The biochemical characterization of aggrecan from normal and tibial-dyschondroplastic chicken growth-plate cartilage

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Tibial dyschondroplasia (TD) is a disorder of endochondral ossification characterized by the presence of an avascular, non-mineralized cartilage lesion extending from the growth plate into the metaphysis. Cells within the TD growth plate fail to differentiate to full hypertrophy, and instead appear to maintain a 'pre-hypertrophic' or 'transitional' status. Studies of the expression and distribution of cartilage matrix macromolecules in the TD growth plate have shown a marked decrease in the levels of aggrecan in the TD matrix. In the present study we compared the biochemical characteristics of the aggrecan molecules extracted from normal epiphyseal and TD cartilage. We have shown three

major differences between normal and TD cartilage aggrecan. These are: (1) increase in molecular mass; (2) increase in the number of keratan sulphate chains; and (3) difference in the pattern of sulphation in TD aggrecan. Such changes in biochemical characteristics of the aggrecan monomers in TD cartilage may be associated with the lack of mineralization of the diseased cartilage. The present study provides a basis for further investigations into the importance of proteoglycans in normal and pathological bone development.

Key words: endochondral, ossification, proteoglycans.

INTRODUCTION

Cartilage plays a central role in the formation and growth of the vertebrate skeleton. In long-bone development, bone replaces cartilage by endochondral ossification, a process which occurs via a specialized segment of hyaline cartilage known as the epiphyseal growth plate [1,2]. Within the growth plate, chondrocytes undergo a sequence of events, including cell proliferation, maturation and hypertrophy. Cell hypertrophy is followed by the mineralization of the hypertrophic cartilage matrix prior to the formation of osteoid by invading osteogenic cells. The extracellular matrix (ECM) constitutes a large, diverse set of molecules, primarily proteoglycans, collagens and glycoproteins, and deviation in composition and organization of the ECM may have a profound effect on tissue integrity and functionality of the epiphyseal plate. As part of the study to investigate the involvement of the ECM in endochondral ossification, we utilized a model of aberrant bone formation, namely avian tibial dyschondroplasia (TD).

In avian TD, the developmental processes of endochondral ossification are disrupted as a result of an apparent arrest of cell hypertrophy. This developmental arrest leads to the accumulation of non-calcified avascular cartilage within the epiphyseal growth plate, which extends from the epiphyseal plate and into the metaphysis [3,4]. Histological studies on TD have shown that the lesion contains a mass of transitional chondrocytes which have failed to differentiate fully [5–7]. TD affects the long bones, particularly the proximal tibiotarsus, causing lameness in affected birds as a consequence of biomechanical forces on a malleable cartilage mass [8] and a focal decrease in growth at the site of the dyschondroplastic lesion [9].

The most abundant cartilage proteoglycan, aggrecan, when extracted from epiphyseal cartilage, has an approximate molecular mass of 2500 kDa, of which the protein core is 220 kDa

[10,11]. Almost 90% of the weight of aggrecan is contributed to by the attached chondroitin sulphate (CS) and keratan sulphate (KS) chains, and N- and O-linked oligosaccharides. In the cartilage ECM, aggrecan is normally bound to hyaluronan molecules, via its N-terminal globular G1 domain, to form huge multi-macromolecular aggregates within the tissue, which endows the tissue with the ability to sustain the large compressive forces exerted upon it with minimal deformation [12]. In addition to providing structural support and the physical properties of cartilage matrix, a number of studies have indicated that proteoglycans in the cartilage matrix act as promoters of mineralization [13,14]. Clear evidence of calcium binding by CS chains has also been reported [15,16]. However, contrasting evidence has also indicated that proteoglycans can be inhibitors of mineralization [17].

We have previously shown, by *in situ* hybridization and immunohistochemistry, that there is atypical expression and distribution of ECM components in the TD lesion, in particular aggrecan [18], implying that alteration of aggrecan expression and biochemical characteristics may be involved in the pathogenesis of TD. We present here findings of the detailed biochemical characterization of aggrecan extracted from normal and dyschondroplastic cartilage by a number of classical methods, including light-scattering, ultracentrifugation and gel-filtration chromatography [19–21] with the aim of gaining insight into the role of aggrecan in bone formation.

EXPERIMENTAL

Extraction of proteoglycans from normal and TD chicken epiphyseal cartilage

Groups of broiler chicks (Cobb strain) were reared under two different dietary regimes in compartments of electrically heated

Abbreviations used: TD, tibial dyschondroplasia; ECM, extracellular matrix; HA, hyaluronic acid; KS, keratan sulphate; CS (C-4-S, C-6-S), chondroitin sulphate (4-sulphate, 6-sulphate); NEM, *N*-ethylmaleimide; GAG, glycosaminoglycan.

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tier brooders. Food and water were available *ad libitum* and diets, based on wheat and soya meal, were either a control diet, containing 12 g of calcium and 7.6 g of phosphorus/kg, or a TD-inducing diet, containing 7.5 g of calcium and 7.6 g of phosphorus/kg [22]. At 3 weeks of age all the birds in each group were killed by cervical dislocation. The right and left proximal tibiotarsi were dissected and scored for the level of severity of TD [9]. Ten normal epiphyseal growth plates and ten grade 5 TD lesions were dissected and washed in PBS/0.02% NaN_3 for 2 h. The tissue was then blot-dried, weighed, frozen in liquid nitrogen and pulverized in a stainless-steel bomb. Individual samples were then freeze-dried, then homogenized in the presence of 50 mM Tris, pH 7.4, containing 4 M guanidinium chloride (GdmCl), 25 mM EDTA, 25 mM ϵ -amino-n-hexanoic acid, 2 mM PMSF and 10 mM *N*-ethylmaleimide (NEM; Sigma), with an IKA-Ultra Turrax (T25) homogenizer. The homogenate was then stirred for 48 h at 4 °C and centrifuged at 2800 *g* for 1 min. The supernatant was retained and the residue was re-extracted with the 4 M GdmCl mixture for a further 24 h. This was then spun at 4000 rev./min for 1 min and the supernatants were pooled.

