Int J Legal Med (2002) 116:340–343
DOI 10.1007/s00414-002-0333-8

ORIGINAL ARTICLE

Y. Açil · I. N. G. Springer · J. G. Prasse · J. Hedderich
S. Jepsen

Concentration of collagen cross-links in human dentin bears no relation to the individual age

Received: 23 April 2002 / Accepted: 15 July 2002 / Published online: 1 October 2002

Abstract Lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) are collagen cross-link residues. HP is present in most tissues, whereas LP is specific for bone and dentin. Based on the current literature there are certain indications that measurement of the concentrations of HP and LP in dentin may be a valuable tool to determine the individual age. The purpose of this investigation was to assess if the concentrations of LP and HP in dentin increase during lifetime. We have investigated 173 molars from 173 individuals (2 through 78 years of age, 31 primary and 142 secondary teeth) in the course of the present study. Levels of LP and HP were measured by HPLC and fluorescence detection. The results show that dentinal concentrations of HP and LP did not increase with age and varied between individuals of the same age and that determination of dentinal concentrations of HP and LP cannot be used to determine the individual age.

Keywords Collagen · Cross-link · Dentin · Forensic medicine · Age-relationship · Human

Introduction

Human dentin is composed of minerals (70%), an organic matrix (20%) and water (10%). Collagen is the major component of the organic matrix in human dentin [8]. Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) are two mature non-reducible cross-links of mature collagen which are formed by a sequence of post-translational modifications [1, 3, 4]. HP is a derivative of three residues of hydroxylysine and is present in almost all mature tissue (e.g. tendons, vessel walls, cartilage, dentine and bone) [1, 3, 4]. LP is a derivative of two residues of hydroxylysine and one residue of lysine and is found in dentine and bone [3, 4, 6, 7, 10, 11, 15, 18, 24, 26, 27].

From the ages of 0 through to 20 years there is a change in the urinary cross-link excretion that allows an estimation of the patients age [3]. Based on a limited number of samples, it was suggested that the ratio of the dentinal concentration of mature compared to immature collagen cross-links, increases with age as dentin is thought to have a minimal collagen turnover [14, 29]. Consequently, this study was designed to examine whether the determination of the dentinal concentration of the collagen cross-links HP and LP can be used to determine or estimate the age of a person in question.

Y. Açil · I.N.G. Springer (✉) · J.G. Prasse
Deptartment of Oral and Maxillofacial Surgery,
University of Kiel,
Arnold-Hellerstrasse 16, 24105 Kiel, Germany
e-mail: springer@mkg.uni-kiel.de, Tel.: /Fax: +49-431-568148

J. Hedderich
Institute of Medical Informatics and Statistics, University of Kiel

S. Jepsen
Department of Conservative Dentistry and Periodontology,
University of Kiel

Materials and methods

Collection and preparation of samples

Extracted molars were immediately stored in normal saline solution containing 0.1% sodium azide. A total of 173 teeth were examined, 31 primary and 142 permanent molars. Enamel and cementum were removed completely with high speed rotating diamond burs (160,000 rpm) with a water spray coolant (50 ml/min). Dentin samples of identical weight (10 mg, analytical scale, error <1/1,000 g, Sartorius, Göttingen, Germany) were taken from the cementoenamel junction and were placed into a solution of 99% ethanol for 12 h. Thereafter, the samples were taken out of the solution and dried at room temperature for 24 h.

Pyridinoline standards and analysis of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP)

The samples of dentin were hydrolysed by 1 ml of 6 M HCl at 110°C for 24 h. The hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) content was quantified using external standards prepared from commercially available adult bovine bone gelatin (Deutsche Gelatine-Fabriken Baden, Germany) prior to sample application onto the chromatography system. HP and LP were purified by a preparative reverse-phase column HPLC. Chromatography was performed on an HPLC system (Dionex, Idstein, Germany) at room temperature as previously described [1, 2, 3, 4].

Statistics

The teeth were divided into the following groups based on the age of the donor: 0–10 years of age (yoa), 11–20 yoa, 21–30 yoa, 31–40 yoa, 41–50 yoa, 51–60 yoa, 61–80 yoa. The arithmetic mean, minimum, maximum, median, 25th and 75th percentile of the dentinal concentrations of HP and LP were calculated. The medians were calculated, since dentinal concentrations of HP and LP varied between different age groups. A correlation coefficient of the dentinal concentrations of LP and HP was calculated according to Spearman [22].

