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Estimation of chronologic age using the aspartic acid racemization method. I. On human rib cartilage

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Abstract Determinations of chronologic age are of great importance in forensic science. At present the aspartic acid racemization method on teeth provides one of the best means in adult individuals. However, if teeth are not available, some other stable tissue has to be used. In this study, the applicability of cartilage from the ribs has been tested. Specimens of rib cartilage were obtained at autopsy from 24 individuals aged 0.2–96 years. An acid-soluble peptide (SP) fraction and an insoluble collagen-rich (IC) fraction were prepared, and the ratio of D/L aspartic acid was determined using the HPLC technique. The correlation coefficient between the D/L ratio and age was r = 0.91 in SP and r = 0.97 in IC. It thus seems as if cartilage from non-weight-bearing areas may be a useful source of tissue for the estimation of chronologic age with the aspartic acid racemization method when teeth are not available.

Key words Age determination · Cartilage · Aspartic acid · High pressure liquid chromatography

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Introduction

A large number of studies have demonstrated that the chronologic age of an individual can be estimated with reasonably good precision by analysis of the degree of racemization of aspartic acid in dental enamel or dentine [1–17]. Studies of dentine have usually demonstrated a high correlation (r up to 0.99) between the D/L ratio of aspartic acid and the chronologic age of the tooth. There is also a close relationship between age and D-aspartic acid accumulation in the white matter of the brain [18], the eye lens [19], elastic fibres of the lung parenchyma [20] and bone [21, 22].

The theory behind this method is that amino acids incorporated into peptides and proteins exist in their L-forms immediately after formation, but then slowly transform into a racemic mixture of L- and D-forms if the tissue is not subjected to physiological renewal. The rate of transformation is about 0.1% per year in stable tissues such as dentine.

There has been controversy about the rate of component turnover in cartilage, but it is now generally agreed that the mean half-life of proteoglycans is 1–2 years, and that there may be pools which turn over more slowly [23]. On the other hand, it has for a long time been believed that collagen has practically no turnover in the adult human cartilage, but recently a very slow turnover has been demonstrated [24]. However, it is questionable if this newly-formed collagen becomes incorporated into the collagenous framework [25].

Ritz and Schütz [26] studied tissues from the nucleus pulposus and different parts of the annulus fibrosus of the intervertebral discs, and found a higher degree of racemization of aspartic acid in levels deeper than superficial. The best relationship to age was found in the anterior peripheral annulus fibrosus (r = 0.97). The test samples consisted of native tissue without separation of tissue compartments. Maroudas et al. [25] studied native cartilage as well as cartilage that had been depleted of proteoglycans. The ratio of D/L aspartic acid was found to be more than twice as high in native as in proteoglycan-depleted tis-

sues. Ritz and Schütz [26] and Maroudas et al. [25] reported lower ratios in diseases rather than normal cartilage.

Since the studies by Maroudas et al. [25] on cartilage and an earlier study by Ohtani and Yamamoto [9] on dentine have shown that there are differences in racemization rates in various tissue fractions, and since Maroudas et al. [25] examined only 3 samples, this study was designed to explore differences in racemization rates in an acid-soluble peptide fraction and an insoluble collagen-rich fraction of cartilage. A further aim was to study cartilage from ribs, which can be regarded as being less subjected to physical stress and pathological changes than the tissues studied previously.

Materials and methods

Material

Samples of cartilage from the fourth rib (junction between the sternum and bony part of the rib) were taken from 24 individuals aged 0.2–96.0 years (mean 48.9 ± 26.2 years) of both sexes (12 males and 12 females) at autopsy. After a brief fixation overnight in 10% neutral formalin at 4° C, the samples were excised from bone and connective tissue, and small pieces from the central area were selected. The samples were washed in distilled water, briefly dried in air an kept at +4° C until used within one week. The tissue was subsequently cut into small pieces and ground to a fine powder in a procelain mortar.