A1D1 preparation from chick epiphyseal cartilage

The A1D1 fractions were prepared from GdmCl cartilage extracts by the method of Hascall and Sajdera [10]. Briefly, the GdmCl concentration of the extract was adjusted to 0.5 M by dialysis, and solid CsCl added to a density of 1.5 g/ml. The samples were centrifuged at 45000 rev./min for 48 h in a Ti70 rotor. The resultant A1 fraction was then dialysed into 4 M GdmCl and subjected to another CsCl-density-gradient centrifugation as described previously. After centrifugation the A1D1 fraction was dialysed into 50 mM Tris/HCl, pH 7.4, and stored at 4 °C, and used for all subsequent analyses.

Agarose-gel electrophoresis of normal and dyschondroplastic A1D1 fractions

Agarose-gel electrophoresis was performed on the basis of methods described by Thornton et al. [21]. Gels were composed of 1% (w/v) agarose in 40 mM Tris/acetate/1 mM Na_2SO_4 /0.1% (w/v) SDS, pH 6.8. To 20 μl of A1D1, an equal volume of 2 $\times$ loading buffer [1 $\times$ loading buffer is 10 mM Tris/acetate (pH 6.8)/0.25 mM Na_2SO_4 /0.01% Bromophenol Blue/0.025% SDS/20% (v/v) glycerol] was added. All samples were denatured for 2 min by boiling prior to electrophoresis at 30 V for 18 h in a Bio-Rad-RNA Subcell horizontal-gel apparatus with the gel submerged in 10 mM Tris/acetate/0.25 mM Na_2SO_4 /0.025% SDS, pH 6.8.

Slot blotting

Samples were transferred on to nitrocellulose membranes (0.2 μm pore size) using a Minifold II 72-well slot-blot apparatus as previously described [23], and the membranes were either processed for Western blotting or stained with Alcian Blue.

Western blotting

Agarose gels after electrophoresis were transferred on to nitrocellulose (0.2 μm pore size) using a Pharmacia Vacugene XL apparatus at 4 MPa (40 mbar) for 1 h with the nitrocellulose and agarose both submerged in 0.6 M NaCl/0.165 M trisodium citrate, pH 7. Following several washes in distilled water the membrane was then treated in an identical fashion as slot-blotted membranes. In the case of Western blotting with antibody

1-C-6 [24], the membrane was initially reduced by immersion in 10 mM dithiothreitol in 6 M GdmCl/0.1 M Tris/HCl/5 mM EDTA, pH 8, for 20 min, and then alkylated by immersion in 25 mM iodoacetamide for 10 min. Membranes were incubated with 5% (w/v) dried skimmed milk, and probed with the following antibodies: (i) 2-B-6 (diluted 1:2000), a mouse IgG antibody reacting with the C-4-S stub generated by digesting aggrecan with chondroitin ABC lyase (this 'stub' contains an epitope consisting of a non-reducing terminal $\Delta^{4,5}$ -unsaturated hexaauronate residue adjacent to *N*-acetylgalactosamine 4-sulphate; (ii) 3-B-3 (diluted 1:2000), a mouse IgM antibody reacting with a C-6-S stub generated after chondroitin ABC lyase digestion of aggrecan (this epitope consists of a non-reducing terminal $\Delta^{4,5}$ -unsaturated hexaauronate residue adjacent to *N*-acetylgalactosamine 6-sulphate) [25]; (iii) 1-B-5 (diluted 1:2000), a mouse IgG reacting with non-sulphated stub [26]; (iv) 5-D-4 (1:4000), a mouse IgG recognizing an oversulphated heptasaccharide domain of KS [27]; (v) 1-C-6 (diluted 1:2000), a mouse IgG recognizing the G1-G2 domain of aggrecan [24]; (vi) CS-56 (diluted 1:2000), a mouse IgM recognizing a native epitope of CS (Sigma) [28]. Primary antibodies were then detected by using the appropriate secondary antibody and an ECL* (enhanced chemiluminescence) detection system (Amersham).

Alcian Blue staining

Membranes were immersed in either 1% (w/v) Alcian Blue in 0.1 M HCl, pH 1, or 1% Alcian Blue in 3% (v/v) acetic acid, pH 2.5, for 10 min, extensively washed with water, then air-dried.

Multi-angle total-intensity light-scattering

Preparations of normal and dyschondroplastic aggrecan in GdmCl were chromatographed on a Superose 6 HR 10/30 column. Samples were eluted with PBS containing 1 mM EDTA and 0.05% NaN_3 , which had been filtered through a 0.2 μm -pore-size filter and degassed. The column effluent was passed through a Dawn DSP laser photometer at a flow rate of 0.5 ml/min. This consists of a flow-cell of pathlength 1 mm, a 15 mW Argon ion laser with a mono modal Gaussian beam profile, 18 detectors which are positioned at angles from 22° to 147° and uses the angular dependence of the total scattered light intensity, combined with theoretical models. The fully automated computer-controlled analysis package (Astra 4) combines the total scattered-light-intensity data with the corresponding concentration values to produce a graph of molecular mass against volume. Providing that there is satisfactory separation, it is possible to estimate a weight-averaged, a number-averaged and a *Z*-averaged molecular mass for the sample.

