Versican gene expression in human articular cartilage and comparison of mRNA splicing variation with aggrecan

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The chondrocytes in human articular cartilage from subjects of all ages express mRNAs for both of the aggregating proteoglycans aggrecan and versican, although the level of expression of versican mRNA is much lower than that of aggrecan mRNA. Aggrecan shows alternative splicing of the epidermal growth factor (EGF)-like domain within its C-terminal globular region, but there is no evidence for a major difference *in situ* in the relative expression of this domain with age. At all ages studied from birth to the mature adult, a greater proportion of transcripts lacked the EGF domain. The relative proportions of the two transcripts did not change upon culture and passage of isolated

chondrocytes. In contrast, the neighbouring complement regulatory protein (CRP)-like domain was predominantly expressed irrespective of age, but cell culture did result in variation of the splicing of this domain. Versican possesses two EGF-like domains and one CRP-like domain, but at all ages the three domains were predominantly present in all transcripts. This situation persisted upon culture and passage of the chondrocytes. Thus, unlike aggrecan, the versican expressed by human articular cartilage does not appear to undergo alternative splicing of its C-terminal globular region, either in cartilage *in situ* or in chondrocytes in culture.

INTRODUCTION

The extracellular matrix of articular cartilage is rich in the aggregating proteoglycan aggrecan. This molecule is characterized by its ability to interact with hyaluronic acid and its abundant content of chondroitin sulphate and keratan sulphate chains [1]. The structure of aggrecan isolated from the cartilage matrix is not constant throughout the life of an organism, but changes due to a combination of synthetic and degradative events [2]. Most notable amongst these is the increased abundance of keratan sulphate and the decreased size of the core protein with age. Analysis of human aggrecan at the cDNA level has revealed that the primary translation product possesses a core protein of at least 2316 amino acids, which may be divided into a series of distinct domains by virtue of their sequence identity with other proteins [3]. Visualization of aggrecan by rotary shadowing has shown that these domains form a series of globular and extended regions [4]. Two globular regions, termed G1 and G2, are present near the N-terminus of the core protein, and one, termed G3, is present near the C-terminus. The globular domains maintain their conformation by the presence of numerous disulphide bonds [5]. A long extended region exists between the G2 and G3 regions and this forms the principal site of attachment for the glycosaminoglycan chains. During ageing, proteolytic processing may occur in the extended domains of the aggrecan molecule, which results in loss of those fragments containing the G2 and G3 regions from the tissue and the accumulation of smaller proteoglycans bearing the G1 domain [2,6].

The general features of the aggrecan core protein appear to be common to a variety of species [7–9], particularly in the domains which form the globular regions. However, there does appear to be species variation within the large extended domain between the G2 and G3 regions [3,7,10], and this may contribute to species variations in the degree to which the mature proteoglycans are substituted by chondroitin sulphate and keratan sulphate

chains. In the human and the rat, the aggrecan gene is composed of 15 exons, with each exon apparently coding for a distinct structural domain [11]. Of particular interest is the G3 region, which is the product of five exons. Two of these exons code for domains showing sequence similarity with epidermal growth factor (EGF) and a complement regulatory protein (CRP). These exons appear to undergo alternative splicing, and may be either present or absent from the mRNA transcripts [3,12]. In the mRNA isolated from juvenile human chondrocytes, the predominant aggrecan message is reported to lack both the EGF-like and the CRP-like domains. Aggrecan molecules possessing the EGF-like domain have been identified in the articular cartilage matrix [13].

The aggregation potential of aggrecan is based on the presence of the G1 region. Other matrix macromolecules having the ability to interact with hyaluronate also possess an analogous region. This has been most extensively studied in link protein [14,15], where it has been shown that one domain is responsible for interaction with aggrecan and adjacent tandemly repeated domains are responsible for interaction with hyaluronic acid [16,17]. Each domain is the product of a distinct exon in the link protein gene [18]. Perhaps surprisingly, the G2 region of aggrecan also contains related hyaluronate-binding domains, yet it shows no interaction with hyaluronate [19]. The large fibroblast proteoglycan versican also bears a G1 region analogous to that in aggrecan, though it lacks the G2 region found in the latter [20,21]. Versican also bears a G3 region, which differs from that of aggrecan in having tandem EGF-like domains. To date, it is not known whether alternative splicing also gives rise to G3 heterogeneity in versican. Versican also possess a long extended region separating its terminal globular regions, but unlike aggrecan this is sparsely substituted with glycosaminoglycan chains because of the low abundance of the consensus sequences necessary for the initiation of glycosylation.

