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Aspartic Acid Racemization in Intervertebral Discs As an Aid to Postmortem Estimation of Age at Death

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ABSTRACT: We investigated whether measurement of aspartic acid racemization in intervertebral discs (IVD) could be used in the postmortem estimation of age at death. The extent of aspartic acid racemization in IVD tissues was found to increase with age. The rate of racemization turned out to be much higher in the nucleus pulposus than in the annulus fibrosus. The relation between age and the D-aspartic acid content in the anterior peripheral annulus fibrosus of IVD was close enough to allow postmortem estimation of age at death based on the extent of aspartic acid racemization in this tissue.

KEYWORDS: forensic science, aspartic acid racemization in intervertebral discs, postmortem estimation of age, age determination

Helfman and Bada [*1,2*] established that during aging a gradual transformation of L-aspartic acid into its D-form (racemization) occurs in tooth enamel and dentin. This produces a measurable, age-dependent increase in the D-aspartic acid concentration in these tissues.

Prompted by these findings, several other groups investigated whether determination of aspartic acid racemization in dentin can be used as an aid to postmortem estimation of age at death [*3–6*]. Their results indicate that this method is superior to most other procedures with regard to accuracy and reproducibility.

Because teeth are not always available to the forensic practitioner, we sought other tissues suitable for estimation of age on the basis of aspartic acid racemization.

Measurable in vivo racemization has been described not only in dentin and tooth enamel [*1,2*], but also in other human and animal tissues containing metabolically stable proteins, for example in the human lens and white matter of the brain [*7–13*].

Age can be determined by measuring the racemization of aspartic acid if the tissue analyzed—dentin for example—contains a sufficiently high concentration of protein that was synthesized early in life and not subsequently exchanged. Such metabolically stable proteins, whose age corresponds roughly to the age of the organism, occur primarily in highly bradytrophic tissues with a low rate of protein turnover.

Intervertebral disc (IVD) tissue is highly bradytrophic. It is avascular and nourished by diffusion. Only in early childhood does the annulus fibrosus possess blood vessels; these however are soon obliterated. The nucleus pulposus is free of vessels for the life of the organism [*14–19*]. Hence, it seemed likely that IVD would contain proteins that remain unchanged from the time of their synthesis until death and that "age" along with

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the organism. We therefore investigated whether measurement of aspartic acid racemization in IVD could be used in the postmortem determination of age at death.

Materials and Methods

Preparation of Tissue Specimens and Measurements

The IVD L4/5 was resected at autopsy from a total of 78 individuals aged 10 to 95 years. Sixty-eight of the IVD showed *no or moderate degeneration*, while 10 exhibited *marked degeneration*. An IVD was considered to be *markedly degenerated* if it had fractures in the annulus fibrosus or if deposits of newly synthesized connective tissue and/or osteophytes were present. Postmortem intervals were between a few hours and 4 weeks in length.

Tissue specimens from both the annulus fibrosus and the nucleus pulposus were investigated.

Seventy-eight annulus fibrosus specimens (68 from IVD with no or moderate degeneration, 10 from IVD with marked degeneration) weighing approximately 250 mg each (wet weight) were taken from the anterior peripheral annulus fibrosus at a point located midway between both end plates and 0.4 to 0.8 cm beneath the anterior longitudinal ligament (layer A, Fig. 1).

Nineteen nucleus pulposus specimens (all from IVD with no or moderate degeneration) weighing 250 mg each were resected from the center of the nucleus pulposus (layer D, Fig. 1).

In five IVD, specimens were taken from six different layers lying between the anterior peripheral annulus fibrosus (layer A, Fig. 1) and the posterior peripheral annulus fibrosus (layer F, Fig. 1).