Dissociative rate-zonal ultracentrifugation

This technique was employed to examine the mass of normal and dyschondroplastic aggrecan monomers. Beckman 13 ml polyallomer tubes (14 mm $\times$ 95 mm) were loaded with a gradient of 6–8 M ultrapure GdmCl using a gradient maker and a Miniplus 2 pump (Gilson). Approx. 4 mg of the sample in 4 M GdmCl (volume less than 500 μl) was then layered gently on to the top of the gradients. The tubes were loaded into a swing-out SW40 rotor and centrifuged at 40 000 rev./min for 4 h and, with the use of a pipette, gently fractionated from the top of the gradient into 20 fractions (600 μl /fraction). The density gradient was checked by using a refractometer, and fractions were analysed by Alcian Blue staining and light-scattering.

Chondroitinase and keratanase digestions

For chondroitin ABC lyase digestion, freeze-dried samples were incubated with 0.1 unit of chondroitin ABC lyase/300 μ g sample in 0.06 M sodium acetate/0.05 M Tris/HCl, pH 8, containing 2 mM EDTA, 2 mM PMSF and 10 mM NEM at 37 °C for 24 h. For keratanase digestion, freeze-dried samples were incubated with 0.05 unit of keratanase/300 μ g sample in 0.15 M Tris/HCl, pH 7.4, containing 2 mM PMSF, 5 mM benzamidine and 10 mM NEM at 37 °C for 10 h. The digestion was stopped by boiling the sample for 2 min.

Aggregation of aggrecan monomer with hyaluronic acid (HA)

Samples of normal A1D1, dyschondroplastic chick A1D1 and bovine femoral-head A1D1 [21] were dialysed into distilled water, freeze-dried and redissolved in PBS at a concentration of 4 mg/ml. Three 250 μ l aliquots (1 mg) of bovine A1D1 were mixed separately with 0.25, 2.5 and 25 μ g of high-molecular-mass HA; (5 mg/ml). Aliquots (250 μ l) of NA1D1 and TDA1D1 were added to 2.5 μ g of HA. These mixtures were incubated for 16 h at 20 °C, after which the samples were directly layered on to the sucrose gradients and analysed by ultracentrifugation under associative conditions.

Associative rate-zonal ultracentrifugation

Gradients were set up as above, but with 10% (w/v) sucrose in PBS and 30% (w/v) sucrose in PBS. Samples (< 500 μ l in PBS) were layered on the top of the gradient and centrifuged in the SW40 rotor at 40 000 rev./min for 90 min. The fractions were analysed using Alcian Blue staining, as described above.

RESULTS

Isolation of aggrecan monomers from normal and TD growth plates

To ascertain that the A1D1 fractions from the normal (NA1D1) and TD (TDA1D1) chick epiphyseal cartilages contain aggrecan monomers, the extracts were analysed by agarose-gel electrophoresis and Western blotting. Figure 1 shows that NA1D1 and

Figure 1 Western blotting of normal (NA1D1) and dyschondroplastic (TDA1D1) A1D1 fractions with antibody 1-C-6

Normal and dyschondroplastic A1D1 samples were electrophoresed on a 1%-agarose gel and blotted on to nitrocellulose membrane. The membrane was then reduced and alkylated and probed with diluted antibody 1-C-6 (1:2000). The bound primary antibodies were localized with alkaline phosphatase-labelled anti-mouse IgG and detected by ECL* (Amersham). Lane 1, NA1D1; lane 2, TDA1D1.

TDA1D1, when subject to agarose-gel electrophoresis and probed with antibody 1-C-6 (which recognizes the G1 and G2 domains of aggrecan), were both positive and the single bands identified in both samples were of similar mobility. Western blottings were performed with a panel of specific antibodies recognizing the glycosaminoglycan (GAG) epitopes of aggrecan and both NA1D1 and TDA1D1 preparations were shown to possess the same epitopes (results not shown).

Molecular-mass and radius of gyration determinations for NA1D1 and TDA1D1

An aliquot of a 0.2 mg/ml solution of NA1D1 containing 0.04 mg of NA1D1 was loaded on to the Superose 6 column and the effluent passed through the Dawn DSP laser photometer. The data were processed using a Zimm plot with a polynomial fit degree of 2 using Astra 4 for Windows. The radius of gyration and the molecular mass for the NA1D1 are shown in Figures 2(a) and 2(c) respectively. Figure 2(a) suggests that there are two different populations of aggrecan molecules. The early eluted (7–7.5 ml) molecules have a much larger radius of gyration and may indicate the presence of multimers, probably due to the absence of dissociating buffer. The majority of the peak (7.5–8.5 ml) appears to be a homogeneous population of molecules, and analysis of this latter portion indicates an average radius of gyration (R_w) of 49.2 ± 2.0 nm. Similar calculations were made for the examination of the molecular weight of the molecules present in NA1D1 (Figure 2c). The front of the peak (7–7.4 ml) appears to represent material of high molecular mass, which may indicate multimers, whereas the remaining molecules appear to have a fairly constant molecular mass. On analysing the data across this peak (7.4–8.4 ml) the average molecular mass was estimated to be 1.99 ± 0.04 MDa.

Calculation of the radius of gyration and the molecular mass of TDA1D1 was determined in the same manner, and profiles are shown in Figures 2(b) and 2(d) respectively. Profiles similar to those of NA1D1 were obtained, and a radius of gyration of 49.4 ± 1.9 nm and molecular mass of 2.81 ± 0.04 MDa were estimated for the portion of molecules representing aggrecan monomers (7.3–8.0 ml). The monomers from both NA1D1 and the TDA1D1 appear to have the same radius of gyration, approx. 49 nm, though the molecular mass of the dyschondroplastic monomer was approx. 0.8 MDa higher.

