Changes with age in the structure of fibromodulin in human articular cartilage

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Summary

An anti-peptide antibody was raised in a rabbit against the carboxy terminal region of the human fibromodulin core protein. The antibody was purified from other components of the resulting antiserum by affinity chromatography using the immobilized peptide, and was used to study the structural heterogeneity of fibromodulin extracted from human articular cartilage of different ages by the use of immunoblotting following sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the extracted macromolecules. In the fetus and neonate, fibromodulin was visualized as a diffuse component with a relative molecular weight of 70-110 kDa, whereas in the mature adult a more discrete component of smaller size was apparent with a relative molecular weight of 67 kDa. The size of the fibromodulin from mature adult cartilage could not be altered by pretreatment of the samples with keratanase II or endo-β-galactosidase before analysis. In contrast, the size of the fibromodulin from younger cartilage could be decreased with both glycosidases, with the endo-β-galactosidase yielding a smaller product than the keratanase. The size of the product resulting from endo-β-galactosidase treatment of the fibromodulin from young cartilage was the same as that of the intact fibromodulin from mature adult cartilage. Thus, fibromodulin is present in human articular cartilage at all ages, but the extracted molecules only appear to exist in a proteoglycan form possessing keratan sulfate chains in the juvenile and young adult, and the size of these chains decreases with age. In the mature adult the fibromodulin does not possess either keratan sulfate or non-sulfated polylactosamine chains, though it appears to possess the same number of N-linked oligosaccharides as its counterparts from the younger tissue, but they are not modified further. The majority of the fibromodulin extracted from arthritic cartilage is of the same size as that found in the normal mature adult, although there is evidence for proteolytic processing. The degree of such processing is greater for the fibromodulin obtained from the cartilage of rheumatoid arthritic joints than osteoarthritic joints.

Key words: Articular cartilage, Proteoglycan, Fibromodulin, Keratan sulfate, Human, Age.

Introduction

Fibromodulin belongs to the family of small leucine-rich repeat proteoglycans, that also includes decorin, biglycan and lumican [1]. The core proteins of these proteoglycans are characterized by a series of central leucine-rich domains flanked by terminal disulfide-bonded domains [2-4]. In the case of decorin and biglycan there are attachment sites for chondroitin sulfate or dermatan sulfate in the extreme amino-terminal region of the core proteins, but these sites are absent from fibromodulin and lumican. The core proteins of all four molecules possess attachment sites for N-linked oligosaccharides in their central regions, and in the case of fibromodulin and lumican these oligosaccharides may be modified to keratan sulfate.

Fibromodulin was first purified from mature bovine articular cartilage as a matrix protein of M, 59 kDa [5]. It was shown to be present in a variety of cartilage types and other connective tissues at a concentration of 1-3 mg/g wet tissue. The name fibromodulin is derived from the ability of the protein to interact with the fibrillar types I and II collagens and influence both the rate of fibrillogenesis and the structure of the resulting fibrils [6]. Interaction with the fibrillar collagens also appears to be a common feature of other family members [7]. Decorin and fibromodulin have been shown to interact at defined sites along the collagen molecules, but do not compete for the same sites [8]. It is likely that fibromodulin interacts with the collagen fibrils at the a or c bands, whereas decorin interacts at the d or e bands [9].
The bovine fibromodulin cDNA sequence indicates that the primary translation product contains 375 amino acids with the first 18 residues representing the signal peptide [2]. The central leucine-rich region possesses 10 repeats of about 23 amino acids, within which reside four consensus sequences for the attachment of N-linked oligosaccharides. A fifth such attachment site lies within the carboxy-terminal disulfide-bonded domain, but unlike the more central sites it does not appear to be substituted [10]. At least in calf cartilage, it appears that all four of the central sites may also serve as acceptors for the attachment of keratan sulfate chains with an average length of 22 disaccharide repeating units.

