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Collagen Turnover in Normal and Degenerate Human Intervertebral Discs as Determined by the Racemization of Aspartic Acid*

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Knowledge of rates of protein turnover is important for a quantitative understanding of tissue synthesis and catabolism. In this work, we have used the racemization of aspartic acid as a marker for the turnover of collagen obtained from healthy and pathological human intervertebral disc matrices. We measured the ratio of the D- and L-isomers in collagen extracted from these tissues as a function of age between 16 and 77 years. For collagen taken from healthy discs, the fractional increase of D-Asp was found to be 6.74×10^{-4}/year; for degenerate discs, the corresponding rate was 5.18×10^{-4}/year. Using the racemization rate found previously for the stable population of collagen molecules in dentin, we found that the rate of collagen turnover (k_T) in discs is not constant but rather a decreasing function of age. The average turnover rate in normal disc between the ages of 20 and 40 is 0.00728 ± 0.00275/year, and that between the ages of 50 and 80 is 0.00323 ± 0.000947/year, which correspond to average half-lives of 95 and 215 years, respectively. Turnover of collagen from degenerate discs may be more rapid than that found for normal discs; however, statistical analysis leaves this point uncertain. The finding of a similar correlation between the accumulation of D-Asp and that of pentosidine for three normal collagenous tissues further supports the idea that the accumulation of pentosidine in a particular tissue can, along with the racemization of aspartic acid, be used as a reliable measure of protein turnover.

The intervertebral disc (IVD),[2] the largest avascular cartilaginous structure (1, 2), plays a primary mechanical role in transmitting loads through the spine and providing flexibility to the spinal column. 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 degradative enzymes, including metalloproteinases and their inhibitors (TIMPs (tissue inhibitors of metalloproteinase)), which are all involved in tissue metabolism and turnover. Thus, normal disc function is dependent on a balance between synthesis and matrix breakdown.

The fine balance between synthesis and degradation determines the concentrations of tissue components and hence the composition of the tissue. *In vivo* (3, 4) and *in vitro* (5) environmental factors such as mechanical stress (6–8) and nutrient levels have been found to affect matrix composition, presumably by modulating rates of macromolecular biosynthesis and degradation. Normally, when a balance is maintained, damaged tissue can be restored by cellular repair responses; otherwise, the matrix composition and organization are altered, and the cellular repair responses become 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 the matrix occur. These processes include, for example, the degradation and formation of lower molecular weight species with different turnover rates, as in the case of aggrecan (9). Such changes in the "molecular age" of protein-containing molecules can, in principle, be probed by measuring the accumulation of the D-aspartic acid isomer and, using this value, calculating the residence time of these molecules. In nature, amino acids are synthesized as L-isomers. Spontaneous racemization slowly converts the L-form of amino acids via a non-enzymatic reaction or sequence of reactions into a racemic mixture of L- and D-forms. Aspartic acid is one of the most rapidly racemizing of the amino acids (10, 11), allowing the concentration of D-isomers to reach measurable levels in proteins that are not renewed or that slowly turn over in living subjects. It is well known that an age-dependent racemization occurs in various human and animal tissues containing metabolically stable, long-lived proteins (12–20). In this work, we assessed the turnover of collagen molecules in healthy and pathological human discs using the racemization of aspartic acid as a probe.

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[2] The abbreviations used are: IVD, intervertebral disc(s); NP, nucleus pulposus; AF, annulus fibrosus; HPLC, high performance liquid chromatography.

EXPERIMENTAL PROCEDURES

Tissue Sampling—Healthy (ages 22–62, $n = 9$) and pathological (ages 16–77, $n = 23$) discs from lumbar and thoracic spines were obtained post mortem or during routine surgical procedures for treatment of scoliosis, disc degeneration, or herniation. In this study, healthy discs will be referred to as "normal" and pathological ones (obtained from cases of scoliosis, IVD herniation, spinal canal stenosis, and fusion procedures) as "degenerate." No scoring of the tissue was performed. All discs were divided into nucleus pulposus (NP) and annulus fibrosus (AF) zones and stored at −20 °C until further analyzed.

