Age-related changes in the synthesis of link protein and aggrecan in human articular cartilage: implications for aggregate stability

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The rates of incorporation of radiolabelled leucine into aggrecan and link protein have been measured in human articular cartilage of different ages. Aggrecan and link protein were purified in the A1 fraction of CsCl gradients as a result of their ability to form high-buoyant-density proteoglycan aggregates with hyaluronic acid. Separation of the aggrecan from the link protein was achieved by Mono Q anion-exchange chromatography. The rates of synthesis of both aggrecan and link protein decreased with age. The age-related decrease in synthesis of aggrecan was paralleled by a decrease in the rate of sulphate incorporation into glycosaminoglycan chains. The synthesis of link protein decreased with age to a greater extent than that of aggrecan such that the ratio of the rates of link protein to aggrecan synthesis decreased from 1 in immature cartilage to 0.2 in mature cartilage. The age-related decrease in link protein synthesis is controlled at

least in part by transcriptional or post-transcriptional mechanisms, as shown by the accompanying age-related decrease in link-protein mRNA. The absence of any age-related decrease in aggrecan mRNA suggests that the decrease in synthesis of aggrecan core protein is controlled by a translational mechanism. Measurement of the total tissue content of aggrecan and link protein by radioimmunoassay revealed an age-related increase in the accumulation of these matrix proteins, even though their *de novo* synthesis was decreasing. This illustrates the importance that the regulation of extracellular post-translational modification also has in controlling the overall turnover of the cartilage matrix.

Key words: protein turnover, mRNA, explant culture.

INTRODUCTION

Articular cartilage is composed principally of large sulphated proteoglycan aggregates embedded in a fibrillar network of type II collagen. The collagen provides cartilage with its tensile strength, while the proteoglycan aggregates enable the tissue to withstand compressive forces. Aggrecan monomers, which form the proteoglycan aggregates, exhibit an extensive age-related heterogeneity in their molecular mass due to extracellular proteolysis of the core protein [1,2] and to differences in the extent to which the core protein is substituted with chondroitin sulphate and keratan sulphate chains [1]. The extracellular matrix is maintained by a small number of cells, the chondrocytes, which are distributed throughout the matrix. In normal cartilage the balance of synthesis and degradation of matrix components is carefully controlled to maintain homeostasis, but in pathological conditions such as osteoarthritis and rheumatoid arthritis, regulatory mechanisms are disrupted and degradation exceeds synthesis, leading to net loss of cartilage. A thorough understanding of the mechanisms which regulate turnover of the matrix is necessary if therapeutic intervention to control cartilage repair is to be effective.

The stoichiometry of the molecular components of proteoglycan aggregates in cartilage is one factor that influences the overall stability of the matrix. Aggregates are formed by the interaction of aggrecan monomers with hyaluronan, and this complex is stabilized by link protein (LP) [3]. A 1:1 molar ratio of aggrecan to LP is generally accepted as being the optimum condition necessary for effective aggregation, and these proportions have been identified in rabbit chondrocyte cultures and

in the Swarm-rat chondrosarcoma [4,5]. However, analysis of *in vitro* aggregation assays showed that LP/aggrecan molar ratios of greater than 1:1 were required to achieve maximum aggregation and aggregate stability [6]. Those authors also suggested that a decline in the concentration of LP could decrease the organization and stability of the extracellular matrix. In this respect it is noteworthy that LP enhanced the extent to which newly synthesized, low-affinity aggrecan interacted with hyaluronan in human articular cartilage [7,8]. Furthermore, recent investigations by Bolton et al. [9] also demonstrated that the level of LP mRNA decreased with the age of the individual and, in an earlier study, Hardingham and Bayliss [10] showed that the concentration of aggrecan G1 domain was in excess of LP at all ages in human articular cartilage. Thus aggregate formation *in vivo* may not be optimal, and a proportion of aggrecan molecules may form a less stable complex with hyaluronan. However, little is known about the formation of stable aggregates *in situ*, and measurements of the concentration of aggrecan and LP in tissue extracts give no indication of the local stoichiometry and organization of these two proteins. These studies also provide no information about the rate of *de novo* synthesis of aggrecan and LP or the extent to which they are incorporated into newly forming aggregates.

The purpose of the present study was to determine whether the previously described age-related decrease in LP mRNA expression [9] was also observed at the level of protein synthesis and to elucidate whether measurements of total accumulated concentrations of aggrecan and LP give an accurate reflection of their relative synthesis rates at a given age.

