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Gelatinase A in Human Dentin as a New Biochemical Marker for Age Estimation*

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ABSTRACT: Dentin proteins from 24 individual permanent molars from patients aged 15 to 73 years were sequentially extracted, with guanidinium chloride (G1-extract) and then with EDTA; after demineralization, the material was again extracted with guanidinium chloride (G2-extract). Extracts were analyzed by SDS-PAGE electrophoresis and the gels were processed for zymography to detect gelatinolytic activities. The patterns of gelatinase A distribution differed in the different dentin protein fractions, and the changes varied with age. Significant differences were detected in gelatinase A in G2-extracts between individual younger than 20 years old and the rest of the sample (χ^2_{exp} = 19.429; 1 d.f.; $p \leq 0.001$). The incidence of true and false positives and negatives, and sensitivity and specificity for the presence of gelatinase A in dentin extracts, were calculated. Determination of gelatinase A in human dentin may be a useful marker to estimate age, especially when other morphological methods are of limited usefulness.

KEYWORDS: forensic science, human dentin, age, matrix metalloproteinase, gelatinase A, forensic anthropology, forensic odontology

Establishing the chronological age of an individual is a recurrent problem in forensic sciences. Studies of mineralized tissues, including bone and teeth, have been proposed as a diagnostic tool in estimating the age of unknown human remains, because these tissues are highly resistant to putrefaction and external agents. In fact, in the last few years, several morphological and biochemical methods have been used to this end. Most recently, interest has been focused on biochemical studies of dental tissues as one of the most precise and reliable methods for estimating age, especially by the determination of aspartic acid racemization (1–10) and the quantification of deoxypyridinoline crosslinks in human dentin (11). Barring destruction by caries, crown abrasion or pathological root resorption, dentin collagen laid down in the developing tooth remains there for the life of the tooth. Dentin seems particularly suitable for the study of biochemical changes in teeth with aging because it is composed of approximately 20% organic matrix (principally collagen) and is thought to have minimal collagen turnover. As in bone, dentin collagen constitutes almost 90% of the organic matrix, and is primarily type I. The central portion, about 95% of the total collagen molecule, is triple-helical in structure, and to stabilize the collagen network, covalent crosslinks exist between the collagen molecules (12,13). Collagen and crosslinks are biochemically important in mineralized tissues, providing plastic, ductile properties, whereas the mineral component confers stiffness. The content of crosslinks in human dentin also changes with age (14); in fact, recent studies of human dentin have detected a linear increase in deoxypyridinoline content (a nonreducible trifunctional crosslink) in relation to the individual's age (11). Matrix metalloproteinases (MMPs) are a family of enzymes which in concert are able to degrade collagen in a variety of tissues, including dental tissues, under diverse physiologic and pathologic situations (15–18). In the formation of a mineralization-competent matrix and during the mineralization process, some of these matrix components undergo remodeling and controlled degradation. In relation to the role of matrix metalloproteinases in mineralized dental tissues, these proteases appear to play an important role in the organized processing of the enamel protein matrix during enamel formation, and the presence of MMPs has been well established in porcine and bovine enamel (19–24). Gelatinase A (MMP-2; gelatinase A; 72 kDa gelatinase/type IV collagenase), in latent and activated forms, was first detected in relatively constant proportions throughout the developing enamel (19). More recently, gelatinolytic activity has been described in human fetal teeth with gelatinase A being implicated in the remodeling and degradation of the dental basement membrane during human tooth morphogenesis (25). Moreover, cultured human odontoblasts derived from adult teeth have recently been shown to synthesize gelatinase A (26). In a recent study, gelatinolytic activity identified as gelatinase A was detected in human dentin and the more recently synthesized nonmineralized dentin matrix (27,28). Therefore, because of the changes we detected in the collagen crosslinks (11), and the evidence of the presence of gelatinase A in human dentin, we determined age-related changes in gelatinase A, an enzyme able to degrade the organic matrix of dentin.

Materials and Methods

Human Dentin Sampling

Healthy erupted human permanent molars, extracted for valid clinical reasons, were obtained from the Department of Oral Surgery at the Hospital Virgen de las Nieves in Granada (Spain) and from private dental clinics. The protocol for collecting samples from human subjects was approved by the Ethics Committee of the

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Virgen de las Nieves Hospital. Molars were taken from 24 patients (15 females and 9 males) ranging in age from 15 to 73 years. Samples were classified in four groups according to patient age (Group 1: ≤20 years of age; Group 2: 21–40 years; Group 3: 41–60 years; Group 4: >60 years). Each group consisted of 6 individual cases.