Fractionation of NA1D1 and TDA1D1 by rate-zonal ultracentrifugation

In order to determine the molecular basis of the more massive aggrecan monomers in TD, rate-zonal ultracentrifugation of NA1D1 and TDA1D1 under dissociative conditions was carried out. Figure 3 shows Alcian Blue staining of an equal portion of each fraction slot-blotted. The most distinct observation from Figure 3 is the presence of a more massive subset of molecules in TD (fractions 16–20). In order to examine the molecular-mass distribution in these gradients, fractions 1–4, 5–8, 9–12, 13–16 and 17–20 from the dissociative-rate-zonal-ultracentrifugation procedures were pooled and each individually analysed by light-scattering. The molecular masses determined for the different pools from normal and dyschondroplastic A1D1 are shown graphically in Figures 4(a) and 4(b) respectively. As expected, the higher the pooled fraction number the more massive the molecules. The average molecular masses for the individual pools were calculated as well as the radii of gyration and are presented in Table 1. Table 1 shows that aggrecan molecules in the NA1D1 range in mass from 1.2 to 3.05 MDa, whereas in the dyschondro-

Figure 2 Determination of the radius of gyration and molecular mass of the NA1D1 and TDA1D1 by multi-angle total-intensity light-scattering using a Dawn DSP laser photometer

(a) Graph showing the radius of gyration of the NA1D1 versus volume eluted from the Superose column. (b) Graph showing the radius of gyration of the TDA1D1 versus volume eluted from the Superose column. The thick broken line indicates change in the angle of gyration, and the continuous line shows the elution profile of the NA1D1 samples. (c) Graph showing the molecular mass of the NA1D1 versus volume eluted from the Superose column. (d) Graph showing the molecular mass of the TDA1D1 versus volume eluted from the Superose column. The broken line indicates the change in molecular mass, and the continuous line shows the elution profile of the TDA1D1 samples. Abbreviation: R.M.S., root mean square.

Figure 3 Fractionation of NA1D1 and TDA1D1 by rate-zonal ultracentrifugation

Rate-zonal ultracentrifugation under dissociative conditions was carried out as described in the Experimental section. Aliquots from each of the 20 collected fractions were slot-blotted on to nitrocellulose filters and then stained with Alcian Blue. The Alcian Blue intensity was measured by densitometric scanning. —●—, Alcian Blue reactivity of the NA1D1 fractions. - - -●- - -, Alcian Blue reactivity of TDA1D1. - - - - - indicates the presence of a linear dissociative gradient as determined by refractive-index measurement of the fractions. GuHCl, GdmCl.

plastic aggregan the molecules range in molecular mass from 1.6 to 3.35 MDa, and the radii of gyration remain constant.

Aggrecan epitopes in NA1D1 and TDA1D1

To determine the reason for the difference in molecular mass between NA1D1 and TDA1D1, initially simple quantification of known epitopes using characterized antibodies was performed. To begin with, differing concentrations of NA1D1 and TDA1D1 (10 ng–10 µg) were slot-blotted and probed with the various aggrecan antibodies and bands quantified by densitometric scanning to ensure dose response. To be able to quantify any difference in epitope reactivity, equal amounts of aggrecan molecules were then subsequently loaded. The criteria for equal loading was to normalize by reactivity with antibody 1-C-6. The presence of G1 would indicate that the molecule was still intact, since any proteolytic degradation would occur between the G1 and G2 domains, and the free G1 domain would then not sediment in the A1D1 preparation.

Slot-blotting was then performed with the following antibodies: 1-B-5, 2-B-6, 3-B-3, 5-D-4, CS-56 and anti-G3. The first three antibodies required chondroitinase digestion of the membrane before immunoblotting. All bands were quantified by densitometric scanning. All blots were repeated in triplicate and an average reactivity for each antibody on TDA1D1 was compared with that obtained for NA1D1, which has been

Figure 4 Molecular-mass determination on pooled fractions from rate-zonal-ultracentrifugation experiments

(a) Fractions of NA1D1 obtained from the rate-zonal-ultracentrifugation experiment were pooled and the molecular masses across the different pooled fractions were determined by light-scattering against volume eluted. ●, Fractions 1–4; 'grey star', fractions 5–8; +, fractions 9–12; ■, fractions 13–16. (b) Fractions of TDA1D1 obtained from the rate-zonal-ultracentrifugation experiment were pooled and the molecular masses across the different pooled fractions were determined by light-scattering against volume eluted. ●, Fractions 1–4; *, fractions 5–8; grey +, fractions 9–12; ■, fractions 13–16; grey ×, fractions 17–20.

assigned an arbitrary unit of 1. Table 2 shows that the reactivity for the G3 domain in TD aggrecan is decreased by 24%. The reactivity of KS in TD was observed to be 22% higher than that of normal, whereas the amount of CS remained unchanged. However, this experiment also showed that the levels of 4-sulphate in the CS chains may be increased in TD aggrecan with a corresponding decrease in the level of unsulphated CS chains.

The level of 6-sulphate remains unchanged. It is therefore apparent that there is an alteration in the CS/KS ratios between normal and dyschondroplastic aggrecan. However, a change in the reactivity of the antibody may also reflect a structural change in the polysaccharide chain. The following experiments were therefore performed to determine whether TD aggrecan contains a higher concentration of KS.

