Age-related changes in protein-related epitopes of human articular-cartilage proteoglycans

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Monoclonal antibodies were prepared that recognize different age-related epitopes on proteoglycan subunits of high buoyant density isolated from human epiphyseal and articular cartilages. Antibody EFG-4 (IgG1) recognizes a proteinase-sensitive segment associated with the core protein. Antibody BCD-4 (IgG1) reacts with keratan sulphate bound to core protein. Both epitopes are minimally expressed in foetal cartilage and increase with age after birth to become maximally expressed in adult cartilage by about 30 years of age. In contrast, monoclonal antibody α HFP-846 (IgM) recognizes a core-protein-related epitope that is maximally expressed in young foetal cartilage, declines up to birth and thereafter and is almost absent after about 30 years of age. Antibody α HFP-846 was used to isolate by immuno-affinity chromatography two subpopulations of proteoglycan subunits from a 16-year-old-human cartilage proteoglycan subunit preparation. Only the antibody-unbound population showed a significant reaction with antibodies EFG-4 and BCD-4. The amino acid and carbohydrate compositions of these proteoglycan fractions were different, and one (antibody-bound) resembled those of foetal and the other (antibody-unbound) resembled those of adult proteoglycans isolated from 24–27-week-old-foetal and 52–56-year-old-adult cartilage respectively. These observations demonstrate that human cartilages contain at least two chemically and immunochemically distinct populations of proteoglycans, the proportions and content of which are age-dependent. It is likely that these populations represent the products of different genes, though their heterogeneity may be compounded by the result of different post-translation modifications.

INTRODUCTION

Proteoglycans are major extracellular components of cartilage matrix. They consist of a central protein core to which chondroitin sulphate and keratan sulphate chains and *N*- and *O*-linked oligosaccharides are covalently attached (Hascall, 1981; Hascall & Kimura, 1982). Changes in proteoglycan structure have been observed in association with the maturation and aging of cartilage (Inerot *et al.*, 1978; Bayliss & Ali, 1978; Sweet *et al.*, 1979; Elliot & Gardener, 1979; Roughley & White, 1980; Garg & Swann, 1981; Roughley, 1986). These studies revealed that in older cartilage the proteoglycans are smaller, have shorter chondroitin sulphate chains and contain more keratan sulphate; they also contain more protein, the amino acid composition of which is different.

These structural changes result in a polydisperse and heterogeneous proteoglycan population in adult cartilage. There is evidence for the existence of more than one core protein, as indicated by studies with antibodies to proteoglycans (Hadházy *et al.*, 1972; Hadházy & Glant, 1975; Champion *et al.*, 1982). The appearance of an additional population of proteoglycan subunits during aging has also been suggested by immunological methods employing cell-mediated immunity (Champion *et al.*, 1982) and by biochemical studies in which two distinct populations have been separated (Bayliss *et al.*, 1983; Heinegård *et al.*, 1985). We have used monoclonal antibodies to identify age-related proteoglycan changes

in human cartilage and to separate from a 16-year-old-human cartilage two distinct proteoglycan populations that have different protein and carbohydrate compositions, one of which exhibits properties of a foetal nature and the other of an adult nature.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following sources: Pronase from Calbiochem-Behring; papain (2 × crystallized, type IV, EC 3.4.22.2), pepstatin A, bovine serum albumin, guanidinium chloride and phenylmethanesulphonyl fluoride from Sigma Chemical Co.; chondroitinase ABC (EC 4.2.2.5), keratanase (lot nos. E83101 and E84101), keratan sulphate, chondroitin, chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate from Miles Laboratories; Sepharose CL-4B from Pharmacia Fine Chemicals; culture media, foetal-calf serum, tissue-culture plates, dishes and immunoplates for enzyme-linked immunosorbent assays from Flow Laboratories; iodoacetamide and chloramine-T from BDH Chemical Co.; CNBr from American Chemicals; CsCl from Accurate Chemical and Scientific Corp.; Bio-Gel HTP from Bio-Rad Laboratories; ^{125}I as Na^{125}I in aqueous solution from New England Nuclear. Rabbit anti-(mouse IgM) serum was produced in our laboratory with the use of IgM purified from pooled ascites fluid.

