF).

Aggrecan standard

Aggrecan was prepared from newborn human cartilage extracted as above. The proteoglycan monomer was then purified by CsCl density gradient centrifugation [34], and its final concentration was determined from the content of hexuronic acid. CS chains were removed from aggrecan by digestion with chondroitinase ABC (0.04 unit/mg of uronic acid) in 0.1 M Tris/acetate, pH 8.0, 0.3 M sodium acetate overnight at 37 °C to give a preparation of aggrecan core protein. Digests were dialysed extensively against water and the concentration of protein was determined by absorbance at 280 nm.

Preparation of the G1 domain

The G1 domain was prepared by trypsin digestion of proteoglycan aggregates from human cartilage as described by Bonnet et al. [35]. G1 domain prepared by this method was used for immunization of rabbits to yield an anti-(human G1 domain) antiserum (anti-hG1). For RIA, the free G1 domain in cartilage extracts was purified from aggrecan core protein by chromatography on a TSK SWG 3000 column (30 cm × 2.1 cm) and the 60 kDa fraction was used in an RIA [36].

Two-step ELISA using JD5

Plastic microtitre plates (Immulon; Dynatech Laboratories) were coated overnight at 4 °C with 200 μl per well of a 1 μg/ml solution of human newborn aggrecan protein core standard in 0.02 M sodium carbonate, pH 9.6. Antigen-coated plates were washed four times with incubation buffer A (containing 0.1 % BSA, 0.1 % Nonidet P-40, 0.15 M NaCl, 0.01 M Tris/HCl, pH 7.4). Serial dilutions of fetal core standard or samples (0.6–80 μg of protein/ml) in 125 μl of buffer A were mixed with 125 μl of JD5 antiserum (1:2000 final dilution) and incubated at room temperature for 1 h, and then overnight at 4 °C. A 200 μl sample of this mixture was then transferred to the antigen-coated plate and mixed with gentle shaking for 90 min at 37 °C. Reference wells contained 200 μl of buffer A. Maximum binding was determined in wells containing 200 μl of diluted JD5 without added competitor. Plates were washed as before, 200 μl/well goat anti-(rabbit IgG)–horseradish peroxidase (Sigma) conjugate was added (diluted 1:1000) and the incubation was continued for a further 90 min at 37 °C. The plates were washed and 200 μl/well peroxidase substrate ABTS was added, and after 20–30 min incubation at 37 °C the absorbance at 405 nm was read on a Bio-Tek Model E-310 EIA plate reader. The data were expressed in terms of percentage inhibition of antibody binding to the antigen-coated plate against the concentration of the G1 domain (μg of G1/ml) in each extract.

Gel electrophoresis and immunoblotting

SDS/PAGE

Electrophoresis was carried out on Laemmi 13 % polyacrylamide gels of extracts containing fusion protein prepared from bacterial cultures. Aliquots were loaded after boiling in buffer containing 0.1 % SDS and 1 % 2-mercaptoethanol. For the analysis of cartilage extracts, samples were separated on 5–15 % polyacrylamide gradient slab gels as follows. Aliquots containing 5 μg of uronic acid in each extract were digested with chondroitinase ABC and keratanase (0.075 unit/ml of each enzyme) in 0.125 M Tris/HCl containing proteinase inhibitors for 2 h at 37 °C. Samples were mixed with an equal volume of sample loading buffer without 2-mercaptoethanol then boiled prior to electrophoresis. Immunoblotting [37] was carried out following electrophoretic transfer of proteins to Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.). The membranes were incubated for 1 h at room temperature in PBS (0.01 M sodium/potassium phosphate buffer, pH 7.2, 0.145 M NaCl and 0.05 % NaN_3) containing 5 % (w/v) dried skimmed milk; then for 1 h in PBS containing 1 % dried skimmed milk and JD5 (diluted 1:300) or anti-hG1 (diluted 1:1000). After extensive washing in PBS, the membranes were incubated for 1 h in PBS containing 1 % dried skimmed milk and goat anti-(rabbit IgG)–horseradish peroxidase (diluted 1:5000), and finally washed as before. Specifically bound antibody was visualized by ECL performed according to the supplier's instructions and membranes were exposed to Hyperfilm-MP.