Analytical procedures

The powder from each individual was divided into 10 mg aliquots and an acid-soluble peptide fraction (SP) was extracted in 1 mL 1 *M* HCL under centrifugation at 5000 g at 4° C for 1 h. The supernatant was removed and dried under reduced pressure. The remaining pellet was used as an insoluble collagen-rich fraction (IC). Both fractions were resuspended in 1 mL 6 *M* HCl and hydrolysed. The samples were placed in hydrolysis tubes, washed several times with argon, evacuated, sealed and heated to 100° C for 6 h. After cooling, the hydrolysate was evaporated to dryness under reduced pressure and finally redissolved in 1 mL 0.06 *M* HCl. To remove particulate matter and inorganic salts, the sample was passed through a BIO-RAD AG 50W-X8 (100–200 mesh) cation exchange column. The column was washed with 6 mL water, after which the amino acids were eluted between 2 and 3.5 mL 8 *M* NH_4OH. The eluate was evaporated to dryness under reduced pressure, and the residue was finally redissolved in 0.5 (SP) or 1 mL (IC) of water. A 100 µL aliquot of each sample was derivatized with a chiral fluorogen according to Aswad [27]. Derivatization was accomplished by thoroughly mixing the sample with 5 µL of OPA-NAC (o-phthaldialdehyde adducted with N-acetyl-L-cysteine) reagent in a polyethylene microfuge tube. After 2.5 min, 475 µL o 50 m*M* sodium acetate (pH 5.2) was added and 25–100 µL of this solution was taken for direct injection into the HPLC system.

HPLC conditions

A Waters HPLC system (Millipore, Milford, Mass.) consisting of 2 model 510 pumps and one model 470 scanning fluorescence detector was used throughout the study. The diastereomeric dipeptides were separated on a reversed phase HPLC column, Kromasil C8 (25 cm and 4.6 mm i.d.; Eka Nobel AB, Bohus, Sweden), placed after a guard column. Isocratic eluation with 90% solvent A and 10% solvent B was carried out for 5 min. Solvent B was then increased linearly to 100% over a period of 5 min. Solvent A was 50 m*M* sodium acetate (pH 5.9) and solvent B was 80% (v/v) methanol and 20% (v/v) solvent A. The flow rate was 1.0 mL/min throughout. The excitation wavelength was set to 340 nm, and the emission wavelength to 420 nm. The data from the detector was collected and processed by Waters' Baseline system (Millipore, Milford, Mass.).

Regression model

The ratio of D- and L-aspartic acid was calculated from the areas under the eluted peaks (mV*s). The racemization of amino acids follows a first-order reversible rate law, where the racemization equation is

$$\ln[(1 + D/L)/(1 - D/L)]_t - \ln[(1 + D/L)/(1 - D/L)]_{t=0} = 2k_t * t$$

A linear regression model was constructed using this expression, where D/L is the ratio of D- and L-aspartic acids, t is any given time during racemization and the logarithmic term at $t = 0$ describes the amount of D-aspartic acid formed during hydrolysis.

Results

Figure 1 shows a representativ chromatogram of hydrolysates of both the acid-soluble peptide fraction (SP) and the insoluble collagen-rich fraction (IC). D- and L-aspartic acid peaks were distinctly separated between 7.5 and 10 min, and the remaining compounds were eluted between 13 and 20 min.

Plots of $\ln[(1 + D/L)/(1 - D/L)]$ of aspartic acid against ages of the individuals are shown in Figs. 2 and 3. The expressions obtained by the linear regression with the ratio of D- and L-forms as the dependent variable were as follows:

The acid-soluble peptide fraction:

$\ln[(1 + D/L)/(1 - D/L)] = 0.0123 + 0.0031 * t.$
r = 0.91, std err est = 0.037165, $n = 23$
(the oldest individual considered as outlier)

The acid-insoluble collagen-rich fraction:

$\ln[(1 + D/L)/(1 - D/L)] = -0.012 + 0.0033 * t.$
r = 0,97, std err est = 0.024149, $n = 24$

To calculate the age of an individual from the insoluble collagen-rich fraction of rib cartilage from the D/L ratio, the following equation was derived:

$AGE = \ln[(1 + D/L)/(1 - D/L)] * 282.78 + 6.72$
std err est = 7.07.