Ethics

The study was conducted in accordance with the standards of the Ethics Committee of the University of Kiel (reference number: D 309/00) and with the Helsinki Declaration of 1983. The patients were informed about the aim and design of the study and written consent was obtained.

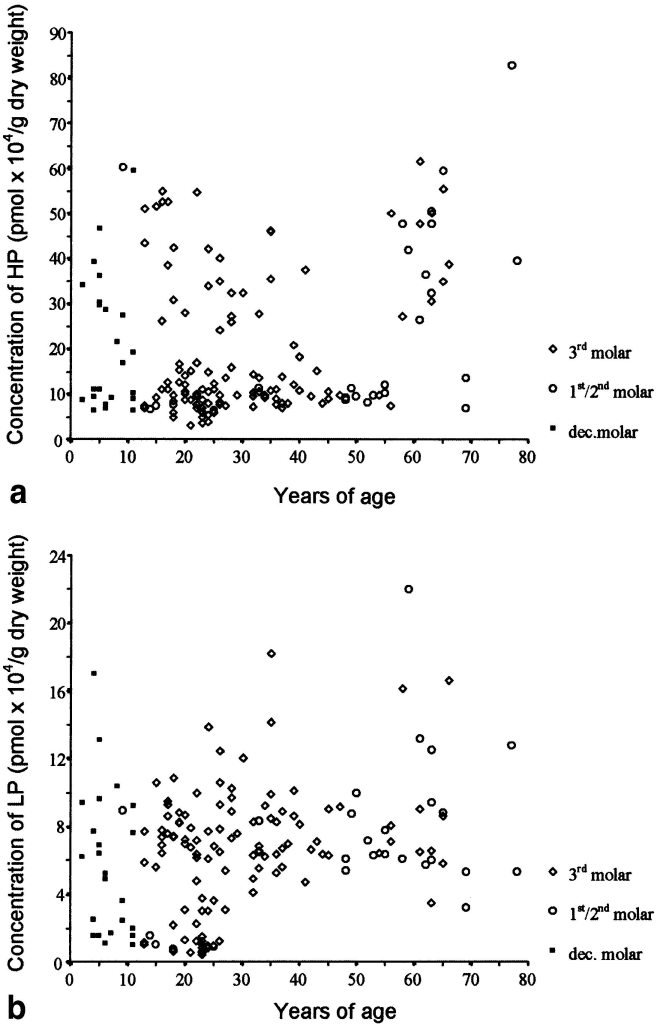

Fig. 1a,b Distribution of the dentinal concentrations of **a** HP and **b** LP in respect to age and type of tooth. 1st, 2nd and 3rd molar of the permanent dentition and 1st and 2nd molars of the deciduous dention (dec. molar) were included in the study. There was no relationship between the dentinal concentrations of HP or LP to age or type of tooth (1st, 2nd and 3rd molar of the permanent dentition and 1st and 2nd molars of the deciduous dention). Also, analysing each type of tooth separately, no relationship to the individual age was found

Results

The dentinal concentrations of HP and LP showed a significant correlation (correlation coefficient: 0.634, $P<0.001$, correlation analysis according to Spearman).

There was no relationship between the dentinal concentrations of HP and LP and the age of the patients or the type of tooth (1st, 2nd and 3rd molar of the permanent dentition and 1st and 2nd molars of the deciduous dention) (Figs. 1a,b, 2). Also, analysing each type of tooth separately, no relationship of the dentinal concentration of HP and LP to the individual age was found (Fig. 1a,b).

The average concentrations of HP in the different age groups decreased in the years 0 through 40 by about 50% to increase thereafter until the age of 80 by about 350% (Table 1). The dentinal concentrations of HP among individuals of the same age group varied considerably (Fig. 1a, Table 1). The average concentrations of LP in the different age groups decreased in the years 0 through 30 by about 30% to increase thereafter by about 200% until the age of 60 and decreased again until the age of 80 (Table 2). Similar to the dentinal concentrations of HP, LP varied noticeably among individuals of the same age group (Fig. 1b, Table 2).