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Isolation of cartilage proteoglycans

Fifty-one specimens of adult human cartilage (foetal from 18 to 40 weeks' gestation, newborn, maturing and adult up to 90 years of age) were obtained (within 3–8 h of death) from the distal femoral condyles of individuals showing no macroscopic evidence of joint trauma or disease. Cartilage slices were extracted with 4 M-guanidinium chloride containing enzyme inhibitors (Roughley & White, 1980) and high-buoyant-density proteoglycans (≥ 1.6 g/ml) were isolated by using dissociative density-gradient centrifugation in 4 M-guanidinium chloride and CsCl (Hascall & Sajdera, 1969; Glant *et al.*, 1986).

Digestions of proteoglycan

Chondroitinase ABC was used at 0.1 unit/mg of proteoglycan in 0.1 M-sodium acetate/0.1 M-Tris/HCl buffer, pH 7.3 (Hascall & Heinegård, 1974), containing 1 mM-iodoacetamide, 1 mM-phenylmethanesulphonyl fluoride, 5 mM-EDTA and 5 μ g of pepstatin/ml to inhibit cysteine, serine, metallo and aspartate proteinases respectively. Keratanase was used at 0.1 unit/mg of proteoglycan in 50 mM-Tris/HCl buffer, pH 7.2, containing 80 mM-NaCl with the same proteinase inhibitors (Conrad *et al.*, 1982). Pronase (5 μ g/mg of proteoglycan) was used in 50 mM-Tris/HCl buffer, pH 7.5 (Glant *et al.*, 1986), and papain (10 μ g/mg of proteoglycan) in 0.2 M-sodium acetate buffer, pH 5.0, containing 5 mM-EDTA and 5 mM-cysteine (Roughley & White, 1980). After 4 h incubation, the enzymes were inactivated by 3 min boiling, except for papain, which was inhibited by the addition of iodoacetamide to a final concentration of 10 mM.

Analytical methods

Uronic acid was determined by the carbazole reaction (Bitter & Muir, 1962), and sialic acid by the periodate/resorcinol method (Jourdan *et al.*, 1971). Samples for hexosamine and amino acid analysis were hydrolysed in sealed tubes under N_2 , evaporated under vacuum, and analysed with a Dionix amino acid analyser (Roughley & White, 1980). Protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Monoclonal antibodies

Antibodies were obtained from fusions of Sp2/0-Ag14 mouse myeloma cells and spleen cells of Balb/c mice immunized with foetal-human or adult-human cartilage proteoglycans as described previously (Glant *et al.*, 1986). The various monoclonal antibodies used in this study recognize different epitopes on foetal-human and/or adult-human cartilage proteoglycan. Immunoglobulin class and subclass specificities of monoclonal antibodies, and the specificity of monoclonal antibodies to various epitopes, were determined by enzyme-linked immunosorbent assay (Glant *et al.*, 1986). Antibody EFG-4 (IgG1) recognizes a proteinase-sensitive peptide segment on the core protein of proteoglycans. The epitope consists of a short peptide on the core protein of both the keratan sulphate-attachment and chondroitin sulphate-attachment regions, and this epitope does not include keratan sulphate, chondroitin sulphate or oligosaccharides. It is specific for adult proteoglycan (Glant *et al.*, 1986). Antibody BCD-4 (IgG1) recognizes an epitope that

is destroyed by alkaline-borohydride cleavage (50 mM-NaOH/1 M-NaBH₄ at 45 °C for 48 h), keratanase, endo- β -galactosidase and proteinase treatments. It represents a peptide substituted with keratan sulphate or keratan sulphate-like structures present only in the chondroitin sulphate-rich fragment(s) of adult-human cartilage proteoglycan. These antibodies have already been described in detail (Glant *et al.*, 1986). Sulphatases, alkaline phosphatase and neuraminidase had no effect on antibody binding to these two (BCD-4 and EFG-4) epitopes. Antibody α HFGP-846 (IgM) was prepared as before (Glant *et al.*, 1986) by using mice immunized with foetal-human proteoglycan.