Discussion

Age determination of teeth with the aid of the degree of racemization of aspartic acid has been shown to be valuable for the estimation of chronologic age of individuals with unknown birth records [3–7]. A series of studies over the last 2 decades [1–17] has shown that both the precision and the reliability of the method are high. Since teeth are not always available in practical forensic cases, it

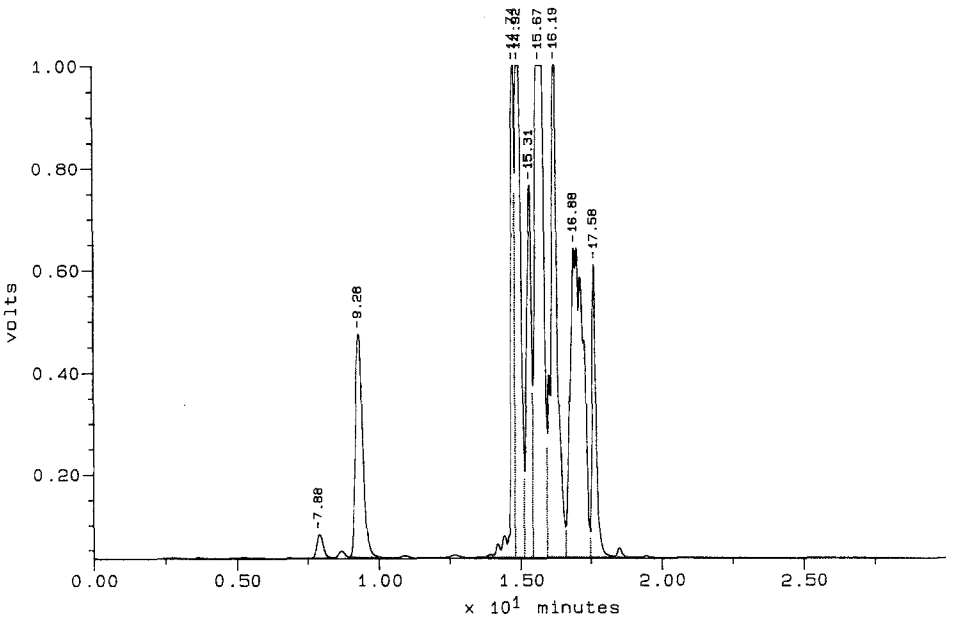

Fig. 1 Reversed phase HPLC separation of OPA-NAC derivatives of D- and L-aspartic acid in the acid soluble peptide fraction of cartilage from the rib, at 7.88 and 9.28 min, respectively. The peaks between 13 and 17 min contain the rest of the injected material. Isocratic eluation with 90% solvent A and 10% solvent B was carried out for 5 min. The content of solvent B was then increased linearly to 100% over a 5 min period. The flow rate was 1 ml/min

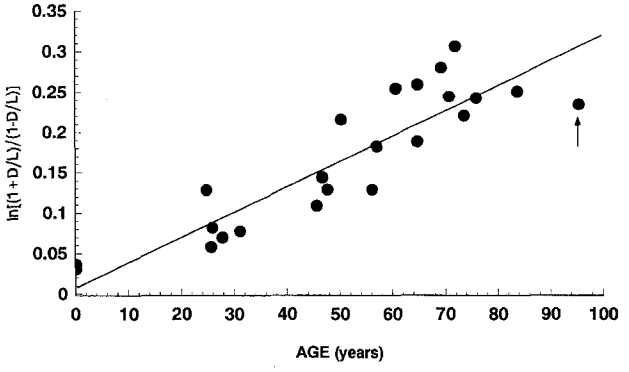

Fig. 2 Plot of $\ln[(1 + D/L)/(1 - D/L)]$ of aspartic acid from the acid-soluble peptide fraction of cartilage from the rib against age. The slope defines the rate constant (2k) of racemization. The arrow indicates an outlier

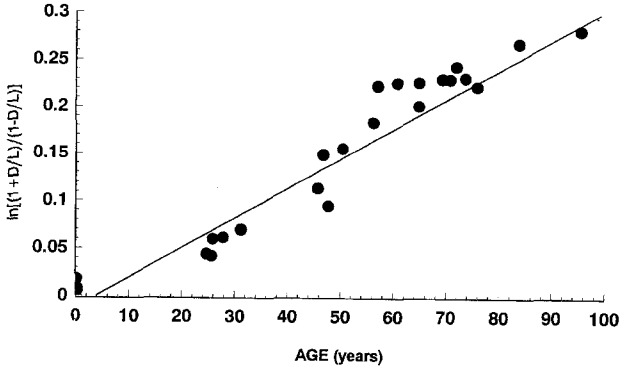

Fig. 3 Plot of $\ln[(1 + D/L)/(1 - D/L)]$ of aspartic acid from the acid-insoluble collagen-rich fraction of cartilage from rib against age. The slope defines the rate constant (2k) of racemization