The purpose of this work was: (1) to determine if versican is also expressed in human articular cartilage and whether it exhibits

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Table 1 Primers for PCR amplification

Nucleotide positions for proteoglycans refer to those described in full-length cDNA sequences cited for aggrecan lacking both EGF- and CRP-like domains [3], and versican possessing all EGF- and CRP-like domains [21]. Nucleotide positions for collagens refer to those described in partial cDNA sequences including the region of COL1A2 covering the C-terminus of the collagen triple helix [24] and the 3' untranslated region of COL2A1 [25]. The upstream primer is shown above the downstream primer in all cases. The expected fragment size upon PCR amplification is given together with any effect upon EGF-like or CRP-like domain splicing.

Molecule	Domain	Primers	Fragment
Aggrecan	6409–6429	5'-CGCATCTAGAAATTGAGTCCT	425 bp or 311 bp (+ EGF, – EGF)
	6699–6719	5'-CCAAGTGTAGTCTTGGGCATT	
Aggrecan	6709–6728	5'-ATGCCCAAGACTACCACTGG	501 bp or 318 bp (+ CRP, – CRP)
	7007–7026	5'-TCCTGGAAGCTCTTCTCAGT	
Versican	1419–1440	5'-TTACCTGAATTCCTGACATAA	780 bp
	2177–2198	5'-TGAGAGCTCTACAACCTTGCTA	
Versican	6377–6400	5'-CACGATAGCAGCATCAGAACAGCA	558 bp, 444 bp or 330 bp (+ 2EGF, + EGF – EGF)
	6911–6934	5'-GACAGGATGATGCTTGAGATGG	
Versican	6911–6934	5'-CCATCTCACAAGCATCCTCTGTC	530 bp or 347 bp (+ CRP, – CRP)
	7417–7440	5'-TTGAATTGCTCTTGCTGATGAGG	
COL1A2	2979–2998	5'-CGGTTACCCTGGCAATATTG	364 bp
	3324–3342	5'-TCCAGTCCGACCATCTTTT	
COL2A1	574–587	5'-CTGGCTCCCAACTGCCAACGTC	414 bp
	964–987	5'-TCCTTTGGGTTTGCAACGGATTGT	

alternative splicing in its G3 domains, and (2) to determine if splicing of aggrecan and versican changes during development and ageing in human articular cartilage.

EXPERIMENTAL

Materials

Guanidine isothiocyanate, penicillin, gentamicin, fungizone, trypsin, Dulbecco's modified Eagles medium (DMEM) and Muloney murine leukaemia virus reverse transcriptase (MMuLV-RT) were from Gibco-BRL, Burlington, Ontario, Canada. Bacterial collagenase (type 1A) and hyaluronidase (type IV) were from Sigma, St. Louis, MO, U.S.A., deoxynucleotides and random hexanucleotide primers were from Pharmacia, Montreal, Quebec, Canada, and T4 polynucleotide kinase and the large fragment of DNA polymerase I (Klenow) were from New England Biolabs, Mississauga, Ontario, Canada. Fetal calf serum (FCS) was from Bocknek, Rexdale, Ontario, Canada, ribonuclease inhibitor (RNasin) was from Promega, through Fisher Scientific, Nepean, Ontario, Canada, Taq DNA polymerase was from Perkin-Elmer Cetus, Montreal, Quebec, Canada, and T7 DNA polymerase (Sequenase) was from United States Biochemical Corp., Cleveland, OH, U.S.A.

Source of cartilage

Human articular cartilage was collected from the distal femur at the time of autopsy, and within 20 h of death. The specimens came from a fetus (24 weeks gestation) and individuals aged 1 month and 8, 28, 49, 68 and 69 years. In all cases the knee joints appeared macroscopically normal and there was no clinical evidence of a connective tissue abnormality.