Purification of Collagen—Collagen was purified by depleting the tissue of all the proteoglycans and non-collagenous protein using sequential enzymatic treatment with chondroitinase ABC (0.125 units/ml in 0.05 M Tris buffer containing 0.06 M sodium acetate, pH 8.0, for 24 h at 25 °C), *Streptomyces* hyaluronidase (1 unit/ml in 0.05 M Tris buffer containing 0.15 M NaCl, pH 6.0, for 24 h at 25 °C) and trypsin (1 mg/ml in 0.05 M $NaHPO_4$ containing 0.15 M NaCl, pH 7.2, for 16 h at 25 °C) as described previously by Schmidt *et al.* (21). Using this method, >97% of the non-collagenous molecules are removed from the tissue (17); this does not include elastin, which is 1.7 ± 0.2% of the total dry weight in human IVD (22). Collagen content on a dry tissue basis was determined by hydroxyproline analysis (23) of papain-digested tissue samples using a conversion factor of 7.6 (24).

Determination of Percentage D-Aspartic Acid—Determination of the D/L-Asp ratio was carried out using high performance liquid chromatography (HPLC) according to Verzijl *et al.* (16) and Aswad (25). 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. Correction was made by subtracting the D/L-Asp ratio measured for young bovine type II collagen.

Calculation of Rates of Protein Turnover—Accumulation of D-Asp depends upon competition between two linear processes. These are described by the rate constants of racemization (k_i) and protein turnover (k_T). When the amount of D-isomer is very small (D/L < 0.15), racemization may be considered irreversible. This is indeed the case in the tissues discussed here. The time rate of change of the amount of D-isomer in a protein can be written as in Equation 1 (26),

$$\mathrm{d}(C_{\mathrm{Asp}}(\mathrm{D})/(\mathrm{D}+\mathrm{L}))/\mathrm{d}t = k_i C_{\mathrm{Asp}}(\mathrm{L})/(\mathrm{D}+\mathrm{L}) - k_T C_{\mathrm{Asp}}(\mathrm{D})/(\mathrm{D}+\mathrm{L}) \quad \text{(Eq. 1)}$$

where C_{Asp} is the molar concentration of aspartic acid in the protein and D/(D + L) and L/(D + L) represent the fractions of the L- and D-isomers, respectively. Assuming that the content of the D-isomer is small enough that D/(D + L) ≈ (D/L) and L/(D + L) ≈ 1, which is the case *in vivo*, Equation 1 can be rewritten as in Equation 2,

$$\mathrm{d}(\mathrm{D}/\mathrm{L})/\mathrm{d}t = k_i - k_T(\mathrm{D}/\mathrm{L}) \quad \text{(Eq. 2)}$$

When the ratio D/L and/or the turnover rate constant k_T is very small, then the rate of change of D/L will be approximately linear with time. Then k_i can be approximated by the accumulation rate. When the amount of D/L becomes relatively large and/or the turnover rate is significant, the time rate of change of D/L will no longer be constant but rather will decrease with age. However, if the turnover rate itself is not constant but rather decreases, then the time rate of change of D/L need not decrease with age. If k_T can be determined, then the half-life ($t_{1/2}$) of the collagen molecules is calculated as in Equation 3,

$$t_{1/2} = \ln(2)/k_T \quad \text{(Eq. 3)}$$

Measurement of in Vitro Rate Constants for Aspartic Acid Racemization—This was based on the procedure by Ohtani (27). Dry collagen samples extracted from the NP (4–7 mg) were placed in glass tubes and heated at 120, 140, 160, or 180 °C for 5–90 min. Samples were digested in papain (5 units/ml in papain buffer, 100 μl/mg of collagen), and the racemization of aspartic acid (*i.e.* the D/L-Asp ratio) was determined after hydrolysis as described above.