Table 1 Summary of the calculated values of the radii of gyration and molecular masses corresponding to the various fractions isolated from the NA1D1 and TDA1D1 samples

Normal			Dyschondroplastic		
Sample	Molecular mass (MDa)	Radius of gyration (nm)	Sample	Molecular mass (MDa)	Radius of gyration (nm)
NRZ1-4	1.2 ± 0.01	39 ± 2.8	TRZ1-4	1.6 ± 0.02	41 ± 2.9
NRZ5-8	2.10 ± 0.03	45 ± 2.9	TRZ5-8	2.11 ± 0.04	39 ± 3.1
NRZ9-12	2.28 ± 0.04	40 ± 3.0	TRZ9-12	2.29 ± 0.04	40 ± 3.2
NRZ13-16	3.05 ± 0.03	45 ± 2.7	TRZ13-16	2.58 ± 0.04	42 ± 3.0
NRZ17-20	Negligible		TRZ17-20	3.35 ± 0.03	44 ± 2.9

Table 2 Summary of the ratios of different aggrecan epitopes in NA1D1 and TDA1D1

Equal amounts of normal and dyschondroplastic aggrecan were slot-blotted on to nitrocellulose and probed with the different aggrecan epitopes. Bands were then quantified using a densitometric scanner and the ratio of normal to dyschondroplastic calculated. For 2-B-6, 3-B-3 and 1-B-5 the membrane was chondroitin ABC lyase-treated before being probed with the antibody.

Antibody	Epitope	Ratio	
		NA1D1	TDA1D1
Anti-G3	G3 domain	1.00	0.76
5-D-4	KS	1.00	1.22
Anti-CS	CS	1.00	1.00
2-B-6	4-Sulphate	1.00	1.22
3-B-3	6-Sulphate	1.00	1.00
1-B-5	No sulphate	1.00	0.80

Determination of CS and KS content in NA1D1 and TDA1D1 by enzymic digestions followed by gel chromatography

To ascertain the possibility that the increased KS content contributes to the increase in the average molecular mass of TD aggrecan, we performed gel chromatography on NA1D1 and TDA1D1 which had been sequentially digested with chondroitin ABC lyase and keratanase. Intact NA1D1 and TDA1D1 (2 mg) in 4 M GdmCl were initially chromatographed on a Sepharose CL-2B column and fractions were slot-blotted on to nitrocellulose and probed for Alcian Blue reactivity. Figure 5 shows that both the intact NA1D1 and TDA1D1 (peaks A) were eluted in the void volume of the Sepharose CL-2B column. Fractions corresponding to the NA1D1 and TDA1D1 peaks were pooled, dialysed extensively into water, freeze-dried and then digested with chondroitinase ABC lyase as described in the Experimental section. The digested sample was rechromatographed in 4 M GdmCl on Sepharose CL-2B under the same conditions.

The results of chondroitin ABC lyase-treated NA1D1 and TDA1D1 are illustrated as peak B in Figure 5, which shows that the chondroitin ABC lyase-treated dyschondroplastic aggrecan has a much broader peak (peak B) than normal. Also, the chondroitin ABC lyase-treated dyschondroplastic aggrecan peaks at fraction 33, whereas the normal aggrecan peaks at fraction 38, suggesting the normal chondroitin ABC lyase-treated aggrecan is smaller in size.

The chondroitin ABC lyase-treated aggrecan was then pooled, digested with keratanase as described in the Experimental section and chromatographed using the same conditions as described above. The elution profiles of the chondroitinase- and keratanase-

digested products were shown as peaks C (Figure 5). Only a small shift on treating the already chondroitin ABC lyase-treated NA1D1 with keratanase was seen, whereas in the TDA1D1 the shift was much greater. This suggests that the dyschondroplastic aggrecan has a greater proportion of KS than the normal A1D1. The chondroitin ABC lyase- and keratanase-treated NA1D1 and TDA1D1 both migrate to the same position, suggesting that these partially deglycosylated aggrecan molecules are similar in size and further indicates that the core protein is not altered in TDA1D1.

Aggregating potential of NA1D1 and TDA1D1 to HA

Previous results indicated that dyschondroplastic aggrecan has intact G1 domain and hence should retain the ability to form macromolecular aggregates with HA. To examine the aggregation of normal and dyschondroplastic aggrecan to HA, associative-rate-zonal-ultracentrifugation experiments were performed initially with bovine femoral-head A1D1 [21] and high-molecular-mass HA under associative conditions. Results of this experiment showed that the most massive multimers formed with 2.5 µg of HA and 1 mg of proteoglycan (Figure 6). This amount of HA was then chosen for subsequent experiments. The experiment was then repeated with NA1D1 and TDA1D1 and with 2.5 µg of HA. Figure 7 indicates that both NA1D1 and TDA1D1 are both able to form aggregates with HA, and the dyschondroplastic aggregates appeared to be slightly more massive than the normal aggregates.

DISCUSSION

Avian TD develops as a consequence of aberrant chondrocyte differentiation, leading to impaired ossification and bone morphogenesis [6–7,29] and is hence often used as a model system for the study of endochondral ossification. Precisely why endochondral ossification fails to proceed normally is to date not fully understood, but a number of studies have indicated that an altered proteoglycan and GAG content in the TD lesion may have a role in the initiation and progression of the disease [18,30]. In particular, we have shown a decrease in aggrecan abundance of approx. 30% in the dyschondroplastic cartilage ([18]; C. Tselepis, A. Kwan, D. Thornton and J. Sheehan, unpublished work) compared with normal. In order to assess the relative importance of this proteoglycan in endochondral ossification, the aggrecan from normal and diseased cartilage was extracted and characterized.