Tissue Preparation

Immediately after extraction, the teeth were cleaned of blood with distilled water and any attached soft tissue was carefully removed with a scalpel. Enamel and cementum were removed from the dentin by diamond burrs under a continuous water spray. After opening the pulp chamber, the pulp content was removed mechanically. All the dentin samples were approximately 1.2 g wet weight. Unless otherwise stated, all steps were carried out at 4°C. Dentin tissue was crushed by pounding with a steel hammer on a cold metallic surface. To remove any tissue remnants and to clean the dentin of associated proteins, the pulverized dentin was washed overnight with 2.5 M NaCl containing proteinase inhibitors (PI) [2.5 mM benzamidine HCl, 50 mM ϵ-amino-n-caproic acid, 0.5 mM N-ethyl maleimide, and 0.3 mM phenylmethylsulfonylfluoride] by constant stirring.

Extraction of Dentin Proteins

Methods for extracting human dentin proteins have been published (11,27). Briefly, the procedure was as follows: dentin proteins were extracted after removing non–specifically bound material by NaCl washes. The pulverized dentin was then rinsed twice with cold distilled water. To elute hydroxyapatite-associated proteins, the dentin was extracted with a 4 M guanidine HCl solution, 65 mM Tris-HCl pH 7.4, plus the PI solution under constant agitation at 4°C. These guanidinium chloride extracts (G1-extracts) were clarified by centrifugation (2000 × g, 10 min) and the supernatants were dialyzed thoroughly in M_r 6000 cut-off Spectrapor dialysis tubing. Aliquots of G1-extracts were then lyophylized and immediately frozen at −20°C until analysis. After guanidinium extraction, proteins within the mineralized matrix (including collagen proteins) were extracted by demineralization with 0.5 M EDTA pH 7.4 and PI solution during a total period of 12 days. The EDTA solution was changed every 3 days and clarified by centrifugation (2000 × g, 10 min). Successive EDTA extracts (E1, E2, E3, and E4) were collected separately. The material was then thoroughly dialyzed at 4°C against large volumes of double-distilled water plus PI, lyophylized after a final solution change in water and then frozen at −20°C until analysis. The remaining dentin tissue was extracted with 4 M guanidine HCl solution, 65 mM Tris-HCl pH 7.4 plus PI to dissociate organic matrix-associated proteins that were not mineral-associated. The guanidinium chloride extracts (G2-extracts) were dialyzed and lyophylized as described previously for the G1-extracts.

SDS-Polyacrylamide Gel Electrophoresis and Zymography

Proteolytic activities in the human dentin extracts (G1-, E-, and G2-extracts) were assayed by a highly sensitive zymography technique described previously (19). Proteins were separated by SDS-polyacrylamide gel electrophoresis (Modular Mini-Protean II vertical electrophoresis system, Bio-Rad Laboratories, Hercules, CA) according to Laemmli (26). For zymography, nonreduced protein samples (-DTT) were electrophoresed on 10% (w/v) polyacrylamide gels containing 125 μg/mL heat-denatured acid-soluble type I collagen (gelatin), prepared as described before (19). Samples were prepared for electrophoresis at either 3 or 6 μg/μL concentrations in Laemmli's sample buffer (29), and 10 μL aliquots were electrophoresed under nonreducing conditions without prior heating. After electrophoresis at 150 V for 1 h, the substrate gels were equilibrated in 5% (v/v) Triton X-100 by two 10 min treatments to remove the SDS, and then incubated in assay buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij 35, 0.02% NaN_3) for 20 h at 37°C. The gels were then fixed for 15 min in 10% acetic acid solution. The undigested substrate and sample proteins were visualized by Coomassie Brillant Blue R-250 staining. Proteolytic activity was detected as cleared bands against the aqua-blue background stain of the undigested substrate in the gel. Activated and latent gelatinase standards (M_r 66 and 59 kDa, respectively) were obtained from conditioned culture medium of rat osteosarcoma cells (ROS 17/2.8) stimulated or not with concanavalin A under serum-free conditions (30). The apparent M_r of the nonreduced enzymes was calculated by using molecular mass markers under reducing conditions (+DTT) (SDS-6H, Sigma Chemical Co, St Louis, MO): rabbit muscle myosin (205 000 Da), *Escherichia coli* β-galactosidase (116 000 Da), rabbit muscle phosphorylase b (97 400 Da), bovine albumin (66 000 Da), egg albumin (45 000 Da) and bovine erythrocyte carbonic anhydrase (29 000 Da).

Statistical Analysis

Differences between age groups were compared using the Chi-squared test. Fischer's exact test was used to assess the significance of the presence of gelatinase A in human dentin. Data were analyzed with the SPSS/PC+ program (SPSS Inc., Chicago, Illinois). The incidence of true and false positives and negatives, and sensitivity and specificity values, were calculated (31).

Results and Discussion

Human dentin extracts were processed by zymography. When G1-extracts from human dentin samples were analyzed (Fig. 1), multiