Contents of Pentosidine in the Tissue of the Intervertebral Disc as an Indicator of the Human Age

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Abstract

The study deals with the post-translational modifications of proteins – glycation of the tissue of the intervertebral disc and determination of one of advanced glycation end's products – pentosidine in the relation to the age. Pentosidine was detected in the hydrolysate of the intervertebral discs from persons between the ages of 16 and 95 years. 142 samples were analysed by high performance liquid chromatography, and the detected amounts of pentosidine were processed statistically. The coefficient of correlation of dependence of the amount of pentosidine on the age amounts to r = 0.92. The results of the work testify to the fact that it is possible to use the detection of pentosidine in the tissue of the intervertebral disc for the estimation of the age. Nevertheless subsequent experiments should be done under different conditions post-mortem decomposition.

Key words: forensic science – age estimation – dead bodies – post-translational modification of proteins – glycation, pentosidine – intervertebral disc

Souhrn

Obsah pentosidinu v tkáních meziobratlové ploténky jako indikátor věku člověka

Studie se zabývá posttranslačními modifikacemi proteinu – glykací tkáně intervertebrální ploténky a stanovením jednoho z pokročilých koncových produktů – pentosidinu ve vztahu k věku. Pentosidin byl detekován v hydrolyzátu meziobratlových plotének u osob ve věku mezi 16 a 95 lety. Sto čtyřicet dva vzorků bylo analyzováno vysoce citlivou kapalinovou chromatografií a zjištěné množství pentosidinu bylo statisticky vyhodnoceno. Koeficient korelace závislosti množství pentosidinu na věku je r = 0,92. Výsledky práce potvrzují skutečnost, že je možné použít stanovení obsahu pentosidinu v tkáni meziobratlové ploténky k přibližnému stanovení věku. Nicméně další experimenty by měly být provedeny za rozdílných podmínek posmrtného rozkladu.

Klíčová slova: soudní lékařství – stanovení věku, těla zemřelých (mrtvoly) – posttranslační modifikace proteinů – glykace – meziobratlová ploténka

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Introduction

The estimation of the age of an individual is an important task when identifying a corpse of unknown identity. There are many methods based on evaluation of morphological changes mainly of hard tissues (as described elsewhere) but methods for age estimation from soft tissue are not frequent. Except morphological methods there is also a menu of biochemical analyses for age estimation whose advantage is standard processing of samples independent of investigator experience. The principle of biochemical methods for the age estimation is analysis of post-translational changes in proteins that result in their structural and functional changes (1). Both intracellular and extracellular

proteins are subject to post-translational modifications (2). The significance of post-translational modifications of intracellular proteins for the determination of the age is small at present, because their characterization is complicated by the autolysis. Thus, from the point of view of estimation of the human age, extracellular proteins and especially collagen, which is the main component of the extracellular matrix and is present in numerous tissues, are significant.

This study deals with the glycation of proteins which is a non-enzymatic reaction of free amino groups (mainly of arginine and lysine) of proteins with glucose or with other reducing carbohydrates. This reaction gives so called Advanced Glycation End products (AGE's) that are irreversible changes (3). Glycation is interesting due to the fact, that in case of some products their accumulation in the tissues depending on the age was established. Many products of non-enzymatic glycation, which were studied in various tissues, were detected (4-9). Pentosidine was studied especially thoroughly (10, 11). Takahashi (12) analysed the bone, cartilage, tendon, ligament, meniscus, muscle and skin in the age groups of very young people (aged 13-16) and very old ones (aged 71-90), and he ascertained a great difference in the contents of pentosidine in the cartilage and tendon of young/old people. Pentosidine was detected in the skin and intervertebral disc of people aged 15-90 within the range which could be applicable for the determination of the age (13, 14). But only a small number of samples were analysed in these studies. The information that pentosidine accumulates in collagen of various tissues is significant from the forensic point of view in those cases, when other methods can not be used like in cases when a part of the body or only soft tissues has been found.

This study deals with assessment of pentosidin in the tissue of annulus fibrosus from intervertebral discs of lumbar spine as a marker of human age for the purposes of age estimation from soft tissues. The study is a part of the project where the content of pentosidine in other tissues was analysed (the dura mater, tendon and myocardium) for same use. Due to the known fact that the amount of pentosidine in tissues increases in case of diabetes mellitus, some analyses were completed with the detection of glycated haemoglobin as an indicator of diabetes mellitus.

Material and methods

Used chemicals

HCl (Penta), pyridoxine (Sigma), acetonitrile (Sigma) for HPLC, methanol for HPLC (Sigma), heptafluorobutyric acid (Fluka) (HFBA), deionised water, the standard of pentosidine (PEN) – a kind gift acquired from Prof. J. de Groot of the TNO Prevention and Health (Netherlands) and from Ing. P. Špaček (The Institute of Rheumatology in Prague) with the concentration of pentosidine 1.19 nM/ml (15).

Equipment

The high pressure liquid chromatography (HPLC) system Waters with the autosampler 717, photo diode array detector (PDA 996), fluorescent detector FD 470 and high-pressure pump 486, controlled by the software Milenium 2000 was used.

Statistical processing

Data processing was carried out with the software Statistica 6.0 CZ (Statsoft CZ) and MS Excel. The correlation coefficient of the amount of pentosidin on age was calculated from all data. The average amount of pentosidine with standard deviation on 95% level of reliability was calculated for every age group to verify the reliability.

Studied tissue

Excisions from intervertebral discs from people autopsied at the Institute of Forensic Medicine and Toxicology were used for the purposes of the study. The set of 142 samples of intervertebral discs of people aged 16-95 was analysed (97 males and 45 females). Only intervertebral discs from the areas L1, L2 or L3 without any evident degenerative changes were used for the analysis. The time since death till excision did not exceed three days, so as to exclude possible influence of post-mortem autolysis. The intervertebral discs were shortly rinsed with running water (to remove blood residues) immediately after the excision from the body, dried on the air and put into a freezer at –90 °C. Excisions from the annulus fibrosus were used for the analysis. The surface layer containing blood vessels was removed carefully. Approximately 50 mg of the tissue from the surface of the first layer of the (AF) were taken for the analysis, rinsed three times in deionised water by intensive shaking for 10 minutes. The rinsed tissue was freeze dried overnight. The dried sample weighing 5±0.3 mg was transferred into a hydrolysation tube. 500 μl of 6M HCl were added into the tube, which were evacuated and flame sealed. Hydrolysis was carried out at 110 °C for 24 hours. Then the hydrolysate was dried under reduced pressure at the temperature of 70 °C.

Detection of pentosidine by HPLC.

The detection of pentosidine was carried out according to Bank et al. (9), slightly modified to our needs. In short: 5 mg of dried hydrolysate were dissolved in 500 μl of standard pyridoxine with the concentration 10 *nM/ml*, (should be prepared

fresh for each bigger sample set) containing 2.4 μ*M*/ml of homoarginine. Of that volume, 300 μl were sampled into a vial for the autosampler and diluted with 600 μl of a solvent containing 0.5 % in 10 % ACN (v/v). The analysis itself was carried out in the column Waters Spherisorb ODS2 at the laboratory temperature with the flow rate amounting to 1 ml/min in two isocratic steps: from 0-17 min. solvent A: 0.15 % HFBA in 24% methanol (v/v); from 17-30 min. solvent B: 0.05 % HFBA in 40% methanol (v/v). This was followed by 30-40 min. washing of the column with solvent C: 0.1% HFBA in 75% ACN and 40-50 min. equilibration of the column with solvent A. The whole analysis lasted for 50 minutes. Each sample was analysed at least twice.

The calibration curve from samples containing 59.5; 119.0; 178.5; 238.0; 357.0; 476.0 and 595.0 pM/ml of pentosidine was set up from the stock standard with the concentration of pentosidine 1.19 nM/ml. The fluorescent detector was programmed as follows: for 0-22 minute λex/λem 295/400 nm and for 22-50 minute λex/λem 328/378 nm.

The amount of pentosidine is expressed as *nM*/mg of tissue.

Glycated haemoglobin.

The analyses of blood for glycated haemoglobin were made using high pressure liquid chromatography on Variant II BioRad analyser, the routine accredited method in the laboratory of the Institute of clinical biochemistry and laboratory diagnostics of General Teaching Hospital in Prague. The glycated haemoglobin was determined in 62 samples of blood taken from the same persons whose tissue samples were analysed for pentosidine. These persons were selected in an appointed time period.