Amino acid racemization *in vitro* follows first-order kinetics as described by Equation 4,

$$\ln((1+\mathrm{D}/\mathrm{L})/(1-\mathrm{D}/\mathrm{L}))_t = 2k_i^* \times t + \ln((1+\mathrm{D}/\mathrm{L})/(1-\mathrm{D}/\mathrm{L}))_{t=0} \quad \text{(Eq. 4)}$$

in which, as defined above, D/L is the ratio of D- to L-Asp, t is the time of heating, k_i^* is the *in vitro* rate constant for aspartic acid racemization, and the logarithmic term evaluated at $t = 0$ describes the amount of D-Asp isomer measured in unheated collagen. The Arrhenius equation was used to determine the activation energy for the racemization of aspartic acid residues. This equation gives a logarithmic relationship between the inverse temperature and the reaction rate (28) as shown in Equation 5,

$$\ln(k_i^*) = -E_a/RT + \text{constant} \quad \text{(Eq. 5)}$$

where E_a is the activation energy, R is the gas constant, and T is the absolute temperature. The racemization rate was evaluated at each temperature using Equation 1. Only data that showed a linear increase in ln((1 + D/L)/(1 − D/L)) with time and a correlation constant $r > 0.98$ were used. Values of $\ln(k_i^*)$ were plotted against the reciprocal of the absolute temperature. A linear fit to Equation 2 gives the activation energy as the slope.

Pentosidine and Amino Acid Contents—These were determined by HPLC as described previously (29, 30). Samples were hydrolyzed in 6 M HCl at 100 °C for 20–24 h and dried. Samples were then dissolved in an internal standard solution containing 10 μM pyridoxine and 2.4 mM homoarginine. For pentosidine analysis, samples were diluted 5-fold with 0.5% (v/v) heptafluorobutyric acid in 10% (v/v) acetonitrile and analyzed by HPLC. An aliquot of the samples used for pentosidine analysis was diluted 50-fold with 1 M sodium borate buffer, pH 11.4, and derivatized with 9-fluorenylmethyl chloroformate for HPLC amino acid analysis. The pentosidine content of the collagen samples was calculated based on hydroxyproline measurements (23, 24) and is expressed as μmol/mol of Lys based on 2×10^{-4} mol of Lys/g of collagen (18, 23).

Statistical Analysis—Linear regression analysis, two-factor analysis of variance, and Student's paired t tests were per-

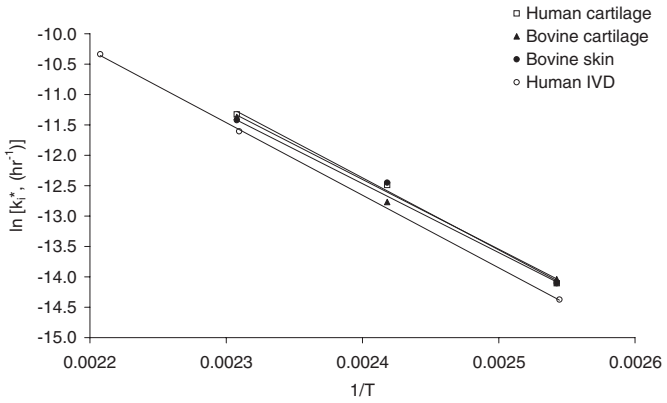

FIGURE 1. ***In vitro* rate constants for racemization of aspartic acid in collagen molecules as obtained from different tissues.** The Arrhenius plot shows the relation between the *in vitro* rate constant for racemization of aspartic acid (k_i*) and the reciprocal of the absolute temperature as measured for human (□) and bovine (▲) cartilage, bovine skin (●) (16), and human IVD (○) (this work). Data were obtained using heating experiments as described under "Experimental Procedures."