Large-pore-agarose/polyacrylamide composite-gel electrophoresis

Composite gels were run under dissociative conditions in 4 M urea, 0.01 M Tris/acetate, pH 6.8, 0.25 mM Na_2SO_4 [38]. Proteoglycan bands were detected by staining with 0.2 % Toluidine Blue. Immunoblotting was carried out following semi-dry electrophoretic transfer (Pharmacia Biotech) in the above buffer without urea.

RESULTS

Preparation of recombinant protein and specificity of antisera

The cDNA clone λC4 isolated from the human chondrocyte library was used in this study. It included all of the globular G3 domain, comprising the type C lectin-like and the complement regulatory protein-like motifs but not the two EGF-like motifs. It extended 350 amino acid residues beyond the G3 domain towards the N-terminus and included approximately 20 % of the

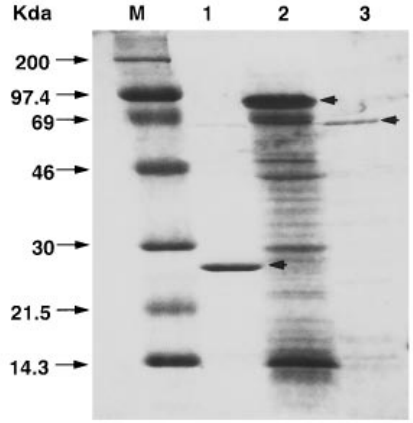

Figure 1 Expression and proteinase cleavage of recombinant protein

SDS/13 %-PAGE of proteins stained with Coomassie Blue R250. Supernatant (100 μl) from IPTG-induced cultures was bound to 10 μl of glutathione–Sephadex affinity matrix which, after six washes, was resuspended in 20 μl of STE buffer containing 20 ng of thrombin and incubated for 18 h at 25 °C. After centrifugation the supernatant was recovered and the matrix was resuspended in 20 μl of STE; 10 μl of each was used for electrophoresis. Lanes 1 and 3, affinity matrix residue and supernatant respectively after thrombin cleavage; lane 2, crude supernatant of cell extracts prepared from IPTG-induced cells. The positions and sizes (kDa) of molecular mass markers (lane M) are indicated. The arrowheads indicate the GST carrier (lane 1), GST–CS-G3 fusion product (lane 2) and CS-G3 protein (lane 3).

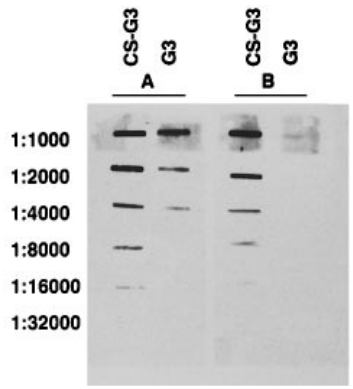

Figure 2 Specificity of antiserum JD5, raised against recombinant CS-G3

Recombinant proteins were purified from *E. coli* cultures of constructs expressing CS-G3 and G3 domains by affinity purification on a glutathione–Sepharose matrix and thrombin cleavage. Slot-blots were prepared containing 10 ng of each protein per slot and treated with serial dilutions of JD5 antiserum (A) and JD5 antiserum that had been preabsorbed with 20 μg of purified recombinant G3 protein for 18 h at 4 °C (B). Antiserum dilution is indicated on the left. In both cases the antiserum had been preabsorbed with an extract of *E. coli* expressing the pGEX-2T parent prior to use.