Results

Pentosidine was proved in all 142 analysed samples. It was found that its amount increases with the age (Fig.1). The amount of pentosidine increased from approximately 0.1 nM/mg of the tissue in young people up to 1.8 nM/mg of the tissue in case of old people (Fig 2). The coefficient of correlation for the average of the values in individual age groups amounts to R =

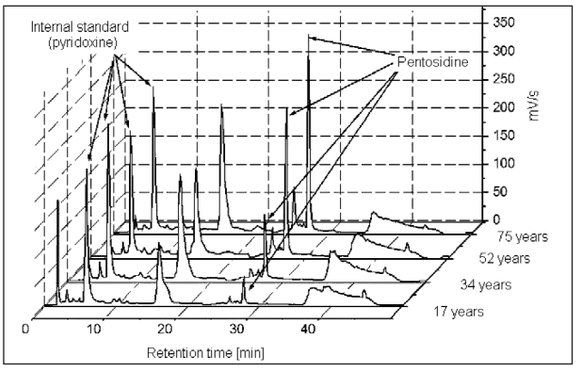

Fig. 1. Chromatograms of HPLC separation of hydrolysates of intervertebral discs tissue. Peaks of pentosidine in persons of different age.

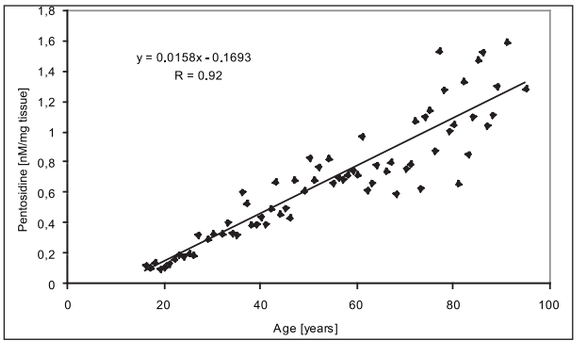

Fig. 2. The values of pentosidine amount in the tissue of intervertebral disc from persons between the ages of 16 and 95 years.

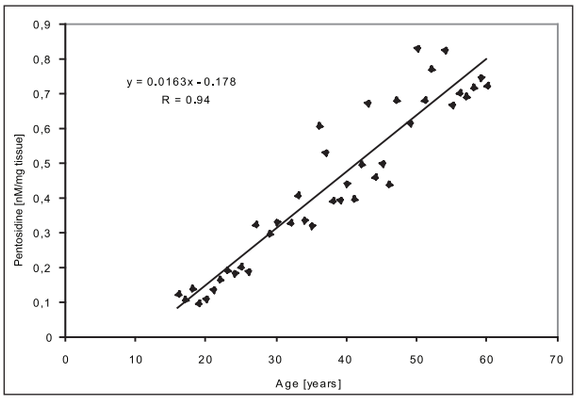

Fig. 3. The values of pentosidine amount in the tissue of intervertebral disc from persons between the ages of 16 and 60 years.

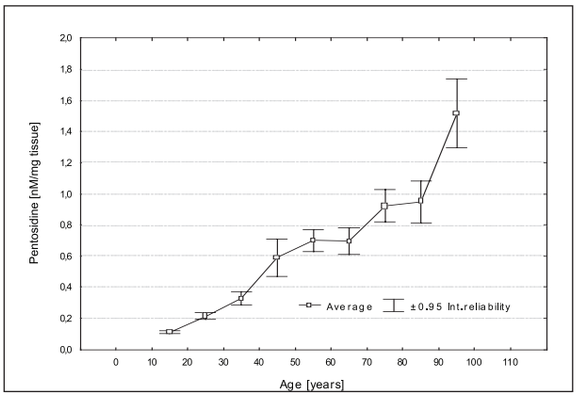

Fig. 4 The average amount of pentosidine in the tissue of intervertebral disc with limits of interval of reliability in age groups from second to tenth decade of life. It follows from the graph that age estimation by assessment of pentosidin in the tissue of intervertebral disc is reliable within the range of 15 to 40 years of age.

0.92. It is evident from this graph that in case of people above 60 years of age the scatter of values is considerable. However, it is evident from the graphs (Fig. 3) and (Fig. 4), that the determination of the age is reliable approximately until the age of 40 years. It is possible to draw the following conclusion from the acquired results: if the amount of pentosidine determined by analysis amounts to 0.4 nM/mg of the tissue, it is an individual, whose approximate age is below 40. The amount of pentosidine above 1 nM/mg of the tissue, however, indicates an elderly person. The amount of glycated haemoglobin (HbA1c) was assessed in 62 people selected. On the whole in 13 cases (i.e. about 21 %) the amount of HbA1c exceeded the upper referential limit (6.1 %). In case of six people, the higher registered amount of HbA1c was also accompanied by the higher amount of pentosidine in the tissue, which was higher than average for the age group in question. On the contrary, we found the value uncommonly high in case of a thirty-five-year-old man (10% HbA1c), whose amount of pentosidine corresponded with the average value typical for his age group.

In the other four cases the higher amount of HbA1c was not accompanied by the higher amount of pentosidine in the tissue than it would correspond to the average of the age group in question.

Discussion

The study proved applicability of assessment of pentosidin in the tissue of intervertebral disc for age estimation. The set is considered as a basic because the amount of pentosidin on sufficient numbers of samples in different age groups is not known. Therefore only samples without advanced autolysis/putrefaction or degenerative changes have been analysed. Both these post-mortem processes are caused by proteolytic enzymes that can break the composition of the tissue. Also, samples with not visible pathologic changes has been analysed because of possible influence on chemical composition. Thus this set represents a group with data about the amount of pentosidin under normal appearance and post-mortem conditions. The analysis of tissue under different post-mortem condition or their appearance may be the topic of next research.

The results show scatter of values after 45 years of age. There are apparently several reasons for the variance in the detected values of pentosidine. The composition of the tissue is one of them. The intervertebral disc is a complicated fibrocartilaginous tissue composed of fibres of collagen and proteoglycans which are formed by glucosamines bound to the core protein that contains a great amount of lysine and arginine residues (16). These two amino acids share in creation of pentosidine. Since the rise of AGE's is a result of a non-enzymatic chemical reaction and is not bound to a certain protein, it is evident that pentosidine is also created in the core protein of proteoglycans. The significant increase amount of pentosidine in the relation with age was proved in non-collagenous proteins (17, 18). It was also found that the amount of pentosidin in agreccan (a non-collagenous protein) from degenerate IVD is lower then in healthy persons (19) This support our opinion that samples without visible pathologic changes should be used for age estimation (it can be assumed that extraction of proteoglycans from the tissue of the intervertebral disc could give more exact results).

Another factor influencing the determination of the age is ageing of the IVD tissue in itself. It is accompanied by a decrease in proteoglycans as a result of slower synthesis and faster degradation of the tissue (20). The decrease in collagen content in annulus fibrosus was found (expressed as contents of hydroxyproline) during the process of ageing (21). These changes in the composition of annulus fibrosus can account for the scatter in the values of detected pentosidine. Although the amount of molecular matrix for pentosidine decreases, pentosidine is still most probably created in several places in the molecule of collagen or proteoglycane. Moreover, eventual disorders of metabolism of carbohydrates (diabetes mellitus) join in formation of AGE's in higher age. From this point of view the accompanying determination of glycated haemoglobin increases the reliability of the age estimation by the assessment of pentosidine. If the amount of glycated haemoglobin exceeds the referential limit and the detected amount of pentosidine is high, it is necessary to evaluate such data in relation to the age by taking into consideration the contingent diabetes mellitus (nevertheless, we recorded low amount of glycated haemoglobin but high amount of pentosidine in case of a treated diabetic patient). The correctness of this procedure is supported by circumstances of a finding of body when no medical history is known which common situation in cases of identification is.

The study proved that the detection of pentosidine in the tissue of the intervertebral disc can be used for the age estimation. The processing of the sample and analysis itself is not difficult, does not require too much time and can be easily done in any laboratory which is the advantage of described method. The result of age assessment is good reliable until the age of 45 years then the reliability diminishes. Due to possible influencing of the values of AGE's by diabetes mellitus, it is appropriate to carry out parallel detection of glycated haemoglobin in blood of the person, from whom samples of tissues will be taken for the detection of

pentosidine. The assessment of pentosidine represents the new method for age estimation from soft tissue which can be used mainly in such cases when only a part of body has been found or age estimation based on other method can not be applied.

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