Direct extraction of cartilage RNA

Approx. 0.1 g of tissue was cut into small pieces of about 4 mm³ in size. Total RNA was extracted directly from the tissue by the acid guanidinium isothiocyanate/phenol/chloroform method [22], with 1 ml of denaturing solution being used to extract RNA from about 0.1 g of tissue. Samples were vortexed for 20 s

immediately after the addition of the complete denaturing solution, and extracted on ice for 15 min before centrifugation. RNA was precipitated from the aqueous phase with an equal volume of propan-2-ol and resuspended in 50 µl of diethyl pyrocarbonate-treated water containing 20 units/ml RNasin. Other than being cut into small pieces, the tissue was not mechanically disrupted prior to the extraction procedure. Samples were stored at –80 °C prior to analysis.

Chondrocyte isolation and RNA extraction

For chondrocyte isolation, tissue was collected in DMEM plus antibiotics (penicillin, gentamicin, fungizone). Where necessary, extraneous connective tissue was dissected off and the cartilage was cut into pieces of approx. 1 mm³ in size. The cartilage pieces were incubated with 5 ml of 0.25 % trypsin, in Hanks' balanced salt solution depleted of calcium and magnesium salts and supplemented with 1 mM EDTA, per g of cartilage, in a spinner flask at 37 °C for 1 h. The trypsin solution was then decanted and the tissue was washed twice with DMEM containing 10 % FCS. The tissue was digested with DMEM containing 0.12 % collagenase and 0.1 % hyaluronidase at 37 °C, for 5–6 h for young tissue or overnight for adult tissue. Following digestion the cell suspension was passed through a tissue sieve to remove any remaining tissue pieces and the cells were recovered by centrifugation for 5 min in a benchtop centrifuge at 1000 rev./min (160 *g*_{av.}) at room temperature. Cells were resuspended in DMEM (high glucose) plus 10 % FCS with penicillin and streptomycin, and seeded at a density of 5 × 10⁶ cells per 150 cm² flask. At 80 % confluence, cells were passaged or total RNA was extracted and stored as described above.

Preparation of fibroblast RNA

Normal human fibroblasts were obtained from a skin biopsy taken from a 32-year-old individual. Fibroblasts were derived from outgrowth from explant culture in minimal essential medium containing 10 % FCS and supplemented with 50 units/ml penicillin-G and 25 µg/ml gentamicin sulphate. Out-

growing cells and subsequent subcultures were passaged using 0.25% trypsin in Puck's medium, with continuing culture in minimal essential medium containing 10% FCS but lacking antibiotics. After passage 6, fibroblasts were grown to 80% confluence and RNA was extracted and stored as described above.

PCR amplification

cDNA was synthesized from 5 μ l of total RNA in a 20 μ l reaction volume, containing 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 200 units of MMuLV-RT, 0.5 mM of each deoxynucleotide, 100 pmol of random hexanucleotide primers and 20 units of RNasin. The mixture was incubated at 37 °C for 60 min. After inactivation at 95 °C for 5 min, 4 μ l of this reaction mixture was used per PCR amplification, using Taq DNA polymerase and standard procedures [23]. Upstream and downstream primers (Table 1), corresponding to regions surrounding the aggrecan [3] and versican [21] EGF-like and CRP-like domains, and adjacent to the versican G1 region, were synthesized on an Applied Biosystems 392A DNA synthesizer. Primers corresponding to regions within the *COL1A2* [24] and *COL2A1* [25] genes were also synthesized. Samples were amplified for 30 cycles in a Perkin-Elmer Cetus DNA Thermal Cycler with denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 1.5 min per cycle. Amplified products were analysed by electrophoresis on a 1.2% agarose gel, and stained with ethidium bromide. For each set of primers, a PCR reaction not containing the cDNA template was performed, and in all cases no reaction product was seen.

Sequencing

PCR amplified products were gel-purified using a DEAE-cellulose membrane [26]. Purified fragments were blunt-ended using the Klenow fragment of *Escherichia coli* DNA polymerase I and phosphorylated with bacteriophage T4 polynucleotide kinase [26]. They were then subcloned into M13 for identification by sequence analysis [26]. Sequencing was carried out using the dideoxy chain-termination method [27] and modified T7 DNA polymerase.