Abbreviations used: RT-PCR, reverse transcription-PCR; GAG, glycosaminoglycan; LP, link protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium.

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MATERIALS AND METHODS

Tissue culture and radiolabelling of proteins

Human articular cartilage from individuals aged 12, 15, 17, 18, 34, 50 and 71 years was obtained from femoral condyles after surgical amputation and was macroscopically normal. The tissue was diced and placed in Ham's F12 medium (Gibco-BRL, Paisley, Renfrewshire, Scotland, U.K.) supplemented with 2 mM glutamine and containing 100 i.u. of penicillin and 100 μ g streptomycin/ml. Approx. 200–300 mg of tissue was pulse-labelled with 100 μ Ci of [4,5- 3 H]leucine and 50 μ Ci of $\text{Na}_2^{35}\text{SO}_4$ (Amersham International, Amersham, Bucks., U.K.) in 1 ml of culture medium for 4–5 h (unless otherwise stated) and the tissue was then transferred to preweighed tubes and stored at -20°C prior to extraction of matrix proteins.

Purification of aggrecan and LP

The frozen cartilage was cryostat-sectioned (20 μ m thickness) [11] and extracted at 4°C for 48 h with 4 M guanidinium chloride/50 mM sodium acetate, pH 6.8, containing protease inhibitors (5 mM benzamidine hydrochloride, 100 mM 6-amino-hexanoic acid, 10 mM EDTA and 1 mM PMSF). The extracts were filtered through glass wool and mixed at 4°C with pig A1 fraction (equivalent to 0.5 mg of uronic acid) for at least 2 h. The extracts were then dialysed to associative conditions using two changes of 100 vol. of 50 mM sodium acetate, pH 6.8, containing inhibitors and subjected to associative CsCl-density-gradient centrifugation at a starting density of 1.62–1.64 g/ml for 60–70 h at 34000 rev./min at 10°C using an 8×25 ml rotor. The addition of pig A1 fraction ensured complete aggregation of human LP and aggrecan and also enabled the majority of these proteins to be recovered in the high-density fractions of the gradients. After centrifugation, the A1 fraction [12] was dialysed against two changes of 100 vol. of water and then freeze-dried. The aggregate was dissociated by resuspension in 4 M guanidinium chloride at 4°C for 48 h. A sample of this extract was dialysed against three changes of 25 vol. of 7 M urea/20 mM Tris, pH 8, and fractionated on a Mono Q column (Pharmacia, St. Albans, Herts., U.K.). In order to ensure that the high levels of free G1, known to be present in adult cartilage, did not interfere with the binding of LP to hyaluronan and therefore impede its recovery in the A1 fraction, the A2, A3 and A4 fractions were subsequently mixed with additional pig A1 and subjected once more to CsCl-density-gradient centrifugation. The presence of LP in the A1 fraction of these gradients was analysed as described below.

The distribution of LP in the A1–A4 fractions was also determined by immunoprecipitation of LP with the monoclonal antibody 8A4, using the method described by Plaas et al. [4]. The control for measuring non-specific binding was done using Protein A–Sepharose beads in the absence of 8A4.

Quantification of aggrecan and LP by Mono Q ion-exchange chromatography

The urea extract was fractionated on a Mono Q column using 7 M urea buffered with 20 mM Tris, pH 8, as the mobile phase (P. Lorenzo, personal communication). Samples (1 ml) were eluted with a gradient of 0–1 M NaCl over a period of 30 min. The 1 M NaCl wash was then maintained for a further 20 min to ensure complete elution of all proteoglycan. Fractions (1 ml) were analysed for their radioactivity by liquid-scintillation counting, and the distribution of aggrecan and LP was confirmed by SDS/PAGE and fluorography of the gels. SDS/PAGE of Mono Q fractions was carried out by precipitating them overnight with

9 vol. of 50 mM sodium acetate/95 % (v/v) ethanol, and the final pellets were re-dissolved in 25 μ l SDS/PAGE sample buffer. Proteins were electrophoresed on a 4–20 % gradient gel and stained with Coomassie Blue. The gels were then soaked in 1.3 M sodium salicylate/5 mM NaH_2PO_4 , pH 7.0, dried, and exposed to preflashed X-ray film for 4 weeks at -70°C .