In the human the structure of the primary translation product for fibromodulin has also been described [11]. It contains 376 amino acids, also with a putative 18 residue signal peptide, and has consensus sequences for N-linked oligosaccharides at identical positions to its bovine counterpart, though at present it is not known whether these sites are also substituted with keratan sulfate. The purpose of the present work was to determine whether keratan sulfate is indeed present on the fibromodulin of human articular cartilage and whether its substitution varies with age.

**Materials and Methods**

**Preparation of Tissue Extracts**

Normal human articular cartilage was obtained from the femoral condyles at the time of autopsy. Specimens were collected within 20 h of death from two fetuses (26 and 32 weeks gestation), two neonates (1 and 6 weeks after birth), three juveniles (2, 3 and 12 years), and eight adults (23, 37, 39, 55, 60, 64, 68 and 74 years). In the case of the 60 and 74 year adults, tissue was also collected from patellar tendon, meniscus, intervertebral disc (L4-5) and skin. In all cases the joints from which the tissue was taken appeared macroscopically normal and there was no clinical history of a connective tissue abnormality. Cartilage was also collected from the femoral condyles of 12 patients ungoing joint replacement surgery. Nine of these individuals (aged 52, 61, 62, 68, 70, 71, 72, 74 and 78 years) had osteoarthritis and three (aged 64, 73 and 74 years) had rheumatoid arthritis. Tissue was stored at -20°C until used for extraction. For the preparation of extracts, cartilage was routinely finely diced then extracted with 10 volumes of 4 M guanidinium chloride, 100 mM sodium acetate, pH 6.0, containing proteinase inhibitors for 48 h at 4°C (12). In the cases where multiple tissues were collected, dicing was replaced by sectioning to 20 μm. The filtered extracts were dialyzed into the appropriate buffer for enzyme treatment, then used directly for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting analysis without further purification.

**Preparation of Anti-Peptide Antiserum**

A peptide (H-CGGLRLASLIEI-OH) corresponding to the carboxy terminal nine amino acids of human fibromodulin [11], plus a linker region consisting of two glycine residues and an amino terminal cysteine (Fig. 1), was synthesized by Fmoc chemistry (Fastmoc) using an Applied Biosystems model 431A solid phase peptide synthesizer. The cysteine residue was used for coupling the peptide to ovalbumin [13], using the bifunctional reagent N-hydroxysuccinimidyl bromoacetate [14]. For production of the anti-peptide antibody, New Zealand White rabbits (3 kg) were injected intramuscularly with 0.5 ml of a suspension comprising a 1:1 mixture of Freund's complete adjuvant and the conjugated peptide in phosphate-buffered saline (PBS, 2 mg/ml).

![Figure 1](image)

**Fig. 1.** Relationship of the peptide sequence used for antibody production to the intact fibromodulin molecule. (a) The core protein of fibromodulin is depicted as a series of central leucine-rich regions with flanking disulfide-bonded domains near the amino (N) and carboxy (C) termini. (b) The amino acid sequence of the carboxy terminal region commencing with the cysteine residue involved in disulfide-bond formation. (c) The sequence of the peptide synthesized to correspond to the carboxy terminal region of fibromodulin, which also possesses an amino terminal cysteine residue and adjacent spacer glycine residues (underlined). (d) The terminal cysteine residue is used for coupling the peptide to ovalbumin for immunization.
Subsequent booster injections were given after 2, 4, 6 and 8 weeks, but using incomplete adjuvant. The animals were killed and bled out 2 weeks after the final boost.

PURIFICATION OF ANTI-FIBROMODULIN IMMUNOGLOBULIN

The specific antibodies in the anti-peptide antiserum were purified by passage through a column of the immobilized immunizing peptide. The peptide was immobilized by coupling to Sulfolink gel (Pierce) via its amino terminal cysteine residue, as described by the manufacturer. Following coupling, unreacted sites on the gel were blocked using cysteine. For antibody purification, a column containing 5 ml peptide-resin was equilibrated with PBS. Twenty-five millilitres of antiserum was then passed through the column at a flow rate of 2 ml/h at room temperature. The column was washed, first with PBS and then with 1 M NaCl to eliminate nonspecific interactions. Finally, salt was removed by washing with PBS, and the antibodies eluted using 0.1 M glycine, pH 2.8. Four milliliter fractions were collected and immediately neutralized by the addition of 200 ~1.0 M Tris/HC1, pH 9.5. Fractions were monitored for protein content by absorbance ~t 280 nm, and the positive fractions were pooled and dialyzed against PBS. The final solution had a volume of 9 ml and an absorbance at 280 nm (A280) of 0.45, equivalent to 0.32 mg/ml of immunoglobulin.