Slot-blot analysis

Preparations of total RNA from either cultured chondrocytes or fibroblasts were denatured by treatment with formaldehyde at 67 °C for 15 min, and then slot-blotted on to a Hybond-N membrane. After cross-linking by u.v. irradiation, the membrane was blocked by prehybridization with denatured herring sperm DNA, followed by hybridization with specific cDNA probes for human aggrecan and versican. Probes were labelled with [³²P]dCTP using the random primer technique. After washing, hybridized probe was detected by exposure of the blot to X-ray film. Both probes were about 1 kb in length; that for aggrecan covered the G1 region of the molecule, whereas that for versican covered the G3 region. Neither probe showed cross-reaction when used for blotting with plasmids containing authentic aggrecan or versican DNA.

RESULTS

Aggrecan

The mRNA encoding aggrecan isolated from human chondrocytes shows sequence heterogeneity in its translated region due to alternative splicing of EGF-like and CRP-like domains [3,12]. However, it is not clear from this previous work

whether the degree of such splicing is dependent upon the procedures used for cell isolation and culture, and whether it therefore reflects the situation occurring *in situ*. It is also unclear whether such splicing is dependent upon the age of the individual from whom the cartilage was obtained. To address these points, RNA was extracted directly from articular cartilage and, following reverse transcription, was used to amplify the regions containing the EGF-like and CRP-like domains.

In the cartilage from subjects of various ages, from the fetus to the mature adult, transcripts were detected which either contained or did not contain the EGF-like domain. The proportions of the two variants did not appear to show any marked age-related trend, and there was always a slight predominance of transcripts lacking the EGF-like domain (Figure 1a). There also appeared to be no effect of the site from which the cartilage was obtained, as the ratio of the two transcripts was independent of the depth from the articular surface of the cartilage from which the RNA was isolated (Figure 1b). Furthermore, there was no major change in the ratio of the two transcripts upon isolation of the chondrocytes from the cartilage matrix or their subsequent growth in cell culture prior to RNA isolation (Figure 1c).

The cartilage RNA preparations also showed no marked age-related variation in expression of the CRP-like domain. At all ages studied, transcripts containing this domain were by far the major species present (Figure 2a). Again, there was no variation observed when the cartilage was sampled at different depths from the articular surface (Figure 2b). In contrast to the EGF-like domain, splicing of the CRP-like domain was significantly affected by chondrocyte isolation and culture. In both primary culture and passaged cells there was considerable expression of the transcripts lacking the CRP-like domain, although those transcripts possessing the CRP-like domain were still more abundant under the conditions used (Figure 2c). Whenever the

Figure 1 Analysis of alternative splicing of the aggrecan EGF-like domain

First-strand cDNA preparations were subjected to PCR amplification using primers flanking the aggrecan EGF-like region. Specimens were obtained from: (a) direct extracts of articular cartilage from a fetus (F), a neonate (N), and individuals aged 8, 28, 68 and 69 years, as indicated; (b) cartilage from the deep (D), mid (M) and superficial (S) zones of an individual aged 69 years; and (c) cartilage from an individual aged 49 years, where cells were isolated and grown in monolayer cell culture (P₀, P₁). In the latter case, material from cultured fibroblasts (Fib) was used as a control. The expected sizes of reaction products (425 and 311 bp) are indicated, together with the sizes of ϕ X174 *Hae*III standards (603 and 310 bp).

Figure 2 Analysis of alternative splicing of the aggrecan CRP-like domain

First-strand cDNA preparations were subjected to PCR amplification using primers flanking the aggrecan CRP-like region. Specimens were obtained from: (a) direct extracts of articular cartilage from a fetus (F), a neonate (N), and individuals aged 8, 28, 68 and 69 years; (b) cartilage from the deep (D), mid (M) and superficial (S) zones of an individual aged 69 years; and (c) cartilage from an individual aged 49 years, where cells were isolated and grown in monolayer cell culture (P_0 , P_1). In the latter case, material from cultured fibroblasts (Fib) was used as a control. The expected sizes of reaction products (501 and 318 bp) are indicated, together with the sizes of Φ X174 *Hae*III standards (603 and 310 bp).

transcript lacking the CRP-like domain was observed, a third component was also observed just below the transcript containing the CRP-like domain. Isolation and sequencing of the third component revealed that it also contained the entire CRP-like domain, and at present we can find no obvious difference in structure to account for the differing mobilities of two transcripts that both contain the CRP-like region.