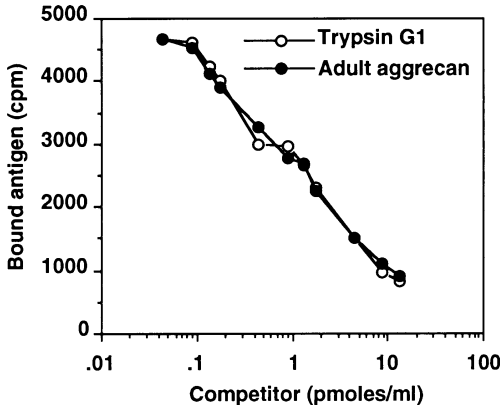

Figure 3 Specificity of anti-hG1 antiserum raised against G1

The reactivity of anti-hG1 against the trypsin-isolated G1 fragment (○; molecular mass 60 kDa) of aggrecan was compared with that against highly purified adult aggrecan (●) [prepared by associative and three sequential dissociative CsCl gradient centrifugation steps; molecular mass 1.14×10^6 Da)] in RIAs using ^{125}I-labelled G1. The molecular mass of purified aggrecan was determined by Sepharose CL-2B chromatography [39].

total CS-attachment domain of aggrecan. The cDNA was expressed in *E. coli* as a fusion product in the construct pGEX1C4 giving a 598-amino-acid protein (CS-G3) linked to GST. SDS/PAGE analysis showed that a band of approximate molecular mass 90 kDa was specifically induced by IPTG and was abundant in the soluble fraction of cell extracts (Figure 1). This protein could bind selectively to glutathione–Sephadex beads, and upon cleavage with thrombin gave two products of approx. 65 and 28 kDa, representing the CS-G3 domain product and GST respectively. Although the fusion product showed binding to the glutathione–Sephadex matrix the affinity was low and the protein could not be purified by this method in amounts that were sufficient for immunization. The IPTG-induced soluble fraction (Figure 1, lane 2) was therefore used to raise an antiserum, JD5.

The specificity of the JD5 antiserum was confirmed by immunoslotblots of recombinant proteins representing the CS-G3 protein used for immunization and the G3 domain. The G3 domain was expressed in a second construct which contained sequence for the terminal 230 amino acid residues of aggrecan, representing the lectin-like and complement-regulatory protein-like motifs but not the CS attachment region. The JD5 antiserum reacted strongly with both recombinant proteins (Figure 2), although the titre was higher for the CS-G3 polypeptide. Antiserum preabsorbed with the G3 protein had a positive titre at 1:8000 for CS-G3, compared with a titre of 1:4000 against G3 with non-preabsorbed antiserum (Figure 2). These results showed that JD5 was able to recognize epitopes within the G3 domain and the adjacent CS attachment segment, although the titre was lower against the G3 domain. The reactivity against the G3 domain was also confirmed by immunoblot after SDS/PAGE of CS-G3 and G3 proteins (results not shown). The reactivity of the G1 domain antiserum (anti-hG1) was tested by RIA with purified aggrecan and isolated G1. Purified aggrecan and isolated G1 showed the same reactivity in their slopes and inhibition on a molar basis (Figure 3). The activity of this antiserum is therefore against epitopes within the G1 sequence, and it is very unlikely that a significant part of the reactivity is against a neo-epitope generated by trypsin cleavage.

ELISA for the C-terminal region of aggrecan

Aggrecan prepared from newborn human cartilage was treated with chondroitinase ABC to make a core protein preparation that was used as the coating antigen on microtitre plates. The concentration of the G1 domain in this preparation and in each cartilage extract was determined by RIA. Aggrecan was quantified by its G1 domain content, and all results from competition assays with the JD5 antiserum were expressed relative to the competition achieved with this aggrecan population. Competition of the core protein standard for binding to JD5 was linear in the range 0.5–10 μg/ml G1, with a 50% inhibition value of 2.2 μg/ml (Figure 4). Newborn cartilage aggrecan that had not previously been treated with chondroitinase ABC produced a