Discussion

We intended to study the usefulness of dentinal HP and LP in the determination of the individual age. Collagen is the major component of the organic matrix of human dentin [8] and in contrast to bone there is minimal collagen turnover in dentin [29]. It appeared likely that components of dentinal collagen might serve as a biological marker of the individual age. Several unsuccessful attempts have been made based on this assumption, one of which

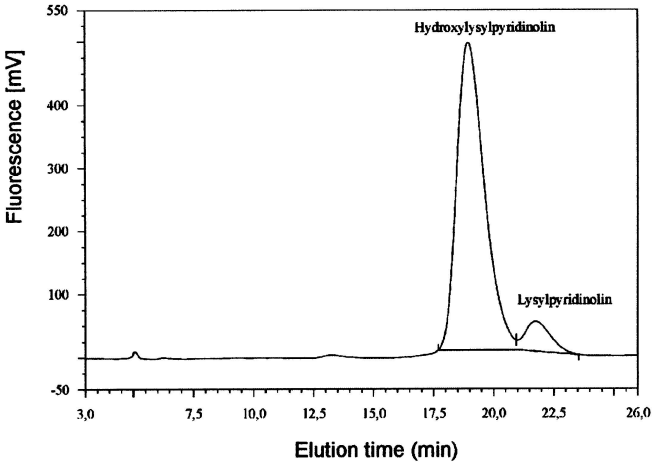

Fig. 2 Representative chromatogram of a 5-year-old patient (1st primary molar). Fluorescence was monitored with excitation at 297 nm and emission at 397 nm. The HP peak appears at 17.5 min after injection, followed by the LP peak. No relationship of the collagen cross-link concentration of human dentin and the patient's age could be observed in the present study

Table 1 Average dentinal concentrations of HP in different age groups (pmol×10^4/g dry weight). HP and LP are not strictly increasing with increasing age. The dentinal concentration of LP varies among individuals of the same age group

Years of age	Mean	StDev	Min	Max	25th percentile	Median	75th percentile
0–10 (*n*=23)	229961.53	144526.71	63758.01	601503.63	92812.09	219147.19	318895.97
11–20 (*n*=38)	208047.31	175208.71	48019.04	595755.06	82247.30	115376.61	326439.20
21–30 (*n*=45)	146002.05	120124.87	31699.63	544878.25	69479.58	98091.34	164577.50
31–40 (*n*=26)	153097.63	111121.96	69357.15	461022.25	91214.81	108480.05	152730.44
41–50 (*n*=14)	118178.95	75838.22	72945.15	374073.81	89909.77	96359.52	107167.47
51–60 (*n*=10)	224213.78	176575.73	73310.57	500937.28	93385.27	111861.37	432898.03
61–80 (*n*=17)	420504.37	182660.71	69375.33	829297.13	313828.25	393976.78	529895.16

Table 2 Average dentinal concentrations of LP in different age groups (pmol×10^4/g dry weight). LP does not strictly increase with increasing age. The dentinal concentration of LP varies among individuals of the same age group

Years of age	Mean	StDev	Min	Max	25th percentile	Median	75th percentile
0–10 (*n*=23)	67675.24	45396.47	11097.76	170438.78	25510.92	62125.21	96422.79
11–20 (*n*=38)	54243.99	34348.96	6441.52	108670.94	14898.00	69475.73	82380.19
21–30 (*n*=45)	49855.66	39045.79	3872.10	138699.45	10150.66	47926.78	78025.92
31–40 (*n*=26)	79441.12	29186.68	41053.46	181721.61	62962.35	75647.91	86764.30
41–50 (*n*=14)	69218.48	16733.67	47457.62	99886.95	57287.47	64697.22	88455.75
51–60 (*n*=10)	93510.92	53558.34	60957.36	220230.70	63492.81	71464.59	100715.76
61–80 (*n*=17)	81836.80	37354.62	32301.91	166049.28	55398.56	65968.57	109719.46

was the determination of the deposition of intratubular collagen fibrils in dentin [9].

In an earlier study it was suggested that the content of mature collagen cross-links in dentin increases with age [29]. However, this hypothesis was supported by the study of only four human individuals of different ages [29]. Other authors studied the dentinal concentration of LP in a group of 22 people between 15 and 73 years of age employing an enzyme immunoassay [14]. The authors confirmed a relationship between dentinal concentrations of LP to the individual age, but had a reliability of only 65% [14].

The present study was designed to establish cut-off points of concentrations of HP and LP for different age groups in a large cohort of 173 individuals aged 0 through 80 years of age. Even though we highly respect the innovative work of the authors cited [14, 29], our results seem to suggest that their results may have been coincidental. We found no relationship between dentinal concentrations of HP and LP and the individual age. The variation of individual concentrations even within the same age group was higher than differences between age groups and the dentinal concentrations of HP and LP did not increase with age.