Light-scattering analyses on normal and TDA1D1 identified that the average molecular mass of dyschondroplastic aggrecan monomer was larger than the normal molecule by approx. 0.8 MDa due to the presence of a more massive subpopulation of

Figure 5 Gel chromatography of intact chondroitin ABC lyase- and keratanase-treated NA1D1 and TDA1D1

Gel filtration was performed on Sepharose CL-2B in 4 M GdmCl at a flow rate of 7.2 ml/min, and fractions (1.2 ml each) were collected. Fractions were slot-blotted on to nitrocellulose and probed for Alcian Blue reactivity at pH 2.5. (i) Shows the intact NA1D1 (peak A), chondroitin ABC lyase-treated NA1D1 (peak B) and the chondroitin ABC lyase- and keratanase-treated NA1D1 (peak C). (ii) Shows the intact TDA1D1 (peak A), chondroitin ABC lyase-digested TDA1D1 (peak B) and chondroitin ABC lyase- and keratanase-digested TDA1D1 (peak C). v_0 (' V_0 ') and v_t (' V_t ') were determined by chromatographing Blue Dextran and sodium dichromate.

aggrecan monomers in the TDA1D1 (3.05–3.35 MDa). Also, in normal cartilage, there are monomers as small as 1.2–1.6 MDa that are not present in the dyschondroplastic lesion. The population of higher-molecular-mass monomers may arise from either an alteration in glycosylation/glycanation or that the normal aggrecan is more susceptible to catabolism. Analyses of molecu-

lar-mass distributions after enzymic digestion of the KS and CS chains indicated that an increased KS content may account for the observed molecular-mass differences. Slot-blotting of the A1D1 fractions with the antibody 5-D-4 also consistently showed that, in dyschondroplastic aggrecan, there was an increase in KS. Since these analyses were performed with the entire A1D1

Figure 6 Aggregation of bovine femoral-head A1D1 with HA under associative conditions

Bovine femoral-head A1D1 (1 mg) was incubated with 0.25 µg (—), 2.5 µg (·····) or 25 µg (---) of HA overnight at 37 °C, then centrifuged in associative gradients. Gradients were then divided into 20 fractions and 10 µl of each was slot-blotted on to nitrocellulose and probed for reactivity with Alcian Blue at pH 2.5.

Figure 7 Aggregation of NA1D1 and TDA1D1 with HA under associative conditions

Normal and dyschondroplastic chick epiphyseal cartilage A1D1 (1 mg) was incubated with either no HA or 2.5 µg of HA. Gradients were then divided into 20 fractions and 10 µl of each was slot-blotted on to nitrocellulose and probed for reactivity with Alcian Blue at pH 2.5. Continuous line, 1 mg of NA1D1; dot-dashed line, 1 mg of TDA1D1; dotted line, 1 mg of NA1D1 + 2.5 µg of HA; dashed line, 1 mg of TDA1D1 + 2.5 µg of HA.

fractions, and because of the small amount of samples available for the investigation, we have not been able to ascertain whether the increase in KS is restricted to the subpopulation of more massive aggrecan in TD, though this is likely, since this subpopulation appears to contain novel GAG epitopes recognized by antibodies 7-D-4 and 3-B-3 (—) [‘(—)’ means ‘not treated with chondotinas ABC’] (C. Tselepis, A. Kwan, D. Thornton and J. Sheehan, unpublished work). Further biochemical characteriz-

ations of the isolated subpopulation will be necessary to answer this question. The higher level of KS in the TD aggrecan can probably be attributed to an increase in chain number rather than length, since gel chromatography of radiolabelled GAG chains showed no difference in GAG length between normal and diseased aggrecans (C. Tselepis, A. Kwan, D. Thornton and J. Sheehan, unpublished work). Immunoblotting with anti-CS antibodies suggested that the amount of CS was unaltered. The role this increase in KS plays in the function of aggrecan is unknown, but increased KS in aggrecan is also found in aging [12,31–34]. This tentatively suggests that the increased KS in aggrecan may lead to an altered mechanical function in the TD growth plate.

Using antibodies 1-B-5, 2-B-6 and 3-B-3 in blotting experiments, we have observed that, whereas the 6-sulphate remained the same, the 4-sulphate increased by 22% and this was compensated for by a decrease of 20% in the 0-sulphate in the TD aggrecan. However, this alteration is only observed in the stub disaccharide, which remains after chondroitin ABC lyase digestion. Whether this alteration is seen throughout the CS chain cannot be ascertained, but the fact that the overall charge density does not differ (results not shown) suggests that this is not the only alteration. It is now widely accepted that changes in the sulphation pattern in proteoglycans are predetermined events which reflect the developmental [35–37] as well as pathological [38–40] stages of the cartilage matrix.

We have previously shown, by immunolocalization with antibody 5-D-4, that the majority of KS reactivity is to intracellular aggrecan in the dyschondroplastic cartilage [18]. This would suggest that the reported biochemical differences arise as a consequence of intracellular accumulation of aggrecan, with the intracellular pool of aggrecan containing a higher number of KS chains. On the basis of our immunoblotting experiments, intracellular accumulation of aggrecan could be due to the absence of a G3 domain, which has previously been implicated in aggrecan secretion [41,42]. Alternatively, the chondrocytes need to be fully hypertrophic for efficient secretion of aggrecan. The fact remains that the aggrecan accumulates in the chondrocytes and may still be atypically further glycosylated as a consequence. This explanation would also account for the absence of the smaller pool of molecules found in normal cartilage. However, further investigation will be required to define molecular structures of the separate pools of aggrecan in TD cartilage. In the present study we have also shown that all of the dyschondroplastic aggrecan is able to bind to exogenous HA in an *in vitro* assay, suggesting that the dyschondroplastic aggrecan molecules have a functional N-terminal domain, though whether *in vivo* aggregation occurs is not known.