seemed of importance to also try some other tissues with slow turn-over to explore if they can be used for age estimations in practice. A recent study [26] of cartilage from the intervertebral discs demonstrated that the D/L ratio of aspartic acid in normal, and near normal cartilage generally correlated well with age. However, the correlation was less good when pathologically changed cartilage was used. The same influence by pathological changes was shown by Maroudas et al. [25] in cartilage from the femoral head. In order to avoid problems with the frequently occurring pathological changes in weight-bearing cartilage we investigated cartilage from the junction between the sternum and the ribs. In this area, which is not subjected to the same amount of physical stress as the weight-bearing intervertebral and femoral head discs, pathological changes are less common. There was a tendency in the SP fraction to a decreased racemization in older individuals, which might be a result of age-dependent pathological changes. Similar decreases were not seen in the IC fraction, which could possibly be interpreted as if renewal of proteins occurs more readily in the ground substance than in the collagen, which is in agreement with some earlier studies [23, 24].

Of the 2 fractions used in this study, the IC fraction is probably the one with a composition closest to the native tissue by Ritz and Schütz [26]. The racemization rates demonstrated in the study by Ritz and Schütz [26] (slope coeficient $2.16 * 10^{-3}$ in the anterior peripheral annulus fibrosus and $4.74 * 10^{-3}$ in nucleus pulposus), and those of both the SP and IC fractions used in this study were of the same order of magnitude, with slope coefficients of $3.1 * 10^{-3}$ and $3.3 * 10^{-3}$, respectively. Similar racemization rates are expected in the 2 fractions, since only minor amounts of the ground substance would be extracted with the preparation method employed, leaving large amounts in the IC-fraction. In the study by Maroudas et al. [25] it was shown that the racemization rate in highly purified collagen, where the proteoglycans had been enzymatically removed, was less than half of that of the native cartilage. However, slope coefficients were not calculated, and detailed comparisons cannot therefore be done.

At present it is not known which of the various tissue components in cartilage has the best age-related correlation with the racemization rate. In lung parenchyma [20] and in bone [21, 22] it has been shown that the correlation is especially related to certain tissue components, elastic fibres and osteocalcin, respectively. Further studies have to be done to explore if the correlation can be increased after separation of specific components in cartilage. The extraction method with acid is rather crude and was used in order to make comparisons with our other studies possible [17, 28].

The racemization rate in cartilage is about three-quarters that in dentine ($4.3 * 10^{-3}$ in the SP fraction) [17]. The difference in racemization rates between dentine and cartilage is most probably caused by the exchange of nutrients by diffusion into the cartilage, and subsequent tissue renewal. This renewal can be expected to be much lower in the highly structurally organized and mineralized dentine. Furthermore, dentine does not contain any cell bodies, only the cellular processes of the odontoblasts, which retract on ageing leaving space for secondary mineralization in the tubules.

This study demonstrated a correlation coefficient of $r = 0.97$ between the D/L aspartic acid ratios in the IC fraction and age, and a 95% confidence interval around an estimated individual age of around 14 years. The correlation and dispersion are about the same as have been shown by Ritz and Schütz [26] in cartilage and by us [17] and Ritz et al. [15, 16] in dentine, but lower than that demonstrated in dentine by others in corresponding preparations ($r = 0.99$) [3, 11]. We found a lower correlation with age in the SP fraction than in the IC fraction. This is in contrast to earlier findings in dentine, where the highest correlations were found in the SP fractions [3, 16]. This difference may again be explained by structural differences between dentine and cartilage. More important may be the fact that there are individual variations in tissue renewal in cartilage, and that newly formed proteoglycans may decrease the relative amount of the D-form.

Conclusions

In conclusion it has been shown that non-weight-bearing cartilage can be used for age estimations with the aspartic acid racemization method, and that the method is sensitive enough for practical use. The collagen fraction appears to be the fraction of choice.

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