This project was focused on primary and permanent molars, because earlier studies found differences of collagen cross-link concentrations in the dentin of incisors, canines, bicuspids and molars [21]. In the present project no significant differences were found between the different types of molars studied. Also, analysing the different types of molars separately, no age-related increase of HP or LP was found. The variation of the dentinal collagen cross-link concentrations in individuals of the same age may be due to genetic disposition and to different forms of functional stress [21, 30].

Earlier studies have shown that the hydroxyproline content increases from the inner surface (pulp cavity) to the outer surface of the tooth, being up to 38% higher in the cementoenamel junction [13]. Therefore, HP and LP variation throughout the dentin of teeth was studied in the course of a pilot study. We found that samples taken from the cementoenamel junction showed the highest and most constant concentrations of HP and LP. The cementoenamel junction is an anatomical site easy to determine in any tooth. There is a certain chance that our results could have been influenced by variation of the sampling site, but we suggest that the number of samples studied should have compensated for the effect of this potential error. Other authors pulverised the whole dentin of a tooth [14]. Again there is a certain chance of systemic error as it appears difficult to remove the enamel cap of a tooth without accidentally removing part of the dentin or leaving parts of the enamel cap. The authors of the paper mentioned [14] employed an enzyme immunoassay technique which appears to be a good option. However, we suggest that our method is very precise. We have employed external standards and repeat measurements showed an error of less than 3%.

In addition to the experiments described in the present paper, hydroxyproline concentrations were measured in dentin of all the teeth studied and could neither be related to dentinal concentrations of HP or LP nor to the individual age. Likewise, HP, LP and hydroxyproline concentrations were measured in the dental pulp and could not be related to dentinal concentrations or to the individual age.

An X-ray of the hand is an important method in forensic science for estimation of the age of juvenile suspects with uncertain date of birth [23]. It could be demonstrated

that urinary HP and LP concentrations are increased between the age of 5 months and 14 years, decrease with age and reach a steady concentration at the age of 15 years and older [3]. During childhood and adolescence there is increased bone turnover related to skeletal growth [3, 25]. However, the measurement of urinary HP and LP appears to be of limited value to determine the age of juvenile subjects. HP and LP values in the urine of adults are independent of gender and age, and are virtually not influenced by nutrition habits or by physical activity [3].

Further fruitless attempts to determine the human age by dentinal probes were based on the peritubular deposition of dentin [12] and on spherical structures of calcium phosphate [5]. At present, the most accurate method for the determination of the individual age of a person is the calculation of the ratio of the dentinal concentrations of D- and L-aspartic acid [16, 17, 19, 20, 23, 28]. This method has an error of less than 3 years of age and probes can be obtained from vital teeth at low risk causing only minor discomfort to the affected person [16, 17, 19, 28].

Based on the results of the present study we suggest that the determination of the mature collagen cross-links HP and LP in dentin has no value in the determination of the individual age.

Acknowledgments We gratefully acknowledge our laboratory technicians Gisela Otto and Mojgan Paymard for their assistance with the analytical procedures. The department of Oral and Maxillofacial Surgery, University of Kiel, Germany, financed this project. We would like to express our gratitude to Prof. Dr. Dr. F. Härle, head of the department, for the support of our work.