SDS-PAGE AND IMMUNOBLOTTING

Cartilage extracts were dialyzed overnight against 0.1 M sodium acetate, 0.1 M Tris/HCl, pH 7.3, then treated with chondroitinase ABC (from Proteus vulgaris, supplied by ICN Biomedicals) at 0.1 U/ml extract for 4 h at 37°C. The treated extracts were mixed 1:1 with SDS-PAGE loading buffer, heated for 3 min at 100°C, and analyzed by SDS-PAGE and immunoblotting.

Results

The presence of fibromodulin was studied in extracts of human articular cartilage, using a combination of SDS-PAGE, electroblotting and immuno-detection with an anti-peptide antibody. The extracts were studied directly without any prior fractionation so that all possible forms of the molecule could be detected without any bias because of the form of glycosylation. The analysis of direct extracts did, however, necessitate the treatment of the samples with chondroitinase ABC before electrophoresis because optimal resolution was not obtained in the presence of large quantities of aggrecan. Using this procedure, the cartilage extracts from a 6-week-old neonate showed a broad band of immunoreactive material, with a size ranging between 70-110 kDa (Fig. 2). The specificity of the immune reaction was demonstrated by using an antibody preparation that had been pretreated with the immunizing peptide. This competition completely abolished the immune staining.

To determine whether the size of the fibromodulin changes with age, extracts from a variety of individuals were examined. A juvenile of 3 years showed a broad size distribution similar to the neonate (Fig. 3), although the average size was slightly smaller with a range of 65–100 kDa. In
In contrast, two adults of 37 and 64 years showed narrower size distribution ranges, with predominant components of about 67 kDa. This was by far the major component in the mature adult, but molecules of larger size were evident in the young adult. Thus, the size of the human cartilage fibromodulin decreases with age, to reach a minimum size in the mature adult. The size change takes place continuously from the juvenile to the young adult (data not shown).

To determine the contribution of keratan sulfate to the observed size heterogeneity, the samples were treated with either keratanase II or endo-β-galactosidase before electrophoretic analysis. The former enzyme will cleave all forms of keratan sulfate, including those that have sulfate groups on both the galactose and N-acetylglucosamine moieties. The latter enzyme will cleave at non-sulfated disaccharides within the keratan sulfate chain or within unmodified polylactosamine chains. Neither the keratanase II nor the endo-β-galactosidase caused any significant change in the size heterogeneity of the fibromodulin from the mature adult cartilage (Fig. 4). In contrast, both glycosidases caused a decrease in size of the juvenile and young adult samples (Figs 5 and 6), with the size diminution being greatest for the endo-β-galactosidase (Fig. 6). This enzyme decreased the average size of the fibromodulin from these tissues to about 67 kDa, to yield a size distribution equivalent to that observed for the intact molecules from the mature adult cartilage. The lack of action of either glycosidase on the fibromodulin from the mature adult cartilage would indicate that the predominant form was substituted with neither keratan sulfate nor polylactosamine chains, whereas the fibromodulin from the juvenile and young adult does appear to possess such glycosylation. Because the size of the fibromodulin core protein is the same for all ages after treatment with endo-β-galactosidase, it is likely that the same number of asparagine residues are involved in N-linked
glycosylation at all ages. The age-related difference in the size of the intact fibromodulin is then a result of the extent to which polylactosamine elongation and sulfation may be occurring.