All tissue specimens were washed ultrasonically in distilled water and hydrolyzed in 6 N HCl for 6 h at 100°C. Hydrochloric acid and water were removed in a vacuum. The residue was esterified with isopropanol/sulfuric acid (10:1) for 1 h at 110°C. After removal of the isopropanol by a stream of air, 2 N ammonium hydroxide was added. The samples were alkaline extracted with dichloromethane and dried again. Acetylation was performed with trifluoracetic anhydride (TFA) at 60°C for 15 min. The amino acids were now present

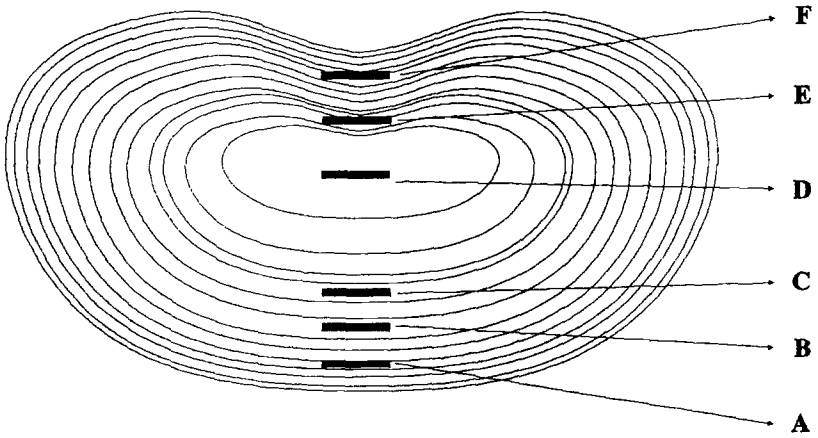

FIG. 1—*Schematic representation of the positions of the 6 layers (A-F) investigated in 5 IVD. A = anterior peripheral annulus fibrosus; D = central portion of the nucleus pulposus; F = posterior peripheral annulus fibrosus.*

as TFA-isopropylesters and could be separated and quantified by gas chromatography on a chiral capillary column (Chirasil-Val) using a flame ionization detector and with hydrogen as carrier gas.

Evaluation

The process of aspartic acid racemization can be described as [*20–22*]:

$$\ln \frac{(1 + D/L)}{(1 - D/L)} = 2\ k(\text{Asp.})\ t + \text{constant}$$

where *D/L* represents the proportion of D-aspartic acid to L-aspartic acid, *k(Asp.)* is the first-order rate constant of the interconversion of enantiomers, and *t* equals time.

The value ln ((1 + D/L)/(1 − D/L)) was calculated for each tissue sample. The relation between age and the racemization of aspartic acid (ln ((1 + D/L)/(1 − D/L))) in IVD with *no or moderate degeneration* was evaluated by linear regression analysis. The values for *markedly degenerated* IVD were interpreted separately.

Results

IVD with no or Moderate Degeneration

Figure 2 shows the extent of aspartic acid racemization in the *anterior peripheral annulus fibrosus* in relation to age in 68 IVD with no or moderate degeneration. The relation between age (*t*) and the extent of racemization (ln ((1 + D/L)/(1 − D/L))) can be represented as (equation (1)):