References

1. Acil Y, Müller PK (1994) A rapid method for the isolation of the mature collagen cross-links, hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP). J Chromatogr A 664:183–188
2. Acil Y, Vetter U, Brenner, R, Muller PK, Brinckmann J (1995) Ehlers-Danlos syndrome type VI: cross-link pattern in tissue and urine sample as a diagnostic marker. J Am Acad Dermatol 33:522–524
3. Acil Y, Brinckmann J, Notbohm H, Muller PK, Batge B (1996) Changes with age in the urinary excretion of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP). Scand J Clin Lab Invest 56:275–283
4. Acil Y, Terheyden H, Dunsche A, Fleiner B, Jepsen S (2000) Three-dimensional cultivation of human osteoblast-like cells on highly porous natural bone mineral. J Biomed Mater Res 51: 703–710
5. Atsu SS, Gokdemir K, Kedici PS (1998) Human dentinal structure as an indicator of age. J Forensic Odontostomatol 16:27–29
6. Body JJ, Delmas PD (1992) Urinary pyridinium cross-links as markers of bone resorption in tumor-associated hypercalcemia. J Clin Endocrinol Metab 74:471–475
7. Branca F, Robins SP, Ferro-Luzzi A, Golden MH (1992) Bone turnover in malnourished children. Lancet 340:1493–1496
8. Butler WT (1992) Dentin extracellular matrix and dentinogenesis. Oper Dent Suppl:18–23
9. Dai XF, Ten Cate AR, Limeback H (1991) The extent and distribution of intratubular collagen fibrils in human dentine. Arch Oral Biol 36:775–778
10. Eyre D (1992) New biomarkers of bone resorption. J Clin Endocrinol Metab 74:470A–470C
11. Hoskin PJ, Stratford MR, Folkes LK, Regan J, Yarnold JR (2000) Effect of local radiotherapy for bone pain on urinary markers of osteoclast activity. Lancet 355:1428–1429
12. Kvaal SI, Koppang HS, Solheim T (1994) Relationship between age and deposit of peritubular dentine. Gerodontology 11:93–98
13. Levine RS (1971) The distribution of hydroxyproline in sound human coronal dentine. Arch Oral Biol 16:127–135
14. Martin-de las Heras S, Valenzuela A, Villanueva E (1999) Deoxypyridinoline crosslinks in human dentin and estimation of age. Int J Legal Med 112:222–226
15. Miyamoto KK, McSherry SA, Robins SP, Besterman JM, Mohler JL (1994) Collagen cross-link metabolites in urine as markers of bone metastases in prostatic carcinoma. J Urol 151: 909–913
16. Mornstad H, Pfeiffer H, Teivens A (1994) Estimation of dental age using HPLC-technique to determine the degree of aspartic acid racemization. J Forensic Sci 39:1425–1431
17. Ohtani S, Yamamoto K (1991) Age estimation using the racemization of amino acid in human dentin. J Forensic Sci 36: 792–800
18. Papatheofanis FJ (1997) Quantitation of biochemical markers of bone resorption following strontium-89-chloride therapy for metastatic prostatic carcinoma. J Nucl Med 38:1175–1179
19. Ritz S, Stock R, Schutz HW, Kaatsch HJ (1995) Age estimation in biopsy specimens of dentin. Int J Legal Med 108:135–139
20. Ritz-Timme S, Rochholz G, Schutz HW, Collins MJ, Waite ER, Cattaneo C, Kaatsch HJ (2000) Quality assurance in age estimation based on aspartic acid racemisation. Int J Legal Med 114:83–86
21. Rivera EM, Yamaguchi ,M (1993) Site comparisons of dentine collagen cross-links from extracted human teeth. Arch Oral Biol 38:541–546
22. Sachs L (1992) Angewandte Statistik. Springer, Berlin: 510–515
23. Schmeling A, Reisinger W, Loreck D, Vendura K, Markus W, Geserick G (2000) Effects of ethnicity on skeletal maturation: consequences for forensic age estimations. Int J Legal Med 113:253–258
24. Springer ING, Açil Y (2002) Urinary levels of lysylpyridinoline and hydroxylysylpyridinoline – marker for the involvement of the bones and for recurrence in cases of oral cancer. J Dent Res 81:1038
25. Springer ING, Suhr M, Fleiner B (2002) Adaptive adjustment of the adolescent porcine mandibular condyle. Bone 31:1190–1195
26. Tamada T, Sone T, Tomomitsu T, Jo Y, Tanaka H, Fukunaga M (2001) Biochemical markers for the detection of bone metastasis in patients with prostate cancer: diagnostic efficacy and the effect of hormonal therapy. J Bone Miner Metab 19: 45–51
27. Vinholes J, Guo CY, Purohit OP, Eastell R, Coleman RE (1996) Metabolic effects of pamidronate in patients with metastatic bone disease. Br J Cancer 73:1089–1095
28. Waite ER, Collins MJ, Duin AC van (1999) Hydroxyproline interference during the gas chromatographic analysis of D/L aspartic acid in human dentine. Int J Legal Med 112:124–131
29. Walters C, Eyre DR (1983) Collagen crosslinks in human dentin: increasing content of hydroxypyridinium residues with age. Calcif Tissue Int 35:401–405
30. Yamaguchi M, Katz EP, Mechanic GL (1986) Intermolecular cross-linking and stereospecific molecular packing in type I collagen fibrils of the periodontal ligament. Biochemistry 25:4907–4913