In all experiments, RNA isolated from a long-term-cultured fibroblast cell line was used as a control in which no aggrecan mRNA was expected. Perhaps surprisingly, amplification of the reverse-transcribed DNA from the fibroblast RNA preparation readily exhibited products having the same size as those obtained for the cartilage aggrecan (Figures 1c and 2c). Isolation and sequencing of these components verified that they were indeed derived from fibroblast aggrecan mRNA. Unlike the cartilage- or chondrocyte-derived aggrecan mRNA, the fibroblast mRNA showed little evidence for the presence of an EGF-like domain (Figure 1c). In contrast, with respect to the CRP-like domain, the fibroblast mRNA showed a similar splicing pattern to that obtained from cultured chondrocytes (Figure 2c).

Versican

The identification of aggrecan mRNA as a product of fibroblasts led us to wonder whether the large fibroblast proteoglycan versican [21] might also be expressed by chondrocytes, and whether its EGF-like and CRP-like domains might also be subject to alternative splicing that could vary with culture or cell of origin. Expression of versican mRNA by human articular cartilage *in situ* was demonstrated using direct tissue extracts and PCR amplification with oligonucleotide primers in the region adjacent to the G1 globular domain. Versican transcripts could be detected in cartilage from subjects of all ages from the fetus to

Figure 3 Expression of cartilage versican

First-strand cDNA preparations were subjected to PCR amplification using primers adjacent to the versican G1 domain. Specimens were obtained from: (a) direct extracts of articular cartilage from a fetus (F), a neonate (N), and individuals aged 8, 28, 68 and 69 years; and (b) cartilage from an individual aged 49 years, where cells were isolated and grown in monolayer cell culture (P_0 , P_1). In the latter case, material from cultured fibroblasts (Fib) was used as a control. The expected size of the reaction product (780 bp) is indicated, together with the sizes of Φ X174 *Hae*III standards (872 and 603 bp).

Figure 4 Analysis of alternative splicing of the versican EGF-like domains

First-strand cDNA preparations were subjected to PCR amplification using primers flanking the versican EGF-like regions. Specimens were obtained from: (a) direct extracts of articular cartilage from a fetus (F), a neonate (N), and individuals aged 8, 28, 68 and 69 years; and (b) cartilage from an individual aged 49 years, where cells were isolated and grown in monolayer cell culture (P_0 , P_1). In the latter case, material from cultured fibroblasts (Fib) was used as a control. The expected sizes of reaction products (558, 444 and 330 bp) are indicated, together with the size of Φ X174 *Hae*III standards (603 and 310 bp).

the mature adult (Figure 3a). Transcript levels appeared to be reduced in the adult cartilage, though this may reflect variation in extraction rather than a true age-related variation in abundance. Following isolation and culture of the chondrocytes, versican mRNA expression was maintained (Figure 3b). Isolation and sequence analysis of the cartilage and chondrocyte products confirmed that they were indeed derived from versican and not through mispriming on the aggrecan transcripts.

While aggrecan possesses only one EGF-like domain, versican may possess two such domains [21], giving rise to the possibility of three different transcripts if alternative splicing occurs in a manner analogous to that with aggrecan. However, upon amplification of the region of versican containing the EGF-like domains by PCR, only a single product was observed, irrespective of whether the original RNA was extracted directly from cartilage (Figure 4a) or from isolated chondrocytes or fibroblasts (Figure 4b). In all cases only the transcript bearing both EGF-like domains was present. This situation remained the same for cartilage at all ages studied.

A similar situation occurred with the single versican CRP-like

Figure 5 Analysis of alternative splicing of the versican CRP-like domains

First-strand cDNA preparations were subjected to PCR amplification using primers flanking the versican CRP-like regions. Specimens were obtained from: (a) direct extracts of articular cartilage from a fetus (F), a neonate (N), and individuals aged 8, 28, 68 and 69 years; and (b) cartilage from an individual aged 49 years, where cells were isolated and grown in monolayer cell culture (P₀, P₁). In the latter case, material from cultured fibroblasts (Fib) was used as a control. The expected sizes of reaction products (530 and 347 bp) are indicated, together with the size of Φ X174 *Hae*III standards (603 and 310 bp).

domain, where isolation of RNA directly from cartilage of different ages, or from cultured chondrocytes or fibroblasts, gave rise to only a single product upon PCR amplification (Figures 5a and 5b). As with the versican EGF-like domains, the versican CRP-like domain was always present. The identity of these products as derivatives of versican was verified by isolation and sequencing. Similar verification was performed for the products containing the versican EGF-like domains.