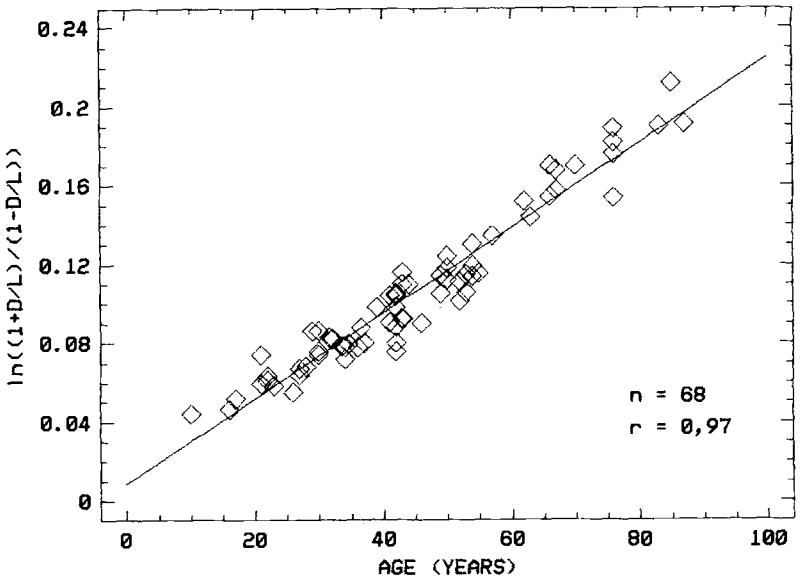

FIG. 2—*Extent of aspartic acid racemization (ln((1 + D/L)/(1 − D/L))) in the anterior peripheral annulus fibrosus in relation to age in 68 IVD with no or moderate degeneration; the calculated linear regression line is shown.*

$$\ln \frac{(1 + D/L)}{(1 - D/L)} = 0.002\ 16\ t + 0.008\ 72 \quad (1)$$

The correlation between the extent of aspartic acid racemization in the anterior peripheral annulus fibrosus and age was close ($r = 0.97$); estimation of age employing Eq 1 has a standard error of estimation of 4.6 years.

The nucleus pulposus displayed a clearly weaker correlation between age and the extent of aspartic acid racemization ($r = 0.89$) (Fig. 3). The relation between age (t) and the extent of aspartic acid racemization (ln ((1 + D/L)/(1 − D/L))) in the nucleus pulposus can be described as (equation (2)):

$$\ln \frac{(1 + D/L)}{(1 - D/L)} = 0.004\ 74\ t + (-0.015\ 39) \quad (2)$$

In five instances, *specimens from six different IVD layers* (layers A to F, Fig. 1) were studied. Figure 4 gives the values for the aspartic acid racemization in layers A to F of one of these IVD (investigation of layers A to F in the four other IVD produced similar results). These analyses revealed that the D-aspartic acid concentration in IVD tissue increases from the periphery inward. Hence, the nucleus pulposus (layer D) had distinctly higher values than the annulus fibrosus; the anterior and posterior peripheral segments of the annulus fibrosus (layers A and F, Fig. 1) had approximately equal D-aspartic acid concentrations.

IVD with Marked Degeneration

In Table 1, the extent of aspartic acid racemization in the annulus fibrosus of 10 IVD with *marked* degeneration is compared with the corresponding "normal values" for the

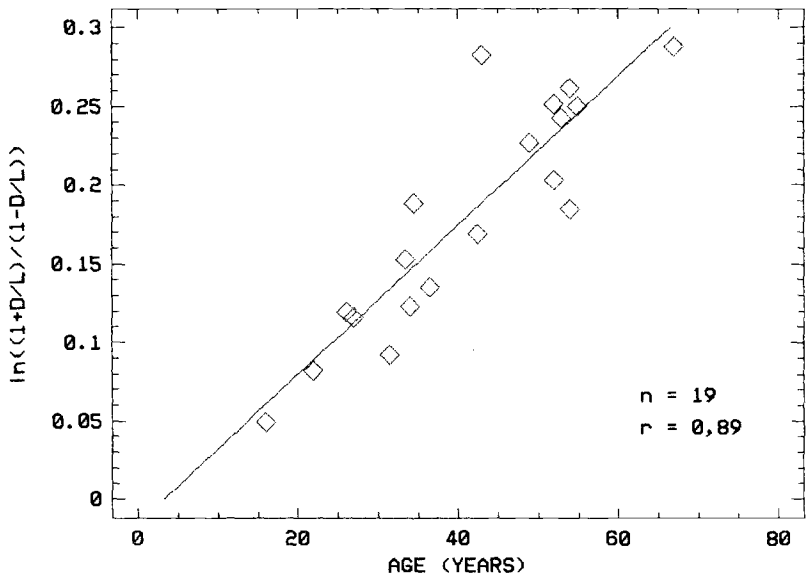

FIG. 3—*Extent of aspartic acid racemization (ln((1 + D/L)/(1 − D/L))) in the nucleus pulposus in relation to age in 19 IVD with no or moderate degeneration; the line represents the calculated linear regression line.*