The roles of biochemically distinct aggrecan in the development of TD are at present unknown. Since proteoglycans have been implicated in the process of mineral deposition [43,44], a decrease in aggrecan concentration and/or the increase in molecular size may have an inhibitory effect on mineralization in the TD cartilage. It is conceivable that either a decrease in concentration or changes in biochemical characteristics of aggrecan in the TD ECM may lead to altered mechanical properties of the growth plate and decreased stability of other matrix macromolecules as a direct consequence of the interruption of intermolecular interactions or cell–matrix interactions. This may lead to the breakdown in tissue integrity, affecting further cellular development, levels of Ca^{2+} and matrix vesicle deposition [45], culminating in the disruption of matrix mineralization. The present study, therefore, provides a basis for further investigations of the structure–function relationship of the ECM in the development of the growth plate in health and disease.

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REFERENCES

- Brighton, C. T. (1987) Morphology and biochemistry of the growth plate. *Rheum. Dis. Clin. North Am.* **13**, 75–100
- Poole, A. R. (1991) The growth plate: cellular physiology, cartilage assembly and mineralization. In *Cartilage Molecular Aspects* (Hall, B. K., ed.), CRC Press, Ann Arbor
- Leach, R. M. and Nesheim, M. C. (1965) Nutritional, genetic and morphological studies of an abnormal cartilage formation in young chicks. *J. Nutr.* **86**, 236–244
- Sauveur, B. and Monglin, P. (1978) Tibial dyschondroplasia, a cartilage abnormality in poultry. *Ann. Biol. Anim. Biochem. Biophys.* **18**, 87–98
- Freedman, B. D., Gay, C. V. and Leach, R. M. (1985) Avian tibial dyschondroplasia: II. biochemical changes. *Am. J. Pathol.* **119**, 191–198
- Farquharson, C., Whitehead, C. C., Rennie, J. S., Thorp, B. H. and Loveridge, N. (1992) Cell proliferation and enzyme activities associated with the development of avian tibial dyschondroplasia: an *in situ* biochemical study. *Bone* **13**, 59–69
- Thorp, B. H., Farquharson, C., Kwan, A. P. L. and Loveridge, N. (1993) Osteochondrosis/dyschondroplasia: a failure of chondrocyte differentiation. *Equine Vet. J. Suppl.* **16**, 13–18
- Thorp, B. H. and Duff, S. R. I. (1988) Effect of unilateral weight-bearing on pelvic limb development in broiler fowls: vascular studies. *Res. Vet. Sci.* **44**, 164–174
- Thorp, B. H. (1988) Relationship between the rate of longitudinal bone growth and physeal thickness in the growing fowl. *Res. Vet. Sci.* **45**, 83–85
- Hascall, V. C. and Sajdera, S. W. (1970) Physical properties and polydispersity of proteoglycan from bovine nasal cartilage. *J. Biol. Chem.* **245**, 4920–4930
- Heinegård, D., Weislander, J., Sheehan, J., Paulsson, M. and Sommarin, Y. (1985) Separation and characterization of two populations of aggregating proteoglycans from cartilage. *Biochem. J.* **225**, 95–106
- Hardingham, T. E. and Bayliss, M. Y. (1990) Proteoglycans of articular cartilage changes in aging and in joint disease. *Semin. Arthritis Rheum., Suppl.* **1**, 12–33
- Shepard, N. and Mitchell, N. (1985) Ultrastructural modifications of proteoglycans coincident with mineralization in local regions of rat growth plate. *J. Bone Joint Surg.* **67A**, 455–464
- Arseneault, A. L. and Ottensmeyer, F. P. (1983) Quantitative spatial distributions of calcium, phosphorus and sulfur in calcifying epiphysis by high resolution electron spectroscopic imaging. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1322–1326
- Hunter, G. K. (1987) An ion-exchange mechanism of cartilage calcification. *Connect. Tissue Res.* **16**, 111–120
- Dziewiatkowski, D. D. (1987) Binding of calcium by proteoglycan *in vitro*. *Calcif. Tissue Int.* **40**, 265–269
- Chen, C. C. and Boskey, A. L. (1985) Mechanisms of proteoglycan inhibition of hydroxyapatite growth. *Calcif. Tissue Int.* **37**, 395–400
- Tselepis, C., Hoyland, J. A., Barber, R. E., Thorp, B. H. and Kwan, A. P. L. (1996) Expression and distribution of cartilage matrix macromolecules in avian tibial dyschondroplasia. *Avian Pathol.* **25**, 305–324
- Pasternack, S. G., Veis, A. and Breen, M. (1974) Solvent-dependent changes in proteoglycan subunit conformation in aqueous guanidine hydrochloride solutions. *J. Biol. Chem.* **249**, 2206–2211
- Sheehan, J. K., Nieduzynski, I. A. and Phelps, C. F. (1978) Self-association of proteoglycans subunits from pig laryngeal cartilage. *Biochem. J.* **171**, 109–114
- Thornton, D. J., Nieduzynski, I. A., Oates, K. and Sheehan, J. K. (1986) Electron-microscopic and electrophoretic studies of bovine femoral-head cartilage proteoglycan fractions. *Biochem. J.* **240**, 41–48
- Rennie, J. S., Whitehead, C. C. and Thorp, B. H. (1993) The effect of dietary 1,25-dihydroxycholecalciferol in preventing tibial dyschondroplasia in broilers fed diets imbalanced in calcium and phosphorus. *Br. J. Nutr.* **69**, 809–816