The electrophoretic profile of fibromodulin in the normal mature adult was compared with that from individuals with arthritic joints (Fig. 7). In patients with osteoarthritis, the major form of fibromodulin was of identical size to that present in the normal cartilage, although in some individuals there was evidence for proteolysis occurring to a minor extent, as products of a smaller size were apparent. In patients with rheumatoid arthritis, the largest product was also the same size as that present in the normal adult, but proteolysis occurred to a much greater extent than in the patients with osteoarthritis. In neither form of arthritis did the fibromodulin show evidence of significant substitution by long polylactosamine chains or keratan sulfate.

The size heterogeneity of fibromodulin was also studied in a variety of different mature adult connective tissues (Fig. 8). The meniscus and the annulus fibrosus of the intervertebral disc showed the presence of fibromodulin having a similar size heterogeneity to that present in the articular cartilage. Fibromodulin was also detected in the patellar tendon, but its size was smaller than that in the other tissues. Two components of similar abundance were observed with molecular sizes of about 60 and 55 kDa. Little immunoreactive fibromodulin was detected in either skin or the nucleus pulposus of the intervertebral disc. Thus, it would appear that fibromodulin is not present at a high level in all adult connective tissues, and that in those tissues where it is present it does not exhibit the size heterogeneity expected for a keratan sulfate-substituted molecule. Further work is needed, however, to determine in which tissues fibromodulin may be present in younger individuals and what its molecular state may be.

Discussion

The data presented in this work suggest that in the juvenile, human articular cartilage possesses...
In the mature adult, fibromodulin from human articular cartilage does not appear to be substituted with keratan sulfate or polylactosamine chains to any great degree because glycosidases expected to cleave such carbohydrate modification had no effect. The size of the adult fibromodulin is, however, larger than one would expect for a non-glycosylated core protein. Based on the deduced amino acid sequence, the mature human fibromodulin core protein without post-translational modification should have a molecular size of about 42 kDa. Thus the size of 67 kDa observed for fibromodulin in the adult is likely due, at least in part, to glycosylation by N-linked oligosaccharides. Preliminary studies with N-glycanase F, which cleaves the asparagine $\rightarrow$ N-acetylglucosamine linkage that attaches all N-linked oligosaccharides to protein, would support the presence of such substitution. Furthermore, it appears likely that the degree to which such substitution is occurring is the same at all ages.

This scenario, of fibromodulin existing as a glycoprotein form in the adult and a proteoglycan fibromodulin that is substituted with polylactosamine chains that, at least in part, are sulfated to form keratan sulfate. The inability of keratanase II to remove these chains to the same degree as endo-$\beta$-galactosidase would indicate that many of the polylactosamine chains are non-sulfated, particularly in the region proximal to their attachment to the core protein. Such a distribution of sulfation has previously been described for corneal keratan sulfate [18]. Thus in the juvenile, fibromodulin exists as a typical keratan sulfate proteoglycan with the keratan sulfate accounting for about 50% of the mass of the molecule. If one assumes that the same four substitution sites are utilized as occurs in bovine fibromodulin [10], then the average size of the keratan sulfate chains would be about 10 kDa assuming a uniform substitution. The size of the chains could be considerably longer, however, if substitution occurred only at a limited number of sites. The size of these chains decreases with juvenile development and in the young adult.

![Figure 6](image1.jpg)

**FIG. 6.** Analysis of fibromodulin from cartilage of different ages after treatment with endo-$\beta$-galactosidase. Cartilage extracts from individuals aged 6 weeks (1), 3 years (2), 37 years (3) and 64 years (4) were analyzed by SDS-PAGE, following their treatment with endo-$\beta$-galactosidase, and subsequent immunoblotting using the anti-peptide antibody against fibromodulin. The migration positions of molecular weight standards are also indicated.

![Figure 7](image2.jpg)

**FIG. 7.** Analysis of fibromodulin from arthritic cartilage. Cartilage extracts from individuals with osteoarthritis (2 and 3) and rheumatoid arthritis (4 and 5) were analyzed by SDS-PAGE and subsequent immunoblotting using the anti-peptide antibody against fibromodulin. The appearance of extracts from normal mature adult (1) and fetal (6) cartilage are also shown for comparison.
form in the juvenile, is compatible with previous studies on bovine cartilage, where fibromodulin was isolated as a 59 kDa matrix protein from 2-year-old animals [5], but as a larger keratan sulfate proteoglycan from 3-week-old calves [10]. It is not, however, certain that this age-change occurs in cartilage from all species, as fibromodulin substituted with keratan sulfate is produced by chondrocytes from both young and adult rabbit cartilage [19].