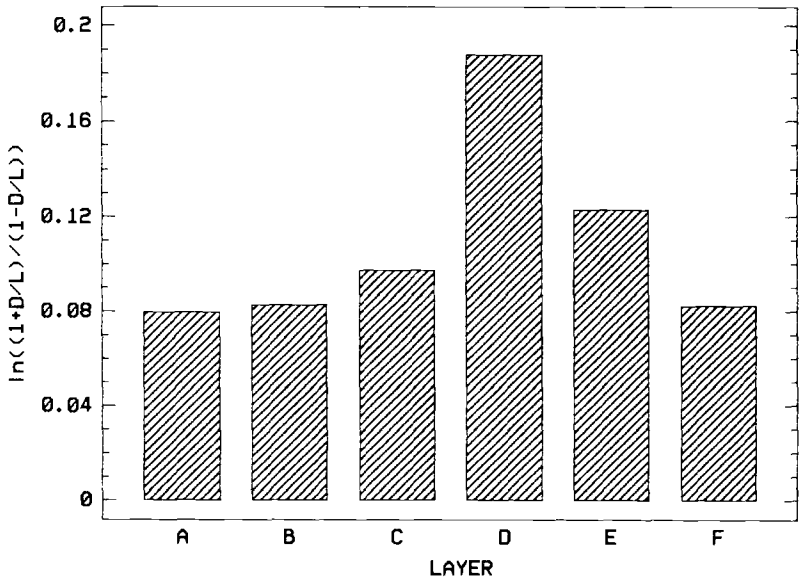

FIG. 4—*Extent of aspartic acid racemization (ln((1 + D/L)/(1 − D/L))) in 6 different layers (A-F) of one IVD. The schematic representation of the 6 layers lying between the anterior peripheral annulus fibrosus (A) and posterior peripheral annulus fibrosus (F) is shown in Fig. 1.*

TABLE 1—*Extent of aspartic acid racemization in the anterior peripheral annulus fibrosus in 10 IVD with marked degeneration (actual values) compared with the "normal values" for the same age in IVD with no or moderate degeneration. The "normal values" were calculated using equation (1).*

Intervertebral discs with marked degeneration		
Age (years)	Extent of racemization (annulus fibrosus) (ln((1 + D/L)/(1 − D/L))	
	Actual values	"Normal values"
65	0.1021	0.1491
68	0.1432	0.1556
71	0.1201	0.1621
75	0.1650	0.1707
76	0.1724	0.1729
76	0.1301	0.1729
81	0.1107	0.1837
83	0.1785	0.1880
83	0.1823	0.1880
95	0.1523	0.2139

same age in IVD with no or moderate degeneration ("normal values" were obtained using Eq 1). Several of the IVD with marked degeneration were found to have D-aspartic acid concentrations in the annulus fibrosus far below the corresponding "normal values."

Discussion

The extent of aspartic acid racemization in IVD was found to increase with age (Figs. 2 and 3). This confirms that IVD contain metabolically stable proteins, in which a measurable in vivo transformation of L-aspartic acid into the D-form takes place. The extent of aspartic acid racemization is a measure of the age of these proteins and hence indirectly of the age of the organism.

The process of aspartic acid racemization in the annulus fibrosus and nucleus pulposus in IVD with no or moderate degeneration can be described by Eqs 1 and 2 given above. According to these equations racemization of aspartic acid proceeds at a faster rate in the nucleus pulposus than in the annulus fibrosus. The D-aspartic acid concentration within a single IVD was found to increase from the periphery inward (Fig. 4).