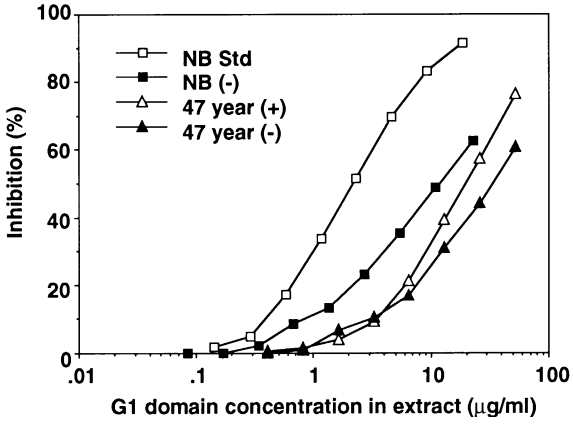

Figure 4 ELISA with the JD5 antiserum using purified aggrecan

Plates coated with 1 μg/ml cartilage core protein standard from newborn (NB Std) were assayed with serial dilutions of purified aggrecan from samples (from newborn and 47-year-old subjects) either treated (+) or not (−) with chondroitinase ABC.

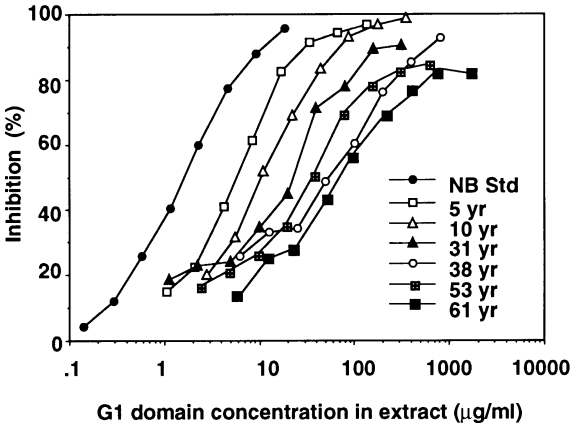

Figure 5 Assays for the C-terminal region of aggrecan in crude extracts of cartilage

Competition curves are shown for cartilage extracts (chondroitinase-treated) from subjects of various ages as indicated. NB Std indicates cartilage core protein standard from newborn. The percentage inhibition values for the C-terminal region are expressed against the total G1 domain concentration in each extract, determined as described in the text. Not all specimens are shown here (see Figure 6).

50% inhibition value of 11.5 μg of G1/ml. There was a similar change in inhibition when an adult (47 year) aggrecan preparation was assayed before (19.0 μg of G1/ml) and after (29.0 μg of G1/ml) removal of the CS chains. This is most likely because JD5 also recognizes epitopes within the terminal CS attachment region, and the CS chains may limit the access of the antiserum to the protein core. For a full analysis, ELISAs for the detection of C-terminal epitopes were carried out on samples of cartilage extracts both with and without prior digestion with chondroitinase ABC.

Changes in aggrecan C-terminal region content with age

These preliminary results suggested that there was a decrease in the content of the C-terminal region of aggrecan in cartilage with age. We investigated this further by assaying cartilage extracts from individuals ranging in age from 5 to 65 years. The competition curves for a representative age spectrum of chondroitinase ABC-treated extracts (Figure 5) showed that, as the age of the cartilage increased, there was a corresponding increase in the 50% inhibition values relative to the total G1 domain content for each sample. This indicated that the content of the C-terminal region of aggrecan decreased with age relative to the G1 domain, with a correlation coefficient of 0.463 for all 21 specimens examined (Figure 6, top panel). The fall in the content of the C-terminal region in cartilage from subjects from newborn to 65 years was approx. 13-fold. Assays done on extracts without chondroitinase ABC digestion had a correlation coefficient of 0.35 (Figure 6, bottom panel). The difference in the 50% inhibition values between the digested and undigested samples was greatest in the specimens from the newborn and 5-year-old subjects, but was less pronounced in specimens from subjects aged 10–65 years (inhibition curves for undigested extracts not shown). This result was compatible with the known change in the composition of aggrecan seen with aging; the CS chains are reduced in size and amount, and they may therefore give less screening of the core protein in the specimens from older subjects.

The content of the C-terminal region was also determined in aggrecan fractions from which all cleaved low-molecular-mass

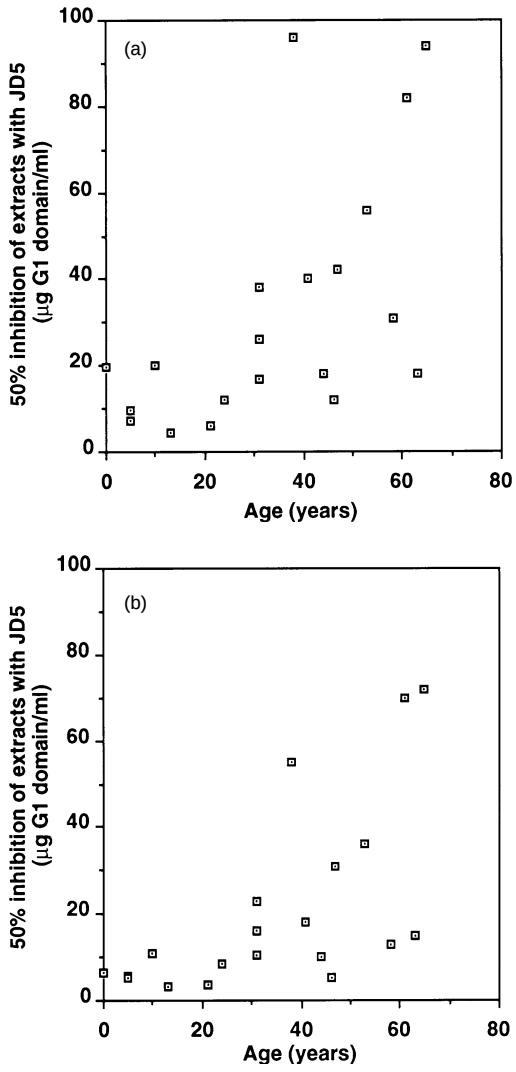

Figure 6 Relationship of the aggrecan C-terminal region content in cartilage with age

The 50% inhibition values (total G1 in μg/ml) from ELISA curves using JD5 are shown for all 21 specimens analysed, before (top panel) and after (bottom panel) chondroitinase treatment. Note that there are two specimens with similar values from 5-year-old subjects.

Table 1 Relationship between the C-terminal region content and the G1 domain in cartilage

Cartilage extracts were provided by subjects of various ages, as indicated. Extracts of cartilage from some specimens were assayed for G1-containing aggrecan, and this was expressed as a proportion of the total G1 domain in the cartilage. The 50% inhibition values (IC_{50}) obtained from the ELISA curves using JD5 are shown before and after taking this proportion of the G1 domain into account. (The total G1 content represents the sum of the G1 domain present on aggrecan and the free G1 retained in the cartilage.)

Age (years)	IC_{50} (μg/ml) of C-terminal region measured against: Total G1	G1 on aggrecan
5	5.4	5.4
20	10.0	8.0
31	10.5	8.6
47	31.0	19.8
53	36.0	23.8
65	72.0	46.4

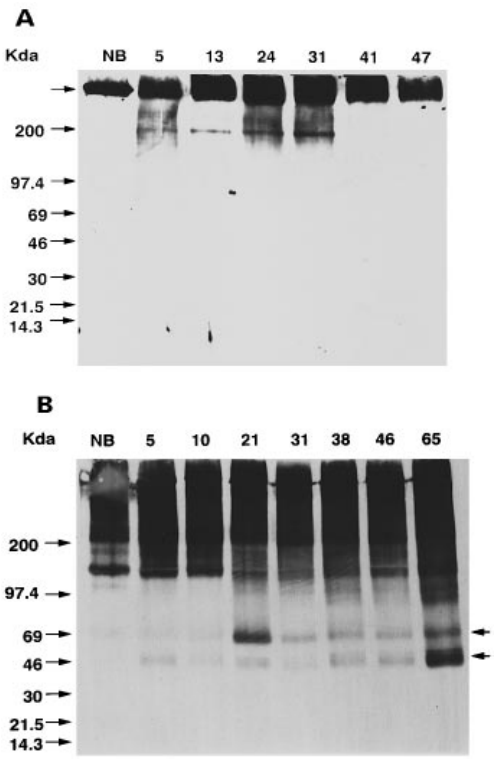

Figure 7 Immunoblot analysis of cartilage extracts

Cartilage extracts equivalent to 5 μg of uronic acid (**A**) or 10 μg of G1 domain (**B**) per lane were digested with chondroitinase ABC and keratanase, followed by electrophoresis under non-reducing conditions. The numbers above each lane refer to the age in years of the subject that provided the cartilage specimen; NB, newborn. Positions of molecular mass markers (kDa) are indicated. Immunoblots are shown (**A**) for the C-terminal region of aggrecan using JD5 and (**B**) for the G1 domain using anti-hG1 antiserum. Fragments resulting from cleavage of the G1 domain are indicated by the two arrows on the right. These two bands were fainter and of lower mobility in the sample from the newborn subject.

G1 fragments (free G1), which accumulate in the matrix, were removed. Extracts from some specimens were fractionated on Sepharose CL-6B to separate aggrecan (V_o fractions) from free G1 (V_t fractions), and the concentration of G1 domain in each fraction was assayed by RIA. When the G1 content of cartilage extracts was adjusted to exclude the free G1, the age-related decrease in the C-terminal region of aggrecan remained pronounced (Table 1).

Analysis of extracts by immunoblotting

Extracts of cartilage were analysed by SDS/PAGE and immunoblotting with JD5 antiserum for the presence of the C-terminal protein fragments which may have been released from aggrecan (Figure 7A). The only protein species immunoreactive with JD5 in specimens from subjects of all ages examined was greater than 200 kDa in size and was homogeneous. Immunoreactive protein fragments of lower electrophoretic mobility were not detected. In contrast, immunoblots probed with the anti-hG1 antiserum showed heterogeneously sized proteins, with a predominant high-molecular-mass protein ($>$ 200 kDa) but also two specific bands of approx. 65 and 46 kDa in high abundance in adult cartilage (Figure 7B). These smaller species correspond to the predicted sizes of the free G1 and G1-G2 fragments that result from proteolytic cleavage of aggrecan during normal turnover in

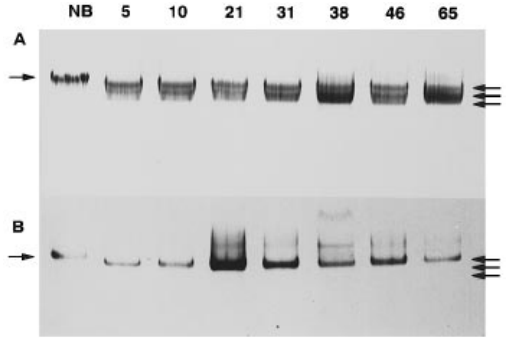

Figure 8 Agarose/polyacrylamide composite gel analysis

(**A**) Cartilage extracts were loaded as whole extracts (without chondroitinase ABC treatment) equivalent to 3 μg of uronic acid per lane and the gel was stained with Toluidine Blue. Numbers above the lanes refer to the age of the subjects that provided the cartilage samples; NB, newborn. The three subpopulations of proteoglycan are marked by the arrows on the right. The single arrow on the left denotes the position of the normally slower migrating proteoglycan population from the newborn. (**B**) Immunoblot of samples (1 μg of uronic acid per lane) probed with JD5 antiserum. The relative positions of the three subpopulations of proteoglycan visualized with Toluidine Blue are marked by the arrows. The top arrow in (**A**) coincides with the slowest migrating population in (**B**).

cartilage. These results suggest that the G3 region, after cleavage from aggrecan, is not retained within the extracellular matrix, presumably because it lacks high affinity for other cartilage components, and therefore it does not accumulate with age.

The heterogeneity of aggrecan was further investigated on composite agarose/polyacrylamide gels to identify those subpopulations that contained the C-terminal region of aggrecan. The number of subpopulations detected by Toluidine Blue staining increased from one in the sample from the newborn subject to three in the adult samples (Figure 8). The single band in the newborn sample was characteristically of particularly low mobility and a similar band was absent from all other samples. This component and only the slowest migrating subpopulation in the adult was immunoreactive with the JD5 antiserum (Figure 8). In contrast, all three subpopulations reacted with the anti-hG1 antiserum (results not shown). A minor unknown component of low mobility was reactive with JD5 in some of the specimens. These results showed that whereas the G1 domain is associated with the increasingly heterogeneous population of aggrecan, the C-terminal region is not.

DISCUSSION

Several studies have shown that aggrecan exists in cartilage in forms that vary widely with respect to the composition and size of its numerous attached carbohydrate chains and its protein core, which generates a polydisperse and heterogeneous population of proteoglycans. The mechanisms responsible for this include age-related changes in biosynthetic and catabolic events [26,27], where the KS content of aggrecan increases and there is a corresponding decrease in the CS content. This is likely to result from a progressive decrease in the length of the CS-bearing domain of the protein from the C-terminus. Until now, the cleavage of this region and its subsequent fate in cartilage have not been described, mainly due to a lack of antibodies specific for epitopes on C-terminal structures. The technique of rotary shadowing electron microscopy has shown the G3 globular structure to be present on most aggrecan molecules prepared from cartilage from young subjects, but it is often missing from

aggrecan prepared from adult cartilage [25], suggesting a loss of this region associated with maturation. A previous method has been described for quantitative analysis of the G3 domain in bovine cartilage using specific marker peptides for G3 [40], but with this method sample preparation and measurement is more complex. Furthermore, the analysis only provides information on aggrecan which bears the G3 domain, and the method would not detect any G3 domain that may be present within the cartilage matrix after cleavage from aggrecan. In our study we determined levels of the C-terminal region, which includes the G3 domain, of aggrecan in human articular cartilage specimens from subjects over an age range. We used recombinant protein to prepare a polyclonal antiserum, because no suitable methods have been developed to purify native C-terminal structures containing the G3 domain from cartilage. The usefulness of our ELISA is that it can be performed on whole extracts of cartilage without further preparative procedures, and numerous samples can be analysed relatively quickly. The results using the antiserum showed that no fragments of the cleaved G3 domain, or of the adjacent CS bearing sequence, were detected, and imply that it does not accumulate in the extracellular matrix either as an intact globular structure or as fragments that are sufficiently large to be immunoreactive. This supports the view of Sandy et al. [40] that the G3 domain is unlikely to be involved in any interactions in the extracellular matrix, but is lost, presumably into the synovial space, once it is free from aggrecan. Our results are also in agreement with the findings of other studies on bovine explant cultures, where two peptides corresponding to the C-terminal region of aggrecan were found to be present in the culture medium, synovial fluid and serum of steers, but were absent from the cartilage matrix [23]. If similar proteolytic peptides were generated in human cartilage they could be lost in the synovial fluid either as complete peptides or after further fragmentation, although we have not investigated this in the present study. It is interesting to note that immunodetection of an EGF-like protein, thought to originate from cartilage aggrecan, in the sera of patients with rheumatoid arthritis and normal controls has been reported [41]. This would suggest that a significant amount of the G3 domain was being cleared from cartilaginous tissues into the circulation, also in agreement with our findings that the domain is lost from cartilage without accumulation. However, it remains to be established at what concentration the G3 domain is present in synovial fluid, and what contribution towards the EGF-like antigen detected in the serum is made by other members of the aggregating proteoglycan family that contain G3-like domains and are expressed in many non-cartilaginous tissues.