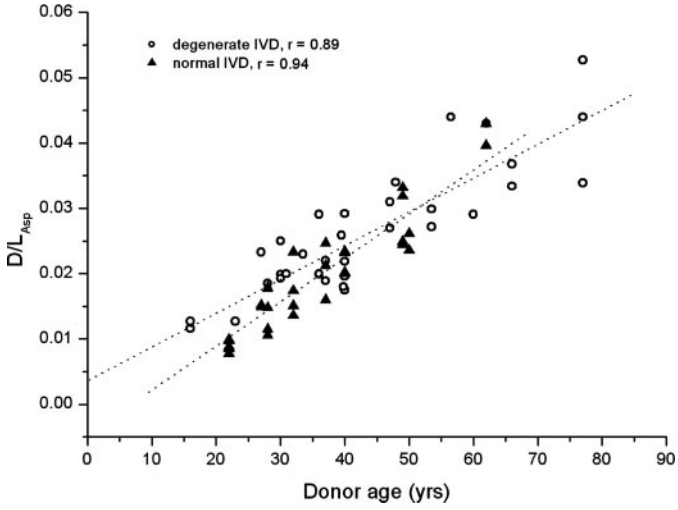

FIGURE 2. **D/L-Asp in collagen obtained from normal (▲) and degenerate (○) human IVD tissues as a function of donor age.** Because of an insignificant difference ($p > 0.05$, *t* test) between NP and AF of the same tissue, the data were pooled. The similar *r* values for the two data sets suggest that there is no additional contribution to the scatter of the degenerate data points due to the pooling of tissues with different pathologies.

formed; $p < 0.05$ was considered to represent a statistically significant difference.

RESULTS AND DISCUSSION

In Vitro Rate Constant of Aspartic Acid Racemization in Human Disc Collagen from NP—Maroudas *et al.* (31) suggested that the rate of racemization for collagen molecules may be *independent* of the tissue of origin. This idea was subsequently supported by heating experiments (16) showing that the *in vitro* activation energies (derived from an Arrhenius plot) for racemization are similar for skin (type I collagen) and cartilage (type II collagen). Our *in vitro* racemization data for collagen from the NP of normal IVD (mostly type II collagen) also are similar. The quantitative relation between the *in vitro* rate constants for racemization of aspartic acid (k_i*) and the reciprocal of the absolute temperature ($1/T$) according to the Arrhenius equation (Equation 5) is shown in Fig. 1. Also shown are data from human and bovine cartilage and bovine skin (16). To compare our values for the *in vitro* rate constant with those obtained earlier for skin and cartilage collagen (16), the linear curve in Fig. 1 was extrapolated to 33 °C. The rate constant obtained for disc collagen is 1.46×10^{-6}/year compared with 1.78×10^{-6}/year and 1.71×10^{-6}/year for skin and cartilage collagen, respectively (16).

It should be noted that measurements carried out at high, non-physiological temperatures for relatively short times (up to 90 min) monitor the thermally driven racemization of collagen with essentially no turnover at all. In this case, the accumulation rate and the racemization rate are the same. The fact that the activation energy is similar for skin, cartilage, and disc NP supports pooling tissue samples containing type I and II collagens (*e.g.* AF and NP in discs) for racemization measurements. The fact that the *in vitro* racemization rates are >2 orders of magnitude smaller than the *in vivo* accumulation rates (16) is likely due to the dry state of the tissue. These results further suggest that under the same environmental conditions, both types of collagen will undergo racemization at the same rate.

TABLE 1

Accumulation rates for the D-isomer of aspartic acid in collagen obtained from normal and degenerate human IVD calculated as described under "Experimental Procedures"

Values for dentin, articular cartilage, and skin are shown for comparison.

Fraction	Accumulation rate	Source
	$\times 10^{-4}$/year	
NP + AF		
Normal	6.74	This work
Degenerate	5.18	This work
Dentin	7.74–8.34	Refs. 9 and 32
Articular cartilage	2.58	Ref. 16
Skin	1.17	Ref. 16

In Vivo Accumulation of D-Asp in Collagen from Normal and Degenerate Discs—This (expressed as the ratio of D- to L-Asp) increased linearly with age in all collagen samples (Fig. 2). Because no significant difference was observed in the rate of D-isomer accumulation in NP compared with AF ($p > 0.05$), data were pooled for both normal and degenerate tissues. Fitting the increase of D-Asp as a function of age to a linear curve resulted in an accumulation rate of 6.74×10^{-4}/year for normal collagen and 5.18×10^{-4}/year for degenerate collagen. Using linear regression analysis, we found no statistical significance in the difference between these two rates. The accumulation rate of the merged data sets is 5.89×10^{-4}/year. There is no indication that the slope of the curve decreases with age for either the normal or degenerate samples. Table 1 shows a comparison of the accumulation rates in human IVD and collagenous tissues for which racemization has been studied (9, 16, 32). The different rates observed may be due to different values of k_i, k_T, or both. Except for dentin, normal collagen in IVD experiences the most rapid accumulation of D-Asp. In Fig. 3, data for D/L-Asp are also pooled by decade. Using two-factor analysis of variance on the histogrammed data in Fig. 3, a significant difference ($p < 0.05$) between the mean values of D/L-Asp in normal and degenerate tissues was found only above 60 years of age ($p > 0.05$). This difference may be due to the fact that fewer samples of

normal tissue up to age 62 were available, whereas samples of degenerate tissue up to age 77 could be analyzed.