Radioimmunoassay

A competitive binding assay was used (Glant *et al.*, 1986). Briefly, optimal dilution of monoclonal antibody was incubated with a serial dilution of unlabelled proteoglycans overnight at 37 °C. ¹²⁵I-labelled foetal or adult-human proteoglycans were then added for 2 h at 37 °C. Antigen-antibody complexes were bound to 25 μ l of 10% (w/v) suspension of Protein A-bearing *Staphylococcus aureus* (Zysorbin; Dimension) as described previously (Glant *et al.*, 1986). In the case of IgM monoclonal antibodies, a tertiary immune complex was formed by rabbit immunoglobulin to mouse IgM (with the use of 100 μ l of 1:25 dilution of immune serum), and this complex was pelleted by the Protein A method. Percentage inhibition of binding was expressed by the following formula:

$$\text{Inhibition (\%)} = 100 - \left[\frac{\text{c.p.m. bound in the presence of inhibiting antigen}}{\text{c.p.m. bound in the absence of inhibiting antigen}} \times 100 \right]$$

Immuno-affinity chromatography

Monoclonal antibody α HFGP-846 was purified on a hydroxyapatite column (Stanker *et al.*, 1985) and coupled to CNBr-activated Sepharose CL-4B as described previously (Glant, 1982). The proteoglycan-binding capacity of the column was determined, and native proteoglycan representing 50% of the maximal antigen-binding capacity was recirculated overnight at room temperature. Unbound proteoglycan was removed by exhaustive washing with 0.1 M-Tris/acetate buffer, pH 7.6, containing 0.5 M-NaCl, 0.05% Tween-20 and 0.1% Triton X-100. Bound proteoglycan was eluted with 0.2 M-glycine/HCl buffer, pH 2.6, and immediately neutralized with 1.0 M-Tris (pH 8.0) and dialysed against deionized water. Samples were freeze-dried and stored at –20 °C.

RESULTS

The preliminary characterization of monoclonal antibody α HFGP-846 is shown in Table 1. Chondroitin sulphate, dermatan sulphate and keratan sulphate either in their native or digested states showed no reaction with this antibody. Native adult cartilage proteoglycan also did not react. In contrast, a strong reaction was seen with native foetal cartilage proteoglycan, though this was abolished when the molecule was treated with chondroitinase ABC, or proteinases, but was unaffected by keratanase. This indicates that the epitope is protein-core-associated, and the loss of reactivity on treatment with chondroitinase ABC suggests that this epitope is (i)

Table 1. Effects of glycosaminoglycans and native and enzymically treated proteoglycans on the binding of monoclonal antibody α HFGP-846 to 125 I-labelled foetal-human proteoglycan monomer

Abbreviations: C-4-S, chondroitin-4-sulphate; C-6-S, chondroitin-6-sulphate; DS, dermatan sulphate.

Proteoglycans or subcomponents	Dry wt. (μ g)	Inhibition (%)
C-4-S, C-6-S and DS	80.0	0
C-4-S, C-6-S and DS digested with chondroitinase ABC	80.0	0
Keratan sulphate	40.0	0
Keratan sulphate digested with keratanase	40.0	0
Adult-human cartilage proteoglycan (D1 fraction)	6.4	4
Foetal-human cartilage proteoglycans (D1 fraction, 24–27 weeks)		
Native (undigested)*	1.6	82
Papain-digested	6.4	1
Pronase-digested	6.4	1
Chondroitinase ABC-digested	6.4	10
Keratanase-digested	1.6	82

* 6.4 μ g of native foetal-human proteoglycan produced 100% inhibition. **Fig. 1. Immuno-inhibition of monoclonal antibodies EFG-4, BCD-4 and α HFGP-846**

Binding of monoclonal antibodies to 125 I-labelled proteoglycan was inhibited with 0.8 μ g of unlabelled human proteoglycan obtained from articular cartilages of different ages. Monoclonal antibodies were diluted to precipitate 50% of 125 I-labelled (8000 c.p.m./50 μ l) foetal (●, α HFGP-846) or adult (■, EFG-4; □, BCD-4) high-buoyant-density proteoglycans from human articular cartilage.

only expressed when chondroitin sulphate and protein are present together and (ii) localized in the chondroitin sulphate-attachment region of the foetal proteoglycan monomer. In the present case the protein component must be foetal-specific unless there are as yet unknown changes in the chondroitin sulphate component during aging.

Proteoglycans isolated from cartilages of different ages were analysed by radioimmunoassay. The relative percentage inhibitions produced by 0.8 μ g of proteoglycan are shown in Fig. 1. The EFG-4 epitope was not found in proteoglycans of foetal cartilages before the third trimester of gestation. Close to its birth its quantity significantly increased, and this continued during the first year after birth until about 30 years of age, when it plateaued. The BCD-4 epitope was present in small

amounts in foetal cartilage, but increased dramatically during development and reached a plateau at about 30 years of age. The latter change reflects the increase in keratan sulphate in human cartilage reported previously in biochemical studies (Bayliss & Ali, 1978; Elliott & Gardner, 1979; Roughley & White, 1980). Epitope α HFGP-846 exhibited a peak up until about 27 weeks of foetal life, after which it decreased gradually, showing a more accelerated decline after birth up to 30 years of age. Only a trace amount could be detected in older cartilage.

This high-buoyant-density proteoglycan isolated from a 16-year-old-human cartilage contains each of the epitopes defined by the three monoclonal antibodies. This proteoglycan fraction was separated into two populations: one that bound to antibody α HFGP-846 (28% of the total) and one that was unbound (72%). Only proteoglycan

that did not bind to antibody α HFPG-846 demonstrated clear binding to antibodies EFG-4 and BCD-4 (Table 2), demonstrating that the epitopes recognized by these latter antibodies were on a population of molecules separate

Table 2. Effects of proteoglycan subpopulations (bound and unbound) from a 16-year-old-human cartilage fractionated by immuno-affinity chromatography with antibody α HFPG-846 on the binding of monoclonal antibodies to 125 I-labelled human proteoglycan monomers

Binding of antibody α HFPG-846 to 125 I-labelled foetal-human proteoglycan and that of antibodies EFG-4 and BCD-4 to 125 I-labelled adult-human proteoglycan were inhibited by the bound and unbound proteoglycan subpopulations.

Proteoglycan (μ g)	Antibody ...	Inhibition (%)		
		α HFPG-846	EFG-4	BCD-4
Bound				
0.1		17.8	−7.1	−5.7
0.2		33.8	−4.0	0.0
0.4		56.6	0.0	1.5
0.8		76.1	0.1	9.4
Unbound				
0.1		0.0	56.1	11.2
0.2		0.7	71.5	16.6
0.4		0.8	81.2	29.8
0.8		1.4	88.6	54.3

from those bearing the epitope recognized by antibody α HFPG-846. The two populations were then subjected to amino acid and carbohydrate analyses. These were compared with those of proteoglycans isolated from foetal cartilage (24-27 weeks' gestation) and adult cartilage (52-56 years of age). The results are shown in Table 3. The proteoglycan that bound to the antibody was similar in its amino acid composition to that of the reference foetal proteoglycan. In contrast, the amino acid composition of the unbound proteoglycan was more similar to that of the adult cartilage proteoglycan, particularly in the content of its serine, glycine and isoleucine residues. In general, there was also a close correspondence between the sialic acid and uronic acid contents and galactosamine/glucosamine ratios for these matching pairs.