Determination of the rate of sulphate incorporation

Explants (30–50 mg) of cartilage were pulse-labelled for 4–5 h in 1 ml of Dulbecco's modified Eagle's medium (DMEM) containing 25 μ Ci of $\text{Na}_2^{35}\text{SO}_4$ and stored at -20°C prior to digestion with 30 μ g of papain in 100 mM sodium acetate, pH 6.0, containing 10 mM cysteine hydrochloride and 2.4 mM EDTA in a total volume of 0.50 ml. Quantification of radiolabel incorporated into glycosaminoglycan (GAG) was performed using an Alcian Blue microplate assay [13].

Quantification of mRNA

Approx. 200 mg of cartilage was placed directly into 0.5 ml guanidinium isothiocyanate solution (Solution D) [14] or was cultured for 4–5 h in Ham's F12 medium prior to addition of the solution D. The tissue was diced and vortex-mixed in the solution D for 30 s. The tissue debris was removed and total RNA was isolated from the extracts using RNeasy columns (Qiagen, Crawley, West Sussex, U.K.). Quantification of mRNA for aggrecan, LP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was accomplished by competitive reverse-transcription PCR (RT-PCR) [9] with modifications as follows. The RT-PCR was carried out using a two step procedure with the *rTth* DNA polymerase (Applied Biosystems, Warrington, Cheshire, U.K.), in which the RT step is carried out in the presence of manganese, which is then chelated and substituted for magnesium prior to PCR. Final reaction conditions for reverse transcription were: 10 mM Tris/HCl, pH 8.3, 90 mM KCl, 1 mM MnCl_2 , 0.25 mM dNTPs, 20 nM of each reverse primer, 4 units of RNAGuard and 5 units of *rTth* DNA polymerase in a total volume of 20 μ l. After incubation at 60°C for 30 min, tubes were placed on ice and aliquots of 5 μ l transferred to a fresh tube for each specific amplification and diluted with 20 μ l PCR buffer [containing 10 mM Tris/HCl, pH 8.3, 5 % (v/v) glycerol, 100 mM KCl, 0.75 mM EGTA, 0.05 % (v/v) Tween 20, 2.5 mM MgCl_2 and 1.6 μ M each of forward and reverse primers]. After an initial denaturation at 95°C for 2 min, PCR was carried out for 30 cycles using a two-step cycling protocol of denaturation at 93°C for 1 min followed by simultaneous annealing and extension at 60°C for 1 min. A final extension step of 7 min at 60°C was carried out. To each 25 μ l PCR reaction mixture was added 2.5 μ l of React buffer 2 (Gibco-BRL) and 12.5 units of restriction endonuclease *Eco*RI (Gibco-BRL) and restriction carried out for 1 h at 37°C . Electrophoresis and quantitative analyses were as described previously [9].

RIA of G1 domain and LP

The RIAs for the G1 domain of aggrecan and of LP were carried out as described previously [15,16], using a rabbit anti-(human G1 domain) antiserum and a rabbit anti-(pig LP) antiserum respectively. Preliminary experiments confirmed that there was greater than 98 % cross-reactivity between pig and human LP in this RIA, whether the LP was included as a complex with aggrecan and hyaluronan or as a purified protein.

RESULTS

Measurement of mRNA and total protein levels for LP and aggrecan

Quantitative, competitive RT-PCR measurements of mRNA extracted directly from cartilage pieces confirmed that, after puberty, the expression of LP mRNA was significantly decreased, whereas that of aggrecan changed much less during aging (Table 1). Thus the ratio of copy numbers for the two mRNAs (LP/aggrecan) was reduced below 1 after 18 years of age (Table 1). The measurement of the LP/aggrecan ratio allows a comparison to be made which is independent of any possible age-related changes in GAPDH mRNA levels. It was also important to show that any observed age-related changes in protein synthesis measured over 5 h were not due to a differential effect of the culture conditions on mRNA levels in tissue of different ages. After culturing explants from five of the seven samples for 5 h it was shown that there was no significant change in mRNA expression (Table 1).

The concentrations of LP and aggrecan G1 domain in cartilage extracts of the same ages were also determined. There was no obvious age-related change in accumulated LP or aggrecan, but the concentration of the total G1 domain did increase after 18 years of age (Table 2). Even in the youngest cartilage samples, there was an excess of G1 domain compared with LP (Table 2). When a correction was made to take account of the 'free' G1 domain which accumulates in articular cartilage during normal aging and the results calculated as nmol of aggrecan/g wet weight, no age-related trends in protein concentrations could be identified, but there was still a deficiency of LP at all ages, with

Table 3 Percentage distribution of radiolabel in the CsCl gradient

After CsCl-density-gradient centrifugation, the samples were frozen and divided into four equal fractions. The radioactivity in 100 μ l aliquots from each fraction was determined and expressed as a percentage of the total radiolabel recovered from the gradient.