Using the accumulation of the D-isomer of aspartic acid (Fig. 2) as a marker, we aimed to calculate the turnover rates of collagen molecules in normal and degenerate human discs. As shown in Equation 2, the amount of D-Asp will grow approximately linearly with age in stable populations of protein where the turnover is very slow, whereas when the turnover is more rapid, the rate of growth will decrease with time. A rapid turnover implies a short residence time and frequent population renewal, whereas a slow turnover implies a long residence time, suggesting that it is more difficult for cells to repair defects, which therefore may accumulate with aging. In proteins with very rapid turnover, such as hemoglobin, accumulation of D-Asp is negligible during the human lifetime (10).

How do the structural and chemical characteristics of the particular tissue influence the rate of racemization of aspartic acid in collagen? The process of racemization depends on time, temperature, and, less strongly, pH (11). Bada (33) derived an empirical equation for the temperature dependence of the racemization constant (Equation 6),

$$k_i(T) = k_i(310) \times \exp(-(16{,}890 \times (310 - T)/(T \times 310))) \quad \text{(Eq. 6)}$$

where the temperature is given in units of Kelvin. This equation predicts, for example, that the racemization constant at 33 °C would be approximately half as large as that at 37 °C. As far as pH is concerned, we are not aware of any systematic study of the dependence of k_i on pH. A low pH environment is known to promote the inversion of L- to D-aspartate (34, 35). The disc has a moderately low pH: it varies from 7.2 to 6.6, the lower limit of this range being found under conditions of degeneration (36). The carboxyl and sulfonic groups of the glycosaminoglycan molecules in the extracellular matrix contribute to the low pH environment of collagen. The relatively low pH in the disc is also due to the production of lactic acid as a by-product of metabolic processes (37). In addition, under conditions of low pH, protein synthesis is significantly reduced (37).

Of the five tissues listed in Table 1, collagen in dentin is considered to be the most stable during the human lifetime (11), *i.e.* turnover is negligible, and therefore the accumulation rate is assumed to be equal to k_i. This value of k_i is thus a characteristic of collagen molecules under the conditions prevailing at the tissue site. To estimate collagen turnover (k_T) as a function of age for normal and degenerate IVD, we make the assumption that the pH and temperature of the disc and tooth are the same. We used the value for k_i of dentin, *i.e.* 7.6×10^{-4}/year (11), in Equation 2 and solved for k_T. Values for D/L-Asp are taken from Fig. 1 and those for accumulation rates (d/d*t*(D/L-Asp)) from the linear fits in Fig. 1. The results are shown in Fig. 4 (*a* and *b*). If the temperature at the site of the dentin is lower than that at the disc, then the k_i value for dentin that we used in Equation 2 must be increased according to Equation 6. The smooth curves describe the expected values for k_T if the mean temperature at the site of the dentin is 1 °C lower

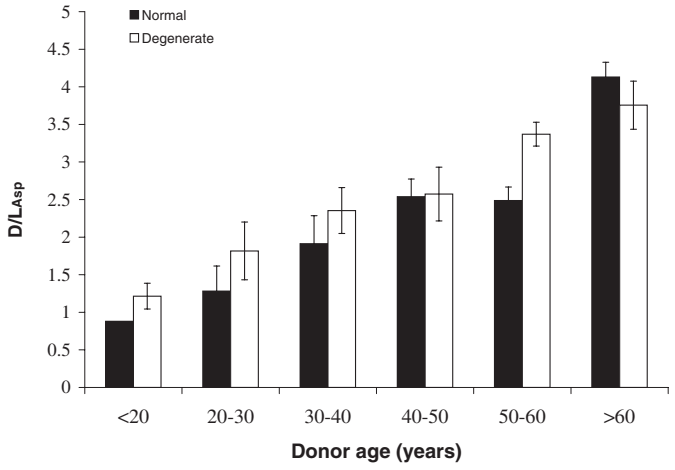

FIGURE 3. **Mean percentage D-Asp for collagen obtained from normal and degenerate human IVD with donor ages grouped according to decade.**