Estimation of mRNA levels following PCR can be misleading, as the degree of amplification can vary with both primer and template sequence for different mRNAs in a given RNA preparation. Therefore, in an attempt to provide comparative levels of aggrecan and versican mRNAs in chondrocytes and fibroblasts, total RNA preparations were subjected to slot-blot analysis. Under the conditions used, aggrecan mRNA was detectable in preparations from both newly isolated and passaged chondrocytes with loadings approaching 0.1 μ g of RNA. In contrast, aggrecan message could not be detected in a preparation from cultured skin fibroblasts at a loading of 10 μ g of RNA (Figure 6a). In the case of versican, mRNA was readily detectable in fibroblasts at loadings approaching 0.1 μ g of RNA. It was also detectable in preparations from both newly isolated and cultured chondrocytes, although at lower relative levels than those detected in the fibroblasts (Figure 6b). With the chondrocyte preparations used, the abundance of versican mRNA was increased in cultured cells. Thus in the first-passage cells it represented at least 10 % of the level observed in the fibroblasts, but in the newly isolated cells the level was nearer 2 %.

Collagen

Identification of versican mRNA in chondrocytes raised the question of whether these cells exhibit any other characteristics normally associated with fibroblast-like cells. One such characteristic is the expression of type I collagen. Direct extraction of cartilage yielded an RNA preparation from which a type II collagen mRNA region could be amplified in small amounts, but in which no type I collagen mRNA product could be detected. Upon placing the chondrocytes in culture, expression of type II collagen mRNA was maintained during primary culture and early passage (Figure 7a). The expression of type I collagen

Figure 6 Comparison of mRNA levels in chondrocytes and fibroblasts

Total RNA preparations were subjected to slot-blot analysis using ³²P-labelled probes specific for (a) aggrecan and (b) versican. Samples were applied in doubling dilutions, commencing with 10 μ g of RNA, and were extracted from chondrocytes directly following cell isolation (C), from chondrocytes after culture to first passage (P), and from cultured skin fibroblasts (F).

Figure 7 Expression of type I and II collagens

First-strand cDNA preparations were subjected to PCR amplification using primers within (a) COL2A1 and (b) COL1A2 cDNA sequences. Specimens were from cartilage, from an individual of 49 years, which was subjected to direct extraction (DE) and chondrocyte isolation and subsequent culture (P₀, P₃). Material from cultured fibroblasts (Fib) was used as a control. The expected sizes of the reaction products (414 and 364 bp) are indicated, together with the sizes of Φ X174 *Hae*III standards (603 and 310 bp).

mRNA was also detectable as early as primary culture and was maintained upon subsequent passage (Figure 7b).

DISCUSSION

The aggrecan gene possesses one exon that encodes an EGF-like domain and one exon that encodes a CRP-like domain, which when present are within the G3 globular region at the C-terminus of the aggrecan core protein [3]. Alternative splicing of these

domains has previously been demonstrated, such that in cultured chondrocytes from young individuals the majority of transcripts lack both of these domains [3]. While other transcripts possess the EGF-like domain, the presence of the CRP-like domain is rare. In the present work, RNA has been extracted directly from human articular cartilage to determine if similar splicing variation occurs *in situ*. Alternative splicing of the EGF-like region was observed, with relative abundances of the two forms similar to those reported previously. However, *in situ* the CRP domain appeared to be predominantly present; a reversal of the situation previously reported. This difference does not appear due to the differing ages of the individuals from whom cartilage was obtained, as *in situ* the age of the cartilage does not have any major effect upon EGF-like or CRP-like domain splicing, at least in the relatively small number of random specimens used in the present study.

The reason for a discrepancy in splicing of the CRP-like domain between different studies may reside in the use of cell culture versus direct extraction. In the present work, splicing of the CRP-like domain appears to change markedly when chondrocytes are isolated from their matrix and placed in culture, with deletion of this domain increasing, although still not predominating. It is not unreasonable to conclude that exposure of the chondrocytes to other culture conditions may enhance this effect even more, and could be an explanation for the previously reported deficit in this domain. Whether these splicing variations in mRNA are reflected at the protein level is currently unknown; nor is it clear whether the EGF-like and the CRP-like regions serve any functional role if they are present. However, the lack of any change in the mRNA expression of these domains with age would suggest that they may not play a regulatory role during cartilage development.