Age (years) ...	12	17	18	50	71	
Fraction Label ...	^3H	^{35}S	^3H	^{35}S	^3H	^{35}S
A1	12	94	11	90	8	85
A2	3	2	2	2	3	4
A3	9	2	9	2	5	4
A4	76	2	78	6	85	9

approx. 2-fold higher levels of aggrecan relative to LP over the age range investigated (Table 2).

Purification of aggrecan and LP

Aggrecan and LP were purified by aggregating them with hyaluronic acid and recovering them in the A1 fraction of a CsCl density gradient. Owing to the known lower buoyant density of aggregates prepared from adult cartilage compared with younger tissue [17], pig A1 was added as a carrier to encourage the formation of high-molecular-mass aggregates and ensure the recovery of all newly synthesized proteoglycan and LP in the A1 fraction. The distribution of newly synthesized LP in all of the gradient fractions was determined by SDS/PAGE and fluorography. To ensure that the LP content of the A2, A3 and A4

Table 1 Quantification of aggrecan and LP mRNA in human articular cartilage of different ages by competitive RT-PCR

Human articular cartilage explants (200 mg) were either transferred directly into guanidinium isothiocyanate or cultured for 5 h in Ham's F12 prior to RNA extraction. Total RNA was extracted using RNeasy spin columns (Qiagen) and was used for competitive RT-PCR using a single competitor copy number for aggrecan (200 000 copies), LP (100 000 copies) and GAPDH (200 000 copies). Results for aggrecan and LP mRNA levels are expressed relative to GAPDH mRNA. Abbreviation: N.D., not determined.

Age (years)	Aggrecan mRNA/GAPDH mRNA		LP mRNA/GAPDH mRNA		LP mRNA/aggrecan mRNA	
	0 h	5 h	0 h	5 h	0 h	5 h
12	0.06	0.05	0.25	0.31	4.16	6.25
15	0.80	1.18	1.91	2.26	2.38	1.92
17	0.65	0.60	1.63	1.21	2.50	2.17
18	0.36	N.D.	0.41	0.49	1.13	N.D.
34	1.25	1.39	0.46	N.D.	0.36	0.35
50	0.37	0.75	0.14	0.50	0.38	0.66
71	0.40	N.D.	0.12	N.D.	0.29	N.D.

Table 2 Quantification of aggrecan and LP by RIA

4 M Guanidinium chloride extracts of articular cartilage were dialysed against distilled water and analysed for their content of G1 domain and LP using RIA procedures. The 'free' G1 domain was also removed from the extracts by size-exclusion chromatography on a Sepharose CL-6B column [24] in order to determine the concentration of G1 that was associated with intact aggrecan.

Age (years)	G1 (nmol/g wet wt.)	Aggrecan G1 (nmol/g wet wt.)	LP (nmol/g wet wt.)	Molar ratio LP/total G1	LP/aggrecan G1
12	53.2	48.2	27.9	0.52	0.58
15	85.2	70.2	41.1	0.48	0.59
17	57.8	42.8	23.2	0.40	0.59
18	67.6	50.6	17.1	0.25	0.34
34	99.4	44.4	20.4	0.23	0.46
50	175.8	75.8	56.4	0.32	0.75
71	234.3	104.3	68.0	0.29	0.65

Figure 1 Purification of aggrecan and LP from human articular cartilage

Human articular cartilage explants (343 mg) obtained from a 15-year-old specimen were pulse-labelled with 100 μ Ci of [4,5- 3 H]leucine and 50 μ Ci of $\text{Na}_2^{35}\text{SO}_4$ for 5 h in Ham's F12 medium. After cryostat sectioning (20 μ m thickness), the tissue was extracted with 4 M guanidinium chloride and dialysed to associative conditions. The A1 fraction from a CsCl-density-gradient centrifugation (starting density 1.62 g/ml) was dialysed against water, freeze-dried, redissociated in 4 M guanidinium chloride and dialysed against 7 M urea. Samples were analysed by HPLC using a Mono Q anion-exchange column. The profiles of ^3H ($\blacktriangle$) and ^{35}S ($\blacksquare$) radioactivity eluted from the HPLC column are shown. Aliquots of 30 μ l from each 1 ml fraction were used for liquid-scintillation counting. The NaCl gradient (0–1 M) is shown (----).

fractions was accurately determined, each fraction was subjected to a further associative CsCl-density-gradient centrifugation in the presence of porcine A1. There was no detectable LP in the A2A1, A3A1 or A4A1 fractions (results not shown). At least 80% of the total $^{35}\text{SO}_4$ radioactivity was recovered in the A1 fraction of the gradient of each specimen, together with approx. 10% of the ^3H -labelled proteins (Table 3). The A1 fractions were dialysed into 7 M urea and fractionated on a Mono Q ion-exchange column eluted with a gradient of 0–1 M NaCl. Three peaks (I, II and III) containing ^3H -labelled proteins were identified (Figure 1). In order to characterize these proteins further, the column fractions were subjected to SDS/PAGE. LP was identified in fractions 10–12 (peak I) by its characteristic banding pattern (three bands) and molecular mass, and proteoglycans were confined to the ^{35}S -rich fractions 19–27 (peak III) (Figure 2A). Fluorography of the gel revealed that newly synthesized LP was also present in peak I and that only the two slowest migrating species were radiolabelled (Figure 2B).

Peak II from the Mono Q column contained a high-molecular-mass protein that was only observed in the fluorograph (Figure 2B). The identity of this protein is not known; however, its high molecular mass, its low abundance, non-sulphation and affinity for hyaluronan (recovered in the A1 fraction) tentatively identify it as a pool of newly synthesized intracellular aggrecan core protein. Supporting evidence for this hypothesis was obtained from a pulse-chase experiment in which cartilage explants from a 17-year-old specimen and a 12-year-old specimen were labelled for 5 h with 100 μ Ci of [4,5- ^3H]leucine and then chased for a further 8 h in DMEM in the absence of radiolabel. This experiment showed that the radioactivity in peak II decreased during the 8 h chase culture (results not shown). These results are consistent with the hypothesis that the radiolabelled protein present in peak II is an intracellular pool of non-glycosylated aggrecan protein core.

The distribution of LP in the A1–A4 fractions of one 12-year-old specimen was also determined by immunoprecipitation of LP with the monoclonal antibody 8A4. Although the fraction

Figure 2 Analysis of Mono Q fractions by SDS/PAGE and fluorography

Aliquots of 400 μ l from each fraction shown in Figure 1 were precipitated and used for SDS/PAGE. (A) Coomassie Blue stain to show total protein. (B) The gel was prepared for fluorography by soaking in sodium salicylate, dried, and exposed to preflashed X-ray film for 4 weeks at -70°C .

volumes used in this experiment were dictated by the volume of antibody that was practicable, i.e. by the concentration of non-labelled LP in the A1 fraction, we were able to show that > 95% of the radioactivity that was associated with LP was present in fraction A1 (results not shown). There was, therefore, no evidence of a 'low-affinity' form of LP as has been found for aggrecan.

Kinetics of incorporation of [^3H]leucine into aggrecan and LP

The ^3H in the Mono Q fractions I and III was used to calculate the kinetics of synthesis of aggrecan and LP during 12 h of culture. The incorporation of radiolabelled leucine into aggrecan (Figure 3A) and LP (Figure 3B) increased linearly with time up to 12 h in culture. The rate of $^{35}\text{SO}_4$ incorporation into GAGs measured as a 4 h pulse after each 4 h of culture was also constant and was used to calculate the amount (nmol) of sulphate incorporated into GAG at each time of culture (Figure 3C). Radiolabelling periods of 4–5 h were chosen for subsequent

Figure 3 Kinetics of aggrecan and LP synthesis

Human articular cartilage explants (200–250 mg) from an 18-year-old specimen were pulse-labelled with 100 μCi of [4,5- ^3H]leucine for 4, 8 and 12 h in duplicate. Aggrecan and LP were purified as described for Figure 1. Results are expressed as ^3H radioactivity (d.p.m.) incorporated per g wet weight of cartilage and are the means of duplicate determinations for aggrecan (A) and LP (B). Cartilage explants (30–50 mg) from the 18-year-old specimen were also cultured for 0–16 h and at 4 h intervals the culture medium was replaced with 1 ml of DMEM containing 25 μCi of $\text{Na}_2^{35}\text{SO}_4$ and the explants were incubated for a further 4 h. The medium was then removed and the tissue was digested with papain and assayed for the incorporation of radiolabel into glycosaminoglycans. Results are shown as nmol of sulphate incorporated into glycosaminoglycans per g wet weight of cartilage (C).

experiments in order to minimize the possibility of protein turnover.

Age-related changes in aggrecan and LP synthesis

The rate of incorporation of [^3H]leucine into LP by cartilage of different ages was calculated from the radioactivity recovered in peak I. Aggrecan synthesis was determined by summation of the ^3H radioactivity associated with peak III. The radioactivity in peak II was an insignificant proportion of the total ^3H radioactivity associated with aggrecan core protein and was therefore not included in the calculations. Incorporation of radiolabelled leucine into both aggrecan and LP decreased with age, but the decrease in LP synthesis was greater than for aggrecan (Table 4). To calculate the ratio of newly synthesized LP to that of aggrecan, incorporation of radiolabel into LP and aggrecan was corrected for the leucine content of each protein [18,19], 26 and 173 respectively. These values are based on the assumption that total cleavage of the signal peptide has occurred in all radiolabelled products.

Table 4 Rates of synthesis of aggrecan and LP in human articular cartilage of different ages

Explants of human articular cartilage (30–50 mg) were pulsed with 25 μCi of $\text{Na}_2^{35}\text{SO}_4$ per ml of DMEM and analysed for rates of sulphate incorporation by the Alcian Blue filtration assay. Further explants (200–300 mg) were pulsed with 100 μCi of [4,5- ^3H]leucine/ml of Ham's F12 medium for 4–5 h and aggrecan and LP extracted and purified by associative CsCl-density-gradient centrifugation. The A1 fraction was analysed by HPLC using Mono Q ion-exchange column and radioactivity in fractions 10–12 and 19–27 used for calculation of rates of synthesis of LP and of aggrecan respectively. The rates of synthesis are expressed as d.p.m. incorporated/h per g wet wt. of cartilage. The rates of $\text{Na}_2^{35}\text{SO}_4$ incorporation are expressed as nmol of sulphate incorporated/h per g wet weight.

Age (years)	Rate of synthesis		Ratio of rates LP/aggrecan	Rate of ^{35}S incorporation
	Aggrecan	LP		
12	193359	20337	0.68	5.65
15	264065	38404	0.97	6.51
17	171714	29018	1.12	4.14
18	56104	4316	0.50	2.15
34	121657	4098	0.21	2.04
50	106603	3815	0.22	2.01
71	96557	3584	0.21	1.78

The human aggrecan gene has an expressed variable-number tandem-repeat polymorphism within the chondroitin sulphate-bearing region [20]. However, the frequency of this polymorphism is rare, and when it occurs its effect on the leucine content of the core protein is negligible.

In younger cartilage the ratio of LP to aggrecan synthesis was close to unity, but after puberty the molar ratio of these proteins was significantly less than 1 (Table 4). The rate of sulphate incorporation into GAG chains also decreased with age (Table 4), suggesting that the sulphation of aggrecan is limited by the availability of the aggrecan core protein and is not due to changes in the number of sulphate residues per individual GAG chain or the number of GAG chains per core protein molecule.

DISCUSSION

In the present study we have used quantitative techniques to determine the extent to which changes in the gene expression and the synthesis of LP and aggrecan could influence the extracellular accumulation of these proteins during normal aging. Subsequent studies will determine how these parameters are altered in cartilage during the development of joint disease.

Previous investigations have described in detail the normal age-related changes that occur in the structure and composition of aggrecan and LP from human cartilage [1,2,21,22]. Many of these are a consequence of proteolytic events which take place during the relatively long time that these molecules are resident in the extracellular matrix of the tissue. For aggrecan, this process results in the accumulation of high concentrations of the G1 domain of the molecule ('free' G1 domain), as the limit-digest product of normal turnover. This protein fragment was first identified in human cartilage by Roughley et al. [23], and its role in matrix assembly was further considered by Bayliss and Roughley [17] and reviewed by Hardingham and Bayliss [24]. Because it remains associated with hyaluronan in the tissue, the G1 domain must influence the packing of the aggregate during aging, and it may also affect the spatial and temporal deposition of newly synthesized aggrecan and its association with LP. LP also undergoes major post-translational modifications and further proteolysis-induced changes in the extracellular matrix during aging [21,22]. This ultimately results in the production of

three proteins (LP1, LP2 and LP3) that retain their functional properties (i.e. they can still bind to hyaluronan and aggrecan) and to the generation of additional protein fragments.

In order to appreciate fully the significance of age-related changes in cartilage, it is necessary to consider separately the two metabolic processes which can influence the final composition of the tissue: (i) gene expression and new synthesis of protein and (ii) extracellular modification, accumulation and turnover of proteins. The first of these is clearly affected by the age of the individual. The synthesis of LP and aggrecan core protein and the sulphation of aggrecan core decrease with age, but it is noteworthy that synthesis of LP decreases to a much greater extent than does that of aggrecan. The present results are consistent with our previous findings showing that LP mRNA decreases with age [9], and they further suggest that changes in LP synthesis are largely a reflection of altered gene expression which is dependent on the availability of mRNA, whereas synthesis of aggrecan core protein is affected more by translational and post-translational events. This is not to say that post-translational modifications do not contribute to the heterogeneity of newly synthesized LP. For example, Hering and Sandell demonstrated that the electrophoretic difference between the two major forms of LP synthesized by bovine cartilage, LP1 and LP2, was an Asn-linked oligosaccharide on LP2 [25,26]. Figure 2(B) shows that human articular chondrocytes also synthesize two major forms of LP that have the same electrophoretic mobility as LP1 and LP2 present in the cartilage matrix.

The results presented here demonstrate that measurements of the accumulated aggrecan and LP in human articular cartilage does not necessarily relate to their biosynthetic rates. Although the biosynthesis of aggrecan and LP decreased with age, the total concentration of aggrecan and LP remained relatively constant. In contrast, the total G1 concentration in cartilage increased with age. Recent studies have shown that, after 20 years, there is an age-related accumulation of 'free G1 domain' of aggrecan in the tissue [24] and that this fragment has a longer half-life than the G1 domain associated with intact aggrecan. Thus, if the calculation of the LP to aggrecan is modified by subtracting the 'free G1 domain', this should theoretically result in a value that is more akin to that measured for the newly synthesized aggregate. When this adjustment is made to the calculation, the ratio of LP to aggrecan remains constant at approx. 0.5. The tissue is therefore deficient in LP at all ages.

LP stabilizes the interaction of aggrecan with hyaluronic acid at a molar ratio which is presumed to be 1:1. Since the ratio of the total concentrations of aggrecan to LP measured in the present study is 2:1, up to 50% of the aggrecan monomers in human tissue cannot be link-stabilized in the classical way. The fact that this ratio is relatively constant regardless of the age of the individual does not necessarily mean that the number of LP stabilized aggregates is the same at different ages. It is possible that the extent of formation of link-stabilized aggregates is dictated by the relative rates of *de novo* synthesis at a particular moment in time. If this is the case, the decreased LP synthesis observed during aging could result in a reduced ability of adult cartilage to form new, stable, aggregates. The production of link-stabilized aggregates by rabbit chondrocytes in monolayer culture did decrease with increasing age of the animal from which the cells were isolated [27]. Furthermore, conversion of low-affinity aggrecan monomers into high-affinity monomers was slower in

older animals *in vivo* [28]. These studies suggest that the availability of LP may regulate the rate at which newly synthesized aggrecan acquires affinity for hyaluronic acid, and it is, therefore, interesting to speculate that this could be the explanation for the slower conversion of newly synthesized, low-affinity aggrecan to high-affinity monomers in adult cartilage [8,29,30].

Although it is interesting to indulge in stoichiometric analyses of this type to try and make some sense of changing patterns of gene expression and protein turnover, they can be very misleading because they are based on the analysis of cartilage extracts and do not take into account any differences in the *in situ* distribution of the molecules or in the metabolic activity of chondrocytes. The challenge of future studies will be to devise quantitative methods to investigate compartmental (pericellular versus intercellular) and zonal changes in the synthesis and deposition of these proteins.

This work was supported by the Arthritis Research Campaign. We are grateful to Dr Audrey McAlinden for performing the sulphate incorporation assays and for advice with the Mono Q HPLC procedure and to Ms Louise McKenna and the Bone Tumour Service at the Royal Orthopaedic Hospital, Birmingham, particularly Mr. S. Carter, Consultant Orthopaedic Surgeon, for supplying some of the human articular cartilage used in this study.

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