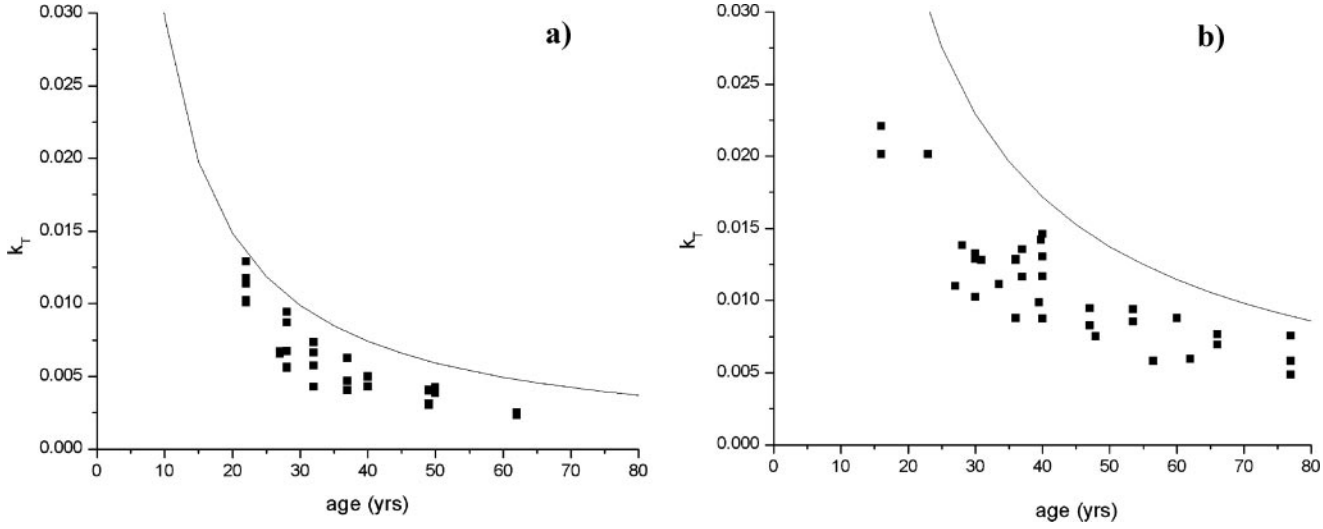

FIGURE 4. **Turnover rates for collagen from normal (*a*) and degenerate (*b*) human IVD as a function of donor age.** Calculations were based on Equation 2 using $k_i = 7.6 \times 10^{-4}$ for dentin (11). Values for D/L-Asp were taken from Fig. 1 and *d*/*d*t (D/L-Asp) from the linear fits in Fig. 1 (also listed in Table 1). The smooth curves describe the expected values for k_T if the mean temperature at the site of the dentin is 1 °C lower than that of the IVD, therefore requiring that k_i be corrected according to Equation 6.