The age-related changes in the content of the C-terminal region of aggrecan showed it to fall by 92% in cartilage from 65-year-old versus newborn subjects when compared with the total G1 domain concentration in the extracellular matrix. This change was rather less marked when the free G1 domain, which accumulated in the matrix, was taken into account. Thus the amount of aggrecan with intact G1 and C-terminal region appeared to fall by 88% between 0 and 65 years of age. The decrease in either case was small up to about 30 years of age, followed by a much sharper decrease thereafter in the specimens examined in this study. The concentration of this region therefore remains relatively constant in the first three decades of life, and this may suggest that the biosynthetic turnover of aggrecan and the progressive cleavage at the C-terminus remains steady during this period. The sharper decrease in the C-terminal region content between 30 and 60 years could be due either to an increase in the C-terminal proteolytic cleavage of aggrecan or to the generally slower turnover of aggrecan lacking G3. This would perhaps reflect the change from maturation of cartilage to events associated with aging, and would agree with the increasing heterogeneity of proteoglycans seen in adult compared with immature cartilage [27].

Following detailed analysis it was noticeable that the aggrecan population bearing the C-terminal region was not heterogeneous by gel electrophoresis and was present in only the slowest moving component in samples from all ages. This component also contained the G1 (binding region) domain. From other biosynthetic evidence this population is representative of newly synthesized proteoglycan [26,27], and the increased mobility of the faster species in samples from older subjects is a consequence of proteolytic processing of the core protein and changes in the KS and CS chains. The results imply that, at every age, aggrecan with an intact C-terminal region has similar electrophoretic mobility to newly synthesized aggrecan. The absence of this region from all of the faster bands suggests that it is removed from aggrecan before there is significant cleavage of the protein core close to the G1 domain or there are changes in the carbohydrate composition (KS and CS chains). The loss of the G3 region may occur soon after synthesis of aggrecan, as experiments on calf chondrocytes in culture showed that there was a rapid removal of G3 from newly synthesized aggrecan associated with the cell layer [42]. Comparison between immature and mature bovine cartilage in that study also showed a decrease in the G3 domain content in the mature cartilage. In the hypertrophic zone of the bovine growth plate the level of G3 is reported to be higher than in the resting zone, which, as the cells in the former zone are more metabolically active, could be correlated with a higher content of newly synthesized aggrecan [43].

The mRNA for aggrecan undergoes alternative splicing within the G3 domain [11,12], which may be another mechanism that results in a population of aggrecan lacking a G3 domain. This could be a source of G3-domain-deficient aggrecan that is found in the extracellular matrix, although evidence in the nanomelic chicken suggests that this is probably not the case because the truncated aggrecan polypeptide lacking G3 is not translocated into the extracellular matrix [20]. Intracellular aggrecan deficient in the G3 domain thus appears to be degraded rapidly after translation, which suggests that splicing of aggrecan mRNA in this region is an unlikely mechanism for producing aggrecan lacking the G3 domain that is found in the matrix. The nanomelia mutation may suggest a function for the G3 domain within certain cellular compartments involved in translocation or degradation pathways. It will now be possible to examine the concentration in the synovial fluid, as well as the function, of the aggrecan G3 domain using the antiserum described in the present study in conjunction with cDNA constructs expressing mutations of the various motifs found in this region.

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