DISCUSSION

By using a monoclonal antibody that recognizes a specific epitope associated with the core protein of foetal proteoglycans we were able to separate two populations of proteoglycans, one of which is of a foetal type and the other of an adult type in their chemical characteristics. Analysis of this epitope in cartilages of different ages has revealed that this foetal epitope is present on a distinct population of proteoglycan molecules, which progressively disappear from cartilage with increasing foetal age after about 27 weeks until it is barely detectable in adult

Table 3. Amino acid analysis and chemical composition of high-buoyant-density proteoglycans from human articular cartilages

Cartilage (age) ...	Foetal (24-27 weeks)	Juvenile (16 years)			Adult (52-56 years)
		Bound	Unbound	Non-separated	
α HFPG-846* ...	Non-separated				Non-separated
Amino acid composition (residues/1000 residues)					
Aspartic acid	70	71	74	68	66
Threonine	74	75	72	74	77
Serine	112	110	95	97	97
Glutamic acid	135	132	129	138	123
Proline	93	91	96	97	102
Glycine	129	127	120	116	114
Alanine	72	75	79	80	80
Valine	70	74	73	77	74
Methionine	2	3	3	3	3
Isoleucine	36	36	49	49	56
Leucine	77	77	80	79	80
Tyrosine	22	24	25	25	28
Phenylalanine	45	42	41	40	43
Histidine	15	14	14	15	17
Lysine	17	17	18	18	19
Arginine	31	32	32	32	21
Chemical composition (μg/mg of proteoglycan)					
Protein	59.2	71.1	104.6	102.2	122.2
Glucosamine	14.4	17.5	49.5	51.1	63.6
Galactosamine	249.7	265.5	178.6	199.5	119.5
Uronic acid	299.6	250.2	182.6	202.8	158.8
Sialic acid	15.2	15.3	32.1	25.6	30.3
Galactosamine/ glucosamine molar ratio	17.3	15.2	3.6	3.9	1.9

* α HFPG-846 monoclonal antibody recognizes protein-related epitope(s) on foetal-human proteoglycans and was used coupled to Sepharose CL-4B.

cartilage by about 30 years of age. As this population declines, a second population of molecules appears, starting in the foetus, bearing both EFG-4- and BCD-4-reactive epitopes. This proteoglycan appears to be dominant in adult cartilage and is characterized by molecules with different amino acid and carbohydrate compositions.

There are other observations demonstrating distinct core protein populations of cartilage proteoglycans. Immunological studies, using polyclonal antibodies together with peptide patterns of trypsin-digested core protein, indicated that the core proteins of two bovine nasal-cartilage proteoglycan populations are similar but not identical (Heinegård *et al.*, 1985). Proteolytic modification of the proteoglycan within the extracellular matrix, or different post-translational glycosylation of the same core protein at different ages, may account for some of these and our observations. Nevertheless, the immunological differences between the two populations in maturing human cartilage seem more likely to represent different gene products. As the EFG-4 epitope is a repeating peptide associated with both the keratan sulphate- and chondroitin sulphate-linkage regions of the core protein, it is unlikely that proteolytic cleavages during aging can produce the differences detected by the monoclonal antibodies. Furthermore, the presence of a foetal-type epitope in the high-buoyant-density proteoglycans of foetal-human cartilage, which disappears completely as the adult-type epitopes accumulate, is difficult to explain by the variation in the activity of glycosyltransferases and sulphotransferases acting post-translationally upon a single core protein. Two other monoclonal antibodies, which are specific for other foetal-human proteoglycan epitopes, have been isolated and demonstrate extremely similar changes to those described here for antibody α HFPG-846 (T. T. Glant, K. Mikecz, P. J. Roughley, E. Buzás & A. R. Poole, unpublished work). Thus we have to suppose that the articular chondrocytes of maturing cartilage may switch to the production of a new core protein, initiating a concomitant change in glycosylation. Whether such a proteoglycan structure is more suitable for the function of the maturing articular cartilage is at present open to question.

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