than that of the IVD. We found that k_T is a decreasing function of age and is consistently higher for degenerate IVD compared with normal IVD. For normal IVD, the average turnover rate between ages 20 and 40 is 0.00728 ± 0.00275/year, and that between ages 50 and 80 is 0.00323 ± 0.000947/year, which correspond to half-lives of 95 and 215 years, respectively. For comparison, in human IVD, Antoniou *et al.* (37) found that between the ages of 15 and 40, there is a progressive reduction in the synthesis of type II procollagen and a small reduction in the prevalence of degradation products of collagen. Between the ages of 40 and 60, collagen synthesis continues to decrease. Similar behavior is observed in the oldest age group (60–80 years), except for a weak increase in synthesis of type I collagen (37). It therefore appears that the rate of collagen turnover in human IVD, as determined here, and collagen synthesis in the disc as determined by Antoniou *et al.* (37) behave qualitatively in a similar manner, as would be expected. For degenerate IVD, the average turnover rate between ages 20 and 40 is 0.01232 ± 0.00247/year, and that between ages 50 and 80 is 0.00729 ± 0.00152/year, which correspond to half-lives of 56 and 95 years, respectively. When considering these results, however, we must recall that the D-Asp accumulation rates for normal and degenerate IVD are not different on a statistical basis, and therefore, the possibility of more rapid turnover in degenerate IVD remains uncertain. Compared with collagen, IVD aggrecan displays a much shorter half-life; there are also significant differences between normal and degenerate tissues. The mean value of the half-life of aggrecan from normal nucleus and annulus is 12.7 years compared with 8.7 years for degenerate tissue (9). This is probably due to the fact that aggrecan is more susceptible to proteolysis because of its less compact structure. Small degraded fragments are produced that are then able to diffuse out of the disc and are subsequently replaced by intact molecules.

Using the racemization of aspartic acid as a marker, Verzijl *et al.* (16) found that between ages 3 and 81, the mean half-life of collagen in articular cartilage is 117 years, which corresponds to a turnover rate of 0.0059/year. Verzijl *et al.* used a racemization rate for dentin of 3×10^{-4}, which is close to what would be obtained from Equation 6, for a temperature of 33 °C at the knee joint. To compare this result for the mean half-life of collagen in articular cartilage with the value for IVD, we used a racemization rate of 5.89×10^{-4}/year, determined, as described above, from the merged data sets for normal and degenerate tissues (Fig. 1). We calculated k_T as a function of age with 7.6×10^{-4}/year as the racemization rate (as above). We obtained a mean turnover rate of collagen of 0.0097/year between the ages of 17 and 77. This corresponds to a mean half-life of 72 years.

In addition to racemization, other changes occur in the structure and composition of human cartilaginous tissues during aging and degeneration. These include accumulation of the advanced glycation end product pentosidine in long-lived proteins (9). It was suggested previously that protein turnover is an important determinant of pentosidine accumulation (16). To support this idea, a correlation was demonstrated between the accumulation of D-Asp and that of pentosidine for both articular cartilage and skin collagen. Using our data from previous work showing that pentosidine increases linearly with age (38), we observed approximately the same correlation in the case of collagen from normal IVD (Fig. 5). The finding of a similar correlation for three normal collagenous tissues further supports the idea that the accumulation of pentosidine in a particular tissue can, along with the racemization of aspartic acid, be used as a reliable measure of local protein turnover.

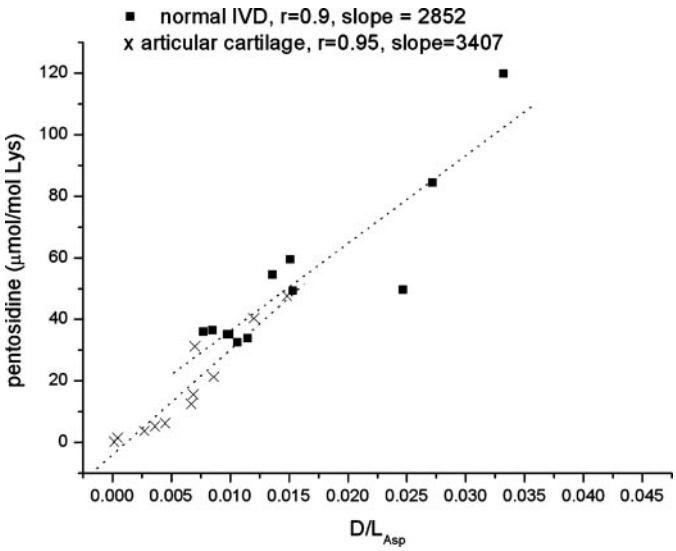

FIGURE 5. **Pentosidine accumulation as a function of D/L-Asp for collagen from normal human IVD compared with normal articular cartilage (taken from Ref. 16).**

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Collagen Turnover in Normal and Degenerate Human Intervertebral Discs as Determined by the Racemization of Aspartic Acid

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