By analogy with the aggrecan gene, it is likely that the versican gene possesses two exons that encode EGF-like domains and one that encodes a CRP-like domain. However, there is no evidence for these domains ever being subject to alternative splicing, irrespective of whether the transcript is derived from a chondrocyte or a fibroblast. This is in marked contrast to the splicing variation in these cell types observed for the aggrecan gene. We must await sequence information from aggrecan and versican genomic DNA at the relevant intron/exon junctions to determine whether this may explain the variation in splicing between the two molecules.

At first sight, it is somewhat surprising that both aggrecan and versican appear to be products of chondrocytes and fibroblasts, as aggrecan is normally associated with connective tissues that are subject to compression, such as cartilage and intervertebral disc [1,28], and versican is associated with the other connective tissues. However, proteoglycans having a structure compatible with that of aggrecan have been identified in meniscus, tendon and sclera [29–31]. Thus, under the correct circumstances, the fibroblast-like cells of these tissues can produce an aggrecan molecule analogous to that of chondrocytes. The identification of matrix versican is less clear. The aggregating proteoglycans isolated from the aorta have a domain structure on rotary shadowing electron microscopy that is compatible with their being versican [32], and a large chondroitin sulphate proteoglycan synthesized by osteoblasts would also appear to be versican [33]. However, the only definitive evidence for versican production as a proteoglycan comes from fibroblasts in culture [34]. With respect to cartilage, it is possible that the large non-aggregating proteoglycan extracted from bovine nasal cartilage could be derived from versican. It is large, contains relatively few chondroitin sulphate chains and is distinct from aggrecan degradation products [35]. Its inability to interact with hyaluronic acid

could be due to proteolytic modification or removal of the G1 domain in a manner analogous to that occurring in aggrecan with age [2].

In relation to relative mRNA levels, it appears that fibroblast aggrecan mRNA is present at levels at least two orders of magnitude lower than that in chondrocytes, based on a slot-blot analysis. This raises the question of whether the product visualized upon PCR amplification is derived from a regulated transcription event, or represents low levels present due to illegitimate transcription [36]. Many genes may exhibit such transcription at a very low level and may not be translated by the cell. Such transcripts can, however, be used for PCR analysis [37]. Whether this is the origin of the aggrecan message observed in the present study is unclear. However, the examples provided above, indicating aggrecan synthesis by the cells of fibrous connective tissues under some circumstances, suggest that this need not necessarily be the case for all cells.

In contrast, it is less likely that illegitimate transcription accounts for the versican mRNA in human chondrocytes, as it is readily detectable by slot-blot analysis. However, its low level compared with fibroblast versican mRNA levels raises two questions. First, are low levels of mRNA characteristic of all of the cells, or do selected cells possess high levels and others none? Secondly, could the detection of versican mRNA be due to contamination from fibroblasts? At present we have no data to categorically answer either question. Preliminary studies have indicated that versican mRNA can be amplified in samples from all depths of articular cartilage, and is therefore not confined to a particular zone, such as the articular surface. However, *in situ* hybridization studies will be necessary to determine whether the mRNA may differ in its relative abundance between different cells. The question of fibroblast contamination is also a difficult question to address, as chondrocytes in monolayer culture readily adopt a fibroblastic phenotype. However, the inability to amplify mRNA for type I collagen from direct cartilage extracts, whereas versican mRNA can be amplified, would support a chondrocyte origin for the versican mRNA. Certainly, care was taken during dissection to wash material of synovial fluid origin from the cartilage and to dissect away any soft connective tissues, particularly from the joint margins in the specimens from young subjects.

Finally, it should be stated that the identification of aggrecan mRNA in fibroblasts and versican mRNA in chondrocytes does not necessarily imply that they are being either translated or post-translationally modified in a normal manner. Verification of such synthesis awaits the generation of a panel of antibodies specific for the core proteins of the two molecules, which can be used to recognize the relevant products in cell culture medium or tissue extracts. Irrespective of this, the ability to readily amplify aggrecan mRNA from cultured fibroblasts might prove extremely useful in the analysis of possible aggrecan gene mutations in chondrodysplasias, as the procuring of skin biopsies is considerably easier than obtaining cartilage biopsies.

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