The rate of amino acid racemization depends not only on the ambient temperature, which in the human organism is a relatively constant 37°C, but also on the "biochemical environment" of the amino acids [*22,23*]. For dentin and human lens it has been shown that the transformation of L-aspartic acid into its D-form proceeds at different rates in different protein fractions [*4,5,9,12,24,25*]. Although both the annulus fibrosus and nucleus pulposus of IVD consist chiefly of collagen fibers and small cell populations lying in a "gel" of proteoglycan and water [*26*], the arrangement and concentration of these components are dissimilar in these two tissues. Hence, the annulus fibrosus and nucleus pulposus differ not only structurally but also in their biochemical composition [*15,18,19,27*]. It can be assumed that differences in the extent of aspartic acid racemization in the layers between the annulus fibrosus and nucleus pulposus are due to differences in the "biochemical environment" of the amino acids.

In the nucleus pulposus the correlation between age and the extent of aspartic acid racemization was much weaker than in annulus fibrosus specimens taken from a IVD with no or moderate degeneration (Figs. 2 and 3).

Care was taken to obtain the nucleus pulposus specimens from a site as near as possible to the center of the IVD. But this was often difficult—especially in older IVD—because the transition from the annulus fibrosus to the nucleus pulposus is not clearly demarcated. Moreover, the considerable mechanical stresses placed on *nucleus pulposus* tissue have been reported to induce structural and biochemical changes even in early adulthood [*15,18*]. This could account for the comparably weak correlation between age and the extent of aspartic acid racemization in the nucleus pulposus.

Stress-induced transformation processes probably also produce structural and biochemical changes in the annulus fibrosus during the course of life [*14,15,18,28,29*]. In old age an invasion by blood vessels can occur with the formation of new (young!) connective tissue in IVD with *marked* degeneration. This is especially likely if radial fractures in the annulus fibrosus extend to a longitudinal ligament [*14,19,28*].

Some of the IVD with *marked degeneration* had D-aspartic acid levels in the anterior peripheral annulus fibrosus that were clearly below "normal values" in IVD of the same age with *no or moderate degeneration* (Table 1). The relatively low D-aspartic acid content in these IVD was primarily due to a "contamination" of the specimens by recently synthesized proteins in newly formed connective tissue.

Our findings permit the following conclusions:

1. The correlation between age and the D-aspartic acid content in the anterior peripheral annulus fibrosus of IVD *with no or moderate degeneration* was close enough to

permit estimation of age at death based on the extent of aspartic acid racemization in this tissue.

The accuracy of the age estimate could be increased by the simultaneous analysis of specimens from *several* IVD. Further studies must determine whether the equation calculated for specimens from IVD L4/5 (Eq 1) can also be applied to tissues from other IVD.

2. If an estimation of age is attempted on the basis of the aspartic acid racemization in the anterior peripheral annulus fibrosus, the following points must be considered:

Analysis of tissue specimens from IVD with *marked degeneration* can produce false low estimations of age. In our experience, such marked degeneration could be diagnosed macroscopically (presence of osteophytes, fractures in the annulus fibrosus, formation of scar tissue).

Specimens must be taken from a *specified* site, since D-aspartic acid levels within an IVD increase from the periphery inward.

If an extensive structural loss of the IVD has resulted due to advanced *postmortem decomposition*, it is not possible to take a specimen from a specified site; an estimation of age in such cases is unreliable. However, so long as IVD macrostructure is maintained, the method described here can be applied even in cases of long postmortem intervals, because *postmortem* even over several years at normal ambient temperatures no considerable racemization occurs [*3*].

3. Estimation of age at death based on the racemization of aspartic acid is much more precise in dentin than in IVD [*3–6*]. Nevertheless, determination of the extent of aspartic acid racemization in the anterior peripheral annulus fibrosus of IVD can aid in postmortem estimation of age in those cases where other methods cannot be applied (for example, when only certain body parts are available). Thus the method described here represents a useful supplement to established procedures for postmortem determination of age at death.

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