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Aggrecan Turnover in Human Intervertebral Disc as Determined by the Racemization of Aspartic Acid*[S]

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Sarit S. Sivan[‡1], Eve Tsitron[‡], Ellen Wachtel[§], Peter J. Roughley[¶], Nico Sakkee[‖], Frits van der Ham[‖], Jeroen DeGroot[‖], Sally Roberts[**], and Alice Maroudas[‡]

*From the [‡]Department of Biomedical Engineering, Technion-Israel Institute of Technology, Haifa 32000, Israel, the [§]Faculty of Chemistry, Weizmann Institute of Science, 76100, Rehovot, Israel, the [¶]Shriners Hospital for Children, Genetics Unit, Montreal, Quebec, H3G 1A6, Canada, [‖]TNO Quality of Life, Business Unit Biomedical Research, P. O. Box 2215, 2301 CE Leiden, The Netherlands, and the **Centre for Spinal Studies, Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, SY10 7AG, United Kingdom*

We have used the racemization of aspartic acid as a marker for the "molecular age" of aggrecan components of the human intervertebral disc matrix (aggregating and non-aggregating proteoglycans as well as the different buoyant density fractions of aggrecan). By measuring the D/L$_{Asp}$ ratio of the various aggrecan species as a function of age and using the values of the racemization constant, k_i, found earlier for aggrecan in articular cartilage, we were able to establish directly the relative residence time of these molecules in human intervertebral disc matrix. For A1 preparations taken from normal tissue, turnover rates of 0.059 ± 0.01 and 0.063 ± 0.01/year correspond to half-life values of 12 ± 2.0 and 11.23 ± 1.9 years for nucleus pulposus and annulus fibrosus, respectively; the turnover rates of 0.084 ± 0.022 and 0.092 ± 0.034/year for degenerate tissue correspond to half-life values of 8.77 ± 2.2 and 8.41 ± 2.8 years, suggesting increased rate of removal of small aggrecan fragments. For the large monomer, fraction A1D1, turnover is 0.13 ± 0.04/year, corresponding to a half-life of 5.56 ± 1.58 years, similar to 3.4 years in human articular cartilage. For the binding region (A1D6), turnover is 0.033 ± 0.0012/year, corresponding to a half-life of 21.53 ± 0.6 years, similar to 23.5 years in articular cartilage. A1 preparations from nucleus pulposus contain a lower proportion of aggregating proteoglycans as compared with annulus fibrosus, suggesting increased proteolytic modification in the nucleus pulposus. D/L$_{Asp}$ values in aggregating and non-aggregating proteoglycans of a 24-year-old individual show similar results, suggesting that the non-aggregating molecules are synthesized initially as aggregating proteoglycans, which thereafter undergo cleavage and detachment from hyaluronan.

The intervertebral disc (IVD)[2] is the largest avascular cartilaginous structure. It lies between the vertebral bodies, anchoring them together. IVD plays a primarily mechanical role in transmitting loads through the spine and providing flexibility to the spinal column. The IVD is highly bradytrophic; it is avascular and nourished by diffusion. Although the outer annulus fibrosus (AF) possesses blood vessels in early childhood, the inner nucleus pulposus (NP) remains avascular for the entire life of the organism (1, 2). The disc has a complex structure and contains very few cells embedded in an extracellular matrix. These cells have the essential function of maintaining and repairing the matrix by synthesizing matrix macromolecules and by producing proteinases for matrix breakdown. Thus, disc function is dependent on a balance between synthesis and matrix breakdown. Normally, when this balance is maintained, damaged tissue can be restored by cellular repair responses. In pathology, when there is imbalance between matrix synthesis and breakdown, the matrix composition and organization are altered, and the cellular repair responses are inadequate. Hence, the degraded matrix can no longer carry loads effectively, which leads to the degeneration of the disc.

During aging, many changes involving the proportions and biochemical properties of aggrecan occur. In particular, the structure and composition of aggrecan changes both with aging and with degeneration. These changes involve an increase in the relative contents of keratan sulfate and protein and a decrease in the molecular weight of aggrecan (3). The important question of whether these changes in composition of the aggrecan with aging represent changes in biosynthesis or are due to the accumulation of degraded aggrecan fragments can be addressed by measuring the accumulation of the D-aspartic acid isomer and hence the residence time or the "molecular age" of these molecules. In nature, amino acids are synthesized as L-isomers. Spontaneous racemization slowly converts the L-form of amino acids into a racemic mixture of L- and D-forms. Aspartic acid is one of the most rapidly racemizing amino acids (4, 5), allowing the measurement of the concentration of D-isomers in living subjects in proteins that are not renewed or that slowly turn over. It is well known that an age-dependent racemization occurs in various human and animal tissues containing metabolically stable, long-lived proteins, *e.g.* in enamel and dentine (4), white matter of the brain (6), eye lens (7, 8), aorta (9), cartilage and skin (10–12), and bone (13, 14).