The occurrence of a predominantly glycoprotein form for fibromodulin in mature normal cartilage also appears to be true for both osteoarthritic and rheumatoid articular cartilage. Although the expression of immature proteoglycan glycosylation patterns has been reported to occur for aggrecan in osteoarthritis [20], there is no evidence that any repair process taking place within the cartilage of the arthritic joints results in the accumulation of proteoglycan forms of the fibromodulin characteristic of the young. However, the fibromodulin that was present in the arthritic joints did show evidence for increased proteolytic processing, as has been reported previously for decorin and biglycan [20]. Thus the increased proteolytic action associated with the arthritic joint appears to affect the structure of all the proteoglycans present in the cartilage.

The presence of a glycoprotein form of fibromodulin in the mature adult does not appear to be unique to articular cartilage, and molecules of identical size are also present in the meniscus and annulus fibrosus of the intervertebral disc. In the patellar tendon, an even smaller sized fibromodulin is present, suggesting that in some tissues a low degree of glycosylation or proteolytic processing may be occurring. In contrast to the annulus fibrosus, the nucleus pulposus of the intervertebral disc showed no evidence for the presence of fibromodulin in either a proteoglycan or a glycoprotein form. A similar situation was previously reported in the bovine intervertebral disc [5], where immunoassay revealed a 20-fold difference in the fibromodulin content of the annulus fibrosus and nucleus pulposus from a 2-year-old animal. In the present work a sample of adult

Fig. 8. Analysis of fibromodulin from different adult connective tissues. Extracts from the meniscus (1), patellar tendon (2), annulus fibrosus (3), nucleus pulposus (4), articular cartilage (5) and skin (6) of an individual aged 60 years were analyzed by SDS-PAGE and subsequent immunoblotting using the anti-peptide antibody against fibromodulin. The migration positions of molecular weight standards are also indicated.
dermis failed also to show the presence of fibromodulin. At present it is not clear why different connective tissues would require different abundances of a collagen-binding proteoglycan, or whether other members of the leucine-rich repeat proteoglycan family may increase in a compensatory manner.

Finally, it is of interest to consider the possible mechanism by which cartilage fibromodulin may be converted from a proteoglycan to a glycoprotein form. In theory this could involve the degradative removal of keratan sulfate following its initial synthesis, or a synthetic failure to extend the N-linked oligosaccharides to sulfated polyactosamine chains. The degradative pathway is unlikely by either proteinase or glycosidase action. Proteolytic conversion of biglycan from a proteoglycan to a glycoprotein form likely occurs in adult human cartilage and intervertebral disc [17]. However, for a similar mechanism to occur in fibromodulin, proteolysis would be required within the central region of the core protein where glycosylation occurs. This would result in a considerable decrease in the core protein size, yet no such change was observed in the normal adult. The action of a keratan sulfate-degrading glycosidase would be expected to affect the keratan sulfate of both fibromodulin and aggrecan, yet it has been reported that the keratan sulfate chains of aggrecan increase in size with age [21]. One would, therefore, seem justified in speculating that the absence of keratan sulfate on fibromodulin is a result of a lack of its synthesis by the mature adult chondrocytes. Because the adult chondrocytes are known to possess the transferases necessary for synthesizing O-linked keratan sulfate on aggrecan, one presumes that the fibromodulin does not proceed through the same intracellular compartment, or that the sites for polylactosamine chain extension on the initial N-linked oligosaccharides are selectively blocked. In this respect, it is interesting to note that lumican, the other keratan sulfate proteoglycan in the leucine-rich proteoglycan family, shows a similar age-related change in keratan sulfate substitution to fibromodulin [22].

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