- Thornton, D. J., Holmes, D. F., Sheehan, J. K. and Carlstedt, I. (1989) Quantitation of mucus glycoproteins blotted onto nitrocellulose membranes. *Anal. Biochem.* **182**, 160–164
- Calabro, A., Hascall, V. C. and Caterson, B. (1992) Monoclonal antibodies directed against epitopes within the core protein structure of the large aggregating proteoglycan (aggrecan) from the swine rat chondrosarcoma. *Arch. Biochem. Biophys.* **298**, 349–360
- Caterson, B., Christner, J. E., Baker, J. R. and Couchman, J. R. (1985) Production and characterisation of monoclonal antibodies directed against connective tissue proteoglycans. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 386–393
- Couchman, J. R., Caterson, B., Christner, J. E. and Baker, J. R. (1984) Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues. *Nature (London)* **307**, 650–652
- Caterson, B., Christner, J. E. and Baker, J. R. (1983) Identification of a monoclonal antibody that specifically recognises corneal and skeletal keratan sulphate. *J. Biol. Chem.* **258**, 8848–8854
- Sobue, M., Nakashima, N., Fukatsu, T., Nagasaka, T., Fukata, S., Ohiwa, N., Nara, Y., Ogur, T., Katoh, T. and Takeuchi, J. (1989) Production and immunohistochemical characterization of a monoclonal antibody raised to proteoglycan purified from a human yolk sac tumor. *Histochem. J.* **21**, 455–460
- Loveridge, N., Farquharson, C., Hesketh, J. E., Jakowlew, S. B., Whitehead, C. C. and Thorp, B. H. (1993) The control of chondrocyte differentiation during endochondral bone growth *in vivo*: involvement of TGF- β and the proto-oncogene *c-myc*. *J. Cell Sci.* **105**, 949–956
- Farquharson, C., Whitehead, C. C. and Loveridge, N. (1994) Alterations in glycosaminoglycan concentration and sulfation during chondrocyte maturation. *Calcif. Tissue Int.* **54**, 296–303
- Bayliss, M. T. and Ali, S. Y. (1978) Age-related changes in the composition and structure of human articular-cartilage proteoglycans. *Biochem. J.* **176**, 683–693
- Sweet, M. B. E., Thonar, E. J.-M. A. and Marsh, J. (1979) Age-related changes in proteoglycan structure. *Arch. Biochem. Biophys.* **198**, 439–448
- Theocharis, D. A., Kalpaxis, D. L. and Tsiganos, C. P. (1985) Cartilage keratan sulphate: changes in chain length with ageing. *Biochim. Biophys. Acta* **841**, 131–134
- Kempson, G. E., Muir, H., Swanson, S. A. V. and Freeman, M. A. R. (1970) *Biochim. Biophys. Acta* **215**, 70–77
- Stocum, D. L., Davis, R. M., Leger, M. and Conrad, H. E. (1979) Development of the tibiotarsus in the chick embryo: biosynthetic activities of histologically distinct regions. *J. Embryol. Exp. Morphol.* **54**, 155–170.
- Harab, R. C. and Mourao, P. A. S. (1989) Increase of chondroitin 4-sulfate concentration in the endochondral ossification cartilage of normal dogs. *Biochim. Biophys. Acta* **992**, 237–240
- Deutsch, A. J., Midura, R. J. and Plaas, A. H. K. (1995) Structure of chondroitin sulphate on aggrecan isolated from bovine tibial and costochondral growth plates. *J. Orthop. Res.* **13**, 230–239
- Caterson, B., Mahmoodian, F., Sorrell, J. M., Hardingham, T. E., Bayliss, M. T., Carney, S. L., Ratcliffe, A. and Muir, H. (1990) Modulation of native chondroitin sulphate structure in tissue development and in disease. *J. Cell. Sci.* **97**, 411–417
- Carney, S. L., Billingham, M. E., Caterson, B., Ratcliffe, A., Bayliss, M. T., Hardingham, T. E. and Muir, H. (1992) Changes in proteoglycan turnover in experimental canine osteoarthritic cartilage. *Matrix* **12**, 137–147
- Visco, D. M., Johnstone, B., Hill, M. A., Jolly, G. A. and Caterson, B. (1993) Immunohistochemical analysis of 3-B-3 (—) and 7-D-4 epitope expression in canine osteoarthritis. *Arth. Rheum.* **3**, 1718–1723
- Li, H., Schwartz, N. B. and Vertel, B. M. (1993) cDNA cloning of chick cartilage chondroitin sulfate (aggrecan) core protein and identification of a stop codon in the aggrecan gene associated with the chondrodystrophy, nanomelia. *J. Biol. Chem.* **268**, 23504–23511
- Vertel, B. M., Grier, B. L., Li, H. and Schwartz, N. B. (1994) The chondrodystrophy nanomelia: biosynthesis and processing of the defective aggrecan precursor. *Biochem. J.* **301**, 211–216
- Matsui, Y., Alini, M., Webber, C. and Poole, R. (1991) Characterization of aggregating proteoglycans from the proliferative, maturing, hypertrophic, and calcifying zones of the cartilaginous physis. *J. Bone Joint Surg.* **73**, 1064–1074
- Hunter, G. K. and Szigety, S. K. (1992) Effects of proteoglycan on hydroxyapatite formation under non-steady-state and pseudo-steady-state conditions. *Matrix* **12**, 362–368
- Nie, D., Genge, B. R., Wu, L. N. and Wuthier, R. E. (1995) Defect in formation of functional matrix vesicles by growth plate chondrocytes in avian tibial dyschondroplasia: evidence of defective tissue vascularization. *J. Bone Miner. Res.* **10**, 1625–1634