In the present work, turnover of the different populations of proteoglycans (PGs) in human IVD (aggregating and non-aggregating PGs, as well as different buoyant density fractions of the aggrecan) has been determined using racemization of aspartic acid as a tool for assessing the molecular age of long-lived proteins. By using the published racemization constant (k_i) earlier found for articular cartilage (15), we were able to determine the turnover rate of the different PG populations and a possible relationship with respect to their molecular age and origin.

EXPERIMENTAL PROCEDURES

Tissue Sampling—Lumbar and thoracic spines from healthy (ages 0–62, $n = 15$) and pathological (ages 30–77, $n = 14$) discs (usually one from each individual) were obtained postmortem or during routine

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental text and equations concerning turnover rates and half-lives, as well as a supplemental table.

[1] To whom correspondence should be addressed: Julius Silver Institute of Biomedical Sciences, Faculty of Biomedical Engineering, Technion, Israel Institute of Technology, IIT, Haifa, 32000, Israel. Tel.: 972-4-8294150; Fax: 972-4-8294599; E-mail: sarit@bm.technion.ac.il.

[2] The abbreviations used are: IVD, intervertebral disc; NP, nucleus pulposus; AF, annulus fibrosus; PG, proteoglycan; GAG, glycosaminoglycan; CS, chondroitin sulfate; ANOVA, analysis of variance.

surgical procedures for treatment of herniation or disc degeneration. In this study, healthy discs will be referred to as "normal," and pathological ones will be referred to as "degenerate." All discs were divided into NP and AF zones, diced or cryosectioned into 20-μm slices, and stored at −20 °C until analyzed.

Extraction of Aggrecan (A1)—For extraction of A1 from NP and AF, 10 volumes of 4 M guanidinium chloride (GuHCl) containing proteinase inhibitors in 50 mM Tris buffer, pH 7.4, at 4 °C for 48 h were used (16). The extracts were clarified by centrifugation at 4 °C, 10,000 rpm for 40 min and exhaustively dialyzed against the same buffer containing no GuHCl. A1 preparations were separated from other tissue proteins by associative cesium chloride (CsCl) equilibrium density gradient centrifugation at a starting density of 1.5 g/ml using 50,000 rpm at 10 °C for 48 h (17). A1 was then recovered from the densest fraction of the gradient (density greater that 1.59 g/ml CsCl). All A1 fractions were assayed for density, exhaustively dialyzed against distilled water, freeze-dried, and analyzed for glycosaminoglycan (GAG), (18) and protein content. The A1 preparations contain aggrecan and its degradation products but not the small leucine-rich repeat PGs, which are also present in the tissue but which sediment at a lower buoyant density.

Isolation of Aggrecan Subfractions (A1D1–A1D6)—A1 fractions were further separated by means of dissociative CsCl density gradient centrifugation in the presence of 4 M GuHCl (50 mM Tris buffer, pH 7.4) at a starting density of 1.5 g/ml (10 °C, 50,000 rpm for 48 h) and subsequently fractionated into aggrecan monomers of decreasing buoyant density and increasing molecular weight (A1D1–A1D6) (15). All fractions were assayed for density, exhaustively dialyzed against distilled water, freeze-dried, and analyzed for GAG (18) and protein content.

In some cases (individuals aged 24 and 56 years), aggrecan subfractions (A1D1–A1D4) were recovered from the aggregating portion alone of the A1 fraction by gel permeation chromatography. Briefly, the A1 preparations (8–12 mg/ml) were fractionated by gel filtration through a Sepharose CL-2B column (100 × 2.5 cm) at a flow rate of 30 ml/h using 0.2 M sodium acetate, pH 5.5, as the running buffer. Fractions were divided into aggregating PGs eluting at the void volume ($A1V_0$ preparation) and non-aggregating PGs eluting in the included volume ($A1V_i1$ and $A1V_i2$ preparations). The aggregating PG preparation ($A1V_0$) alone was further fractionated to yield the aggrecan subfractions (A1D1–A1D4) by means of dissociative CsCl density gradient centrifugation in the presence of 4 M GuHCl at a starting density of 1.5 g/ml. The resulting fractions were assayed for GAG and protein contents.

Determination of GAG Content—For determination of sulfated GAG content derived predominantly from aggrecan, 100-μl samples of the tissue digests were analyzed using the 1,9 dimethylmethylene blue dye binding assay (18), with chondroitin sulfate as a standard.

Chondroitin Sulfate (CS) Measurements—Uronic acid content as a measure of CS was determined by the carbazole reaction (19). Hyaluronic acid is also present in the human IVD at between 1 and 4% of the total GAG content, depending on age and anatomical site (20), and will contribute to the estimation of CS by uronic acid content. However, its incomplete extraction and lower sedimentation position will minimize its presence in the high buoyant density A1 preparations. It is therefore unlikely that hyaluronic acid contributes greatly to the evaluation of CS by the carbazole reaction.

Determination of Percentage of D-Aspartic Acid—The D/L_{Asp} ratio was determined by high performance liquid chromatography according to Verzijl and Aswad (10, 21). All D/L_{Asp} data were corrected for the amount of D- and L-Asp present in papain and for racemization during the hydrolysis step. Corrections were made either by subtracting the intercept of the graph for D/L_{Asp} *versus* donor age or by subtracting the D/L_{Asp} ratio measured for A1 preparations purified from human neonatal or from young fetal bone (~17 weeks old).

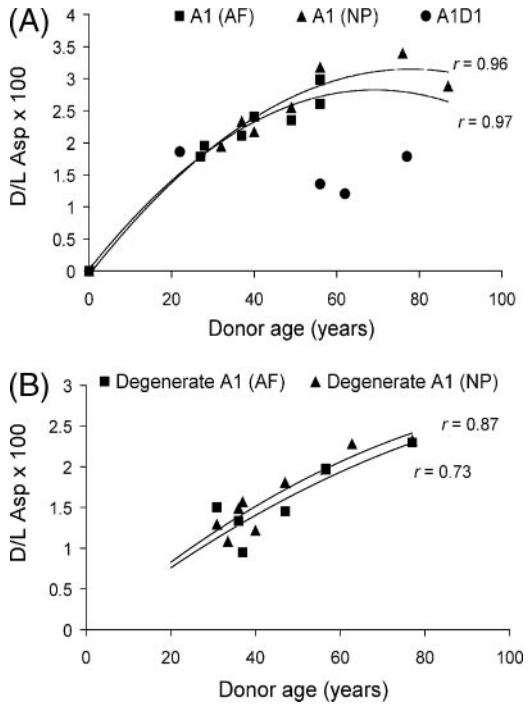

FIGURE 1. **D/L_{Asp} ratio for A1 (NP and AF) preparations obtained from normal (*A*) and degenerate (*B*) human IVD as a function of donor age.** A quadratic curve was found to give the best fit ($n = 8$, $r = 0.96$ and $n = 8$, $r = 0.97$ for normal NP and AF respectively; and $n = 9$, $r = 0.87$ and $n = 6$, $r = 0.73$ for degenerate NP and AF, respectively). No significant difference was found in the rate of accumulation of the D-isomer with age in NP as compared with AF in normal or degenerate discs ($p > 0.05$, *t* test). For comparison, A1D1 (average of normal NP and AF) is presented in *panel A* of this figure.

Calculation of Turnover Rates and Corresponding Half-lives—Turnover rates and half-lives of A1 and A1D1–A1D6 fractions were calculated based on previous work by Maroudas *et al.* (15, 22) on collagen turnover with some modifications suitable for aggrecan and are discussed in detail in the Supplemental Data.

Statistical Analysis—The statistical significance of the differences in D-Asp accumulation between (i) NP and AF and (ii) aggregating and non-aggregating PGs was determined using Student's *t* test (assuming unequal variances, $p < 0.05$). The statistical significance of the differences in D-Asp accumulation between normal and degenerate tissue and between age groups (or young and old specimens) from A1 or A1D1–A1D6 fractions was analyzed using two-factor ANOVA including the Tukey post hoc test and interactions; $p < 0.05$ was considered to represent statistically significant difference. Linear regression analysis of the age-related increase in protein content, normalized to A1, for normal and degenerate tissue (both NP and AF) was performed. The limit of significance of the difference in slopes between NP and AF was set at the $p = 0.05$ level.

RESULTS

Accumulation of D-Asp in Aggrecan from Normal and Degenerate IVD—Fig. 1 shows the experimental values of D/L_{Asp} for the A1 fractions obtained from NP and AF of normal and degenerate human IVD as a function of donor age. Corrections to the D/L_{Asp} values were applied as described under "Experimental Procedures." Racemization of aspartic acid (expressed as the ratio D/L_{Asp}) in the A1 preparations clearly increases with age in a non-linear fashion ($n = 8$, $r = 0.96$ and $n = 8$, $r = 0.97$ for normal NP and AF, respectively, Fig. 1*A*; and $n = 9$, $r = 0.87$ and $n = 6$, $r = 0.73$ for degenerate NP and AF, respectively, Fig. 1*B*). No significant difference was observed in the rate of accumulation of the

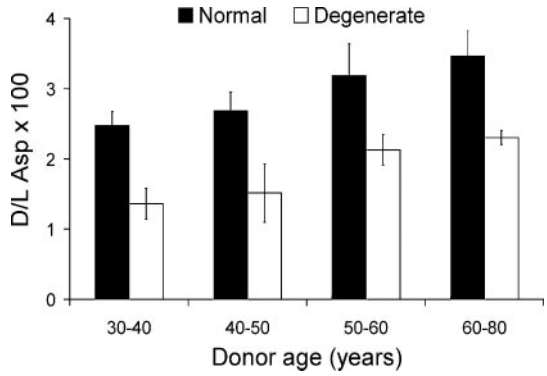

FIGURE 2. **D/L_{Asp} for A1 preparations obtained from NP of normal and degenerate human IVD as a function of donor age.** Similar results were obtained for AF (data not shown). A significant difference ($p < 0.05$) was observed between mean values of normal and degenerate tissues averaged over all age groups using two-factor ANOVA including the Tukey post hoc test and interaction. However, a significant difference in D-Asp accumulation between age groups was noted only between 30–40 and 50–60 and between 30–40 and 60–80.

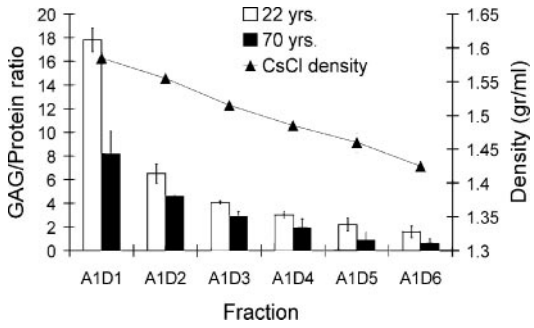

FIGURE 3. **Distribution of GAG to protein ratio (g/g) in a dissociative CsCl density gradient of PGs (A1 fraction) obtained from normal young (22 years) and old (70 years) human IVD.**

D-isomer with age in NP as compared with AF ($p > 0.05$) for both normal and degenerate tissue. For comparison, D/L_{Asp} ratios for representative samples of A1D1 obtained from normal tissue (mean values of normal NP and AF) are shown in the same figure. In contrast to the entire aggrecan (A1), no age-related change is observed for A1D1 after maturity. Since aggrecan is known to be heterogeneous with respect to molecular size and composition (23, 24), the observed age-related increase of D/L_{Asp} for A1 taken together with the constancy of the A1D1 values indicate that there is a differential turnover of the various aggrecan subfractions.

Fig. 2 shows the accumulation of D-Asp in A1 preparation obtained from NP of normal and degenerate human IVD as a function of donor age. Using two-factor ANOVA, a significant difference ($p < 0.05$) was observed between the mean values of D/L_{Asp} for normal and degenerate tissues averaged over all age groups. The measured values of D/L_{Asp} are consistently lower in degenerate aggrecan as compared with normal. For example, in the 30–40-year age group, accumulation of the D-isomer was nearly twice as high in the normal discs as compared with the degenerate discs. A significant difference in D-Asp accumulation between age groups (both normal and degenerate tissue) was noted *only* between 30–40 and 50–60 and between 30–40 and 60–80. Since differences in D-Asp accumulation are more marked between normal and degenerate tissue and less as a function of age, we conclude that the alterations occurring during aging are slow and statistically less significant than those occurring during degeneration. Similar results were obtained for AF (data not shown).

To examine the significance of the constancy of A1D1, the D/L_{Asp} ratio was determined for each of the different buoyant density fractions (A1D1–A1D6) isolated from the dissociative CsCl gradient of A1. It is well known that cartilage PGs distribute themselves within the CsCl density gradient according to their molecular composition, *i.e.* the higher the GAG content, the greater the density at which the PGs equilibrate (23). Fig. 3 shows the distribution of the GAG/protein ratio (w/w) of A1D1–A1D6, taken from normal NP of young (22 years) and old (70 years) individuals, in a dissociative CsCl density gradient at a starting density of 1.5 g/ml. Thus, the A1D1 fraction contains the highest content of GAG per weight of protein, whereas the intermediate fractions (A1D2–A1D6) represent mixtures of aggrecan of decreasing carbohydrate content. The A1D6 fraction contains mainly free G_1 domain and link protein. Moreover, the decrease in GAG content per weight of protein with age is also demonstrated. The chemical composition of A1D1 to A1D6 subfractions from a NP of a 62-year-old individual is shown in Table 1.

Fig. 4 shows a comparison between D/L_{Asp} of normal and degenerate A1D1–A1D6 for three different age groups: young normal, elderly normal, and elderly degenerate. Data presented are the average between NP and AF. The averaging procedure was necessary due to the small amounts of material available and was justified due to the fact that no significant difference was found between the values of D/L_{Asp} for NP as compared with AF (Fig. 1*A*, $p > 0.05$). Using two-factor ANOVA, a comparison of means between the different subfractions, revealed significant differences in D-Asp accumulation ($p < 0.05$) only between A1D1 and A1D3–A1D6. Significant difference was also found between young and elderly normal groups as well as between elderly degenerate and elderly normal groups. Accumulation of D-Asp in the elderly degenerate specimens is similar to that in the young normal age group, due to increased protein turnover in the degenerate tissue (Table 2).

As was found for the A1 preparation, an age-related increase in accumulation of the D-isomer is noted in A1D2–A1D6 subfractions (Fig. 4). It was found to be the highest in A1D6 from both normal and degenerate discs, as in the case of articular cartilage (12); this is also the case for A1D1–A1D4 from the *aggregating* PG fraction isolated by chromatography of the A1 preparations on Sepharose CL-2B (data not shown). The amount of D-isomer in A1D1 remained approximately unchanged with age (Fig. 1*A*), as in A1D1 obtained from femoral head and femoral condyle cartilage (15). An inverse correlation between GAG content and the extent of racemization is noted (Table 1 and Fig. 4). Assuming that the racemization rate is a constant for each subfraction, these results imply that the large heterogeneity in size and composition among these lower density fractions (A1D2–A1D6) is accompanied by a differential turnover. The maximum and age-independent turnover rate is inferred for the large monomer fraction, A1D1. As expected, the lowest values of D/L_{Asp} were observed for the young and normal discs (ages 22 and 24 years). In the case of the older specimens, lower accumulation was observed for the degenerate tissue as compared with the normal tissue (Fig. 4).

To learn more about the extent of modification of the aggrecan structure and accumulation of specific products, non-denaturing gel permeation chromatography (Sepharose CL-2B) was employed to separate the A1 preparations. Fig. 5 shows a typical elution profile (A1 prepared from a healthy 24-year-old donor) of three fractions representing aggregating PGs (*Peak* $A1V_o$o, fractions 33–50), large non-aggregating PGs (*Peak* $A1V_i1$, fractions 51–68), and small non-aggregating PGs (*Peak* $A1V_i2$, fractions 69–88). For the AF A1 preparations derived from 24- and 56-year-old individuals, the aggregating PGs accounted for about 42% of the total PGs. For the NP A1 preparations derived from the same individuals, the aggregating PGs accounted for 24 and 31% of the total PGs,

TABLE 1

Composition of aggrecan monomers of different buoyant density obtained from nucleus pulposus of a 62-year-old individual

Composition	A1D1	A1D2	A1D3	A1D4	A1D5	A1D6
Dry weight recovered (mg)	74.2	61.4	48.8	34.2	16	8.5
Protein (% of dry weight)	6.7	9.4	12.2	16.7	36.6	32.6
CS (% of dry weight)	47.03	36.6	19.9	14.2	12.14	7.3
CS/protein (dry weight ratio)	7.02	3.9	1.63	0.85	0.33	0.22
Total GAG (% of dry weight)	81.67	68.29	53.43	43.99	37.87	36.02
Buoyant density (g/ml)	>1.59	1.55–1.59	1.51–1.55	1.49–1.51	1.46–1.49	1.42–1.46

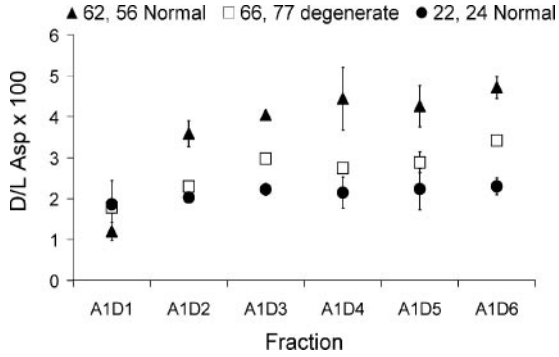

FIGURE 4. **D/L_{Asp} ratio for aggrecan subfractions (A1D1–A1D6) obtained from young normal and elderly normal and degenerate human IVD.** Data represent average values between AF and NP. Two-factor ANOVA analysis with post hoc Tukey test and interactions showed that the mean accumulation of D/L_{Asp} for the various subfractions was significantly different ($p < 0.05$) only between A1D1 and A1D3–A1D6. Significant difference in accumulation was found between young and elderly normal groups as well as between elderly degenerate and elderly normal groups.

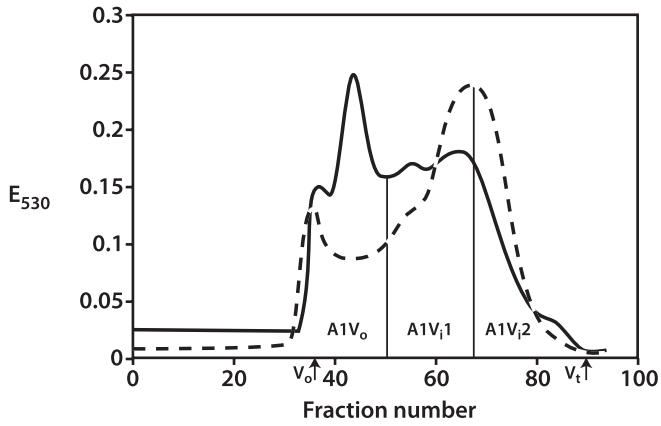

FIGURE 5. **Analysis of IVD A1 preparations by gel filtration chromatography.** A1 preparations from normal AF (*solid line*) and NP (*dashed line*) of a 24-year-old individual were analyzed by Sepharose CL-2B chromatography. PGs elution was assessed by the dimethylmethylene blue dye binding assay and monitored by absorbance at 530 nm. The void (V_o) and total (V_t) volumes of the column are indicated, together with the fractions that were pooled to yield A1V0, A1V$_i$1, and A1V$_i$2 preparations.

TABLE 2

Half-life values of A1-preparations obtained from NP and AF of normal and degenerate human intervertebral discs

Using the experimental D/L_{Asp} data and the racemization rate constant for aspartic acid, k_i, of 1.873×10^{-3} per year, previously found for articular cartilage, half-life values of aggrecan preparations were calculated (Equations 5 and 6 in "Supplemental Data"). The values of α represent the increase in protein with age as obtained from the linear fits in Fig. 7.

Fraction	α	k_T	Half-life
	year^{-1}	*year*$^{-1}$	*years*
NP (normal)	0.0047	0.059 ± 0.01	12.0 ± 2
AF (normal)	0.011	0.063 ± 0.01	11.23 ± 1.9
NP (degenerate)	0.063	0.084 ± 0.022	8.77 ± 2.2
AF (degenerate)	0.086	0.092 ± 0.034	8.41 ± 2.8

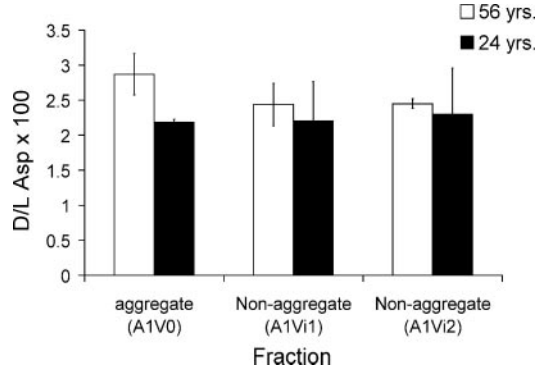

FIGURE 6. **D/L_{Asp} ratio for aggregating (A1V$_o$) and non-aggregating PGs (A1V$_i$1 and A1V$_i$2) obtained from normal young (24 years) and old (56 years) human IVD after gel filtration chromatography through Sepharose CL-2B.** A significant difference ($p < 0.012$) was observed only between aggregating and non-aggregating PGs of 56 years, using Student's *t* test.

respectively. Thus, the A1 preparations derived from the NP contain a lower proportion of aggregating PGs than those from the AF, suggesting a greater degree of proteolytic modification in the NP.

The above fractions were characterized with respect to their D/L_{Asp} content. The fact that no significant difference ($p > 0.05$) was observed between D/L_{Asp} ratios of aggregating (A1V$_0$) and non-aggregating fractions (A1V$_i$1 and A1V$_i$2), from the 24-year-old individual (Fig. 6), suggests that the non-aggregating PGs were synthesized at the same time as the aggregating PGs and that both PG types exhibit similar retention times in the IVD. The fact that the D/L_{Asp} ratio of the non-aggregating PGs is significantly lower ($p < 0.012$) than that of aggregating PGs in the 56-year-old individual implies that some of the non-aggregating PGs are preferentially lost, and it is most likely that these are the smaller fragments, with larger amounts of D-Asp. Throughout life, there is probably continual degradation of the non-aggregating PGs, and once they reach a critically small size, they will be lost from the disc by diffusion. This implies that the smallest non-aggregating molecules will be derived from the oldest aggrecan molecules and hence will be of the highest D-Asp content. The fact that similar D/L_{Asp} values were obtained for the non-aggregating pools of the 56-year-old individual suggests that a balance exists between the loss of small fragments from A1V$_i$2 and cleavage of the larger fragments from the A1V$_i$1 pool to smaller fragments present in A1V$_i$2.

Turnover of Aggrecan and Its Subfractions (A1D1–A1D6)—Despite the fact that the A1 preparations are very heterogeneous in nature (composed of aggregating and non-aggregating PGs), we nevertheless considered it useful to calculate the mean half-life values of the protein moiety in these preparations obtained from normal and degenerate specimens. Using the racemization rate constant of L- to D-conversion of aspartic acid (k_i) of 1.87×10^{-3} year^{-1} found previously for articular cartilage (15), and the D/L data obtained from the racemization measurements, we were able to calculate the rate constant for protein turnover (k_T) and the corresponding half-life for A1 preparations as well as for the aggrecan subfractions (A1D1–A1D6) as described in the Supplemental Data. Values for α, which relate to the increase in the amount of protein with age, are derived from Fig. 7. For A1 preparations taken

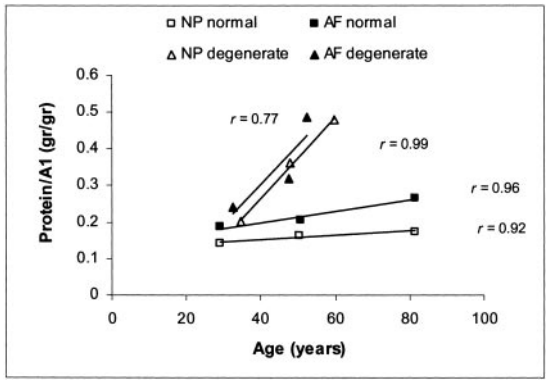

FIGURE 7. **Increase in protein content of aggrecan as a function of donor age.** The protein/A1 ratio was determined by dividing protein content by the dry weight of the total A1 preparation (g/g of dry weight of A1). Measurements were grouped as follows: young, middle-aged ,and elderly for normal and degenerate tissue. The slope/Y-intercept, α, is used to describe the increase in protein per year. Linear regression analysis was performed on the normal and degenerate NP and AF. No significant difference in protein increase with age is observed between AF and NP in both the normal and the degenerate tissue ($p < 0.05$).

from normal tissue, turnover rates of 0.059 ± 0.01 and 0.063 ± 0.01/year correspond to half-life values of 12 ± 2.0 and 11.23 ± 1.9 years for NP and AF, respectively; the turnover rates of 0.084 ± 0.022 and 0.092 ± 0.034/year for degenerate tissue correspond to half-life values of 8.77 ± 2.2 and 8.41 ± 2.8 years (Table 2), suggesting an increased rate of removal of small aggrecan fragments. The large scatter is probably due to the heterogeneity of the A1 preparations.

The protein turnover rate for aggrecan subfractions (A1D1–A1D6) from normal tissue was calculated after averaging NP and AF (Table 3). The mean value of the turnover rate constant (k_T) for the large monomer in fraction A1D1 is 0.13 ± 0.042/year, which corresponds to a half-life of 5.56 ± 1.58 years as compared with 3.4 years in human articular cartilage (15), whereas the turnover for the binding region (A1D6) is 21.53 ± 0.6 years as compared with 23.5 years previously found for articular cartilage (15).

DISCUSSION

In this research, an attempt was made to determine the turnover rates and resulting half-life values of different PG populations in human IVD by using the accumulation of the D-Asp isomer as a marker for residence time in the tissue. A rapid turnover of a given species in the tissue implies a short residence time and hence a possibility of renewal and repair of matrix components. A slow turnover, on the other hand, means that it is more difficult for cells to repair defects, which therefore may accumulate with aging. Determination of the residence time of a molecular species is one way to assign age-related changes in composition to either alteration of synthesis or degradation.

Aggrecan from human IVD exists in forms that vary with respect to the size and composition of the protein moiety as well as the size of the covalently bound carbohydrate polymers. This heterogeneity is very similar to that of aggrecan in articular cartilage (15). Many of these variations occur during maturation and degeneration of the tissue and give rise to a broad distribution of molecular sizes (25). A1 subfractions (A1D1–A1D6) isolated by means of dissociative CsCl gradient centrifugation show continuous changes from the fraction isolated at the highest buoyant density (A1D1) to that recovered from the lowest buoyant density (A1D6). These changes include: (*a*) an increase in the relative amount of protein; (*b*) a decrease in the relative amount of uronic acid and hence of CS; (*c*) a decrease in the relative amounts of GAG and hence in the sum of keratan sulfate and CS; (*d*) a decrease in the ratio of CS to protein.

TABLE 3

Half-life values of aggrecan monomers (A1D1-A1D6) taken from normal human IVD

Using the experimental D/L_{Asp} data and the racemization rate constant for aspartic acid, k_i, of 1.873×10^{-3} per year, previously found for articular cartilage, half lives of aggrecan monomers were calculated (Equations 5 and 6 in "Supplemental Data"). For A1D1, the value of α used, which relates to the increase of protein with age, is zero, whereas for A1D2-A1D6, α equals 0.008 $year^{-1}$ (this value is a mean of the slopes obtained for normal NP and AF, Table 2).

Aggrecan monomers	k_T	Half-life
	$year^{-1}$	*years*
A1D1	0.134 ± 0.042	5.56 ± 1.58
A1D2	0.054 ± 0.0002	12.85 ± 0.05
A1D3	0.042 ± 0.003	16.51 ± 1.20
A1D4	0.0427 ± 0.004	17.28 ± 1.14
A1D5	0.039 ± 0.0035	17.92 ± 1.29
A1D6	0.033 ± 0.0012	21.53 ± 0.6

A1 preparation, as a whole, shows an increase in the amount of protein with age. This is due to a very slow turnover of A1D6 (half-life of 21.53 ± 0.6 years) as compared with a relatively rapid turnover of the large monomer, A1D1 (half-life of 5.56 ± 1.58 years). It is therefore the small molecular species that are rich in protein that accumulate with age. These turnover products may be attached to hyaluronan or be non-aggregated.

For the aggrecan species in the A1D1 fraction, representing the GAG-rich intact monomer, the D/L_{Asp} ratio remains approximately constant throughout adult life; on the other hand, for the A1 preparation as a whole, there is an increase with age in the D/L_{Asp} ratio after maturity (>20 years) until a plateau is reached above 60 years of age. This implies that between 20 and 60 years, the rate of racemization is higher than the rate of turnover, whereas above 60 years of age, these processes reach equilibrium. There are two main metabolic processes that may account for why the content of D-Asp in disc PGs does not continue to increase after age 60. First, the rate of synthesis of new PGs (with low D-Asp) is unlikely to increase as disc PG content decreases with age. Second, the rate of loss of the older PGs (with high D-Asp) may well increase, which is likely as the proportion of non-aggregating PGs increases with age, suggesting ongoing catabolism (due to lower D-Asp value). It is the loss of these small fragments coupled with the inability of the disc to replace them by synthesis of new aggrecan that results in the age-related decrease in disc PG content. Catabolism may not only result in loss of the smallest non-aggregating PGs but also in the loss of some free G_1 and link protein due to hyaluronan depolymerization (26). Furthermore, due to differences between accumulation of the D-isomer in A1 and A1D1, we deduced that the A1 preparation must contain subpopulations with a lower turnover rate and hence longer residence time than that of the monomer in the A1D1 fraction. The data in Tables 1 and 3 confirm the existence of low buoyant density aggrecan subfractions with longer residence time in the tissue. It is for these fractions that the ratio D/L_{Asp} increases with age. It is apparent from Fig. 1 that the mean turnover rate constant for A1 is lower than for A1D1. Indeed, for A1D1, the turnover rate constant is found to be 0.134/year, which corresponds to a half-life of 5.56 ± 1.58 years, whereas for A1, a mean turnover rate constant (average of NP and AF) of 0.062 ± 0.01/year corresponds to a half-life of 11.62 ± 2.0 years. The finding that the turnover rate constant, k_T, was the same ($p > 0.05$) for the NP and AF in both normal and degenerate discs is surprising since these tissue zones have different cells, which produce different amounts as well as different morphologies of the matrix. We have no explanation for this finding. It is important to note that in all cases studied, D/L_{Asp} content was lower in aggrecan from degenerate tissue as compared with normal, *i.e.* degenerate aggrecan is "younger"; this is in accordance with previously published data for osteoarthritic articular cartilage (12). This suggests that higher levels of turnover and hence lower levels of accumulation are present in degenerate tissue.

It is important to note that the validity of the comparison between the molecular ages of different PG species from the determination of D/L_{Asp} was based on the implicit assumption that the racemization rate constant, k_i, of aspartyl and asparaginyl is the same throughout the protein core. This was indeed validated by the fact that there were only statistically minor differences between the D/L_{Asp} ratios of the A1D1 and A1D6 fractions of young subjects (22 and 24 years), in which age-related changes are small and would not have masked the differences in the intrinsic racemization rate constants, had these been present (Fig. 4).

An attempt was made to address the question regarding the origin of the small aggregating PGs as well as the non-aggregating PGs in human IVD, using the racemization of aspartic acid as a tool for determining *in vivo* turnover rates of different PG populations, in both normal and pathological tissues. Our finding that the non-aggregating PGs of a normal 24-year-old specimen have residence times that are similar to those of the aggregating PGs ($p > 0.05$, Fig. 6) and hence are of similar molecular age suggests that the non-aggregating PGs were synthesized at the same time as aggregating PGs. It is likely that the non-aggregating PGs were synthesized as aggregating PGs that were later cleaved by proteolysis. The PG fragments that were no longer bound to hyaluronan, but were retained within the disc, then formed the non-aggregating PGs (27). Such retention of non-aggregating fragments of aggrecan appears to be unique to the IVD and does not occur in articular cartilage. This probably reflects the large size and avascular nature of the disc, which prevents ready loss of large macromolecules by diffusion. The alternative explanation that the non-aggregating PGs were synthesized as such *de novo* is unlikely as there is no evidence to support the existence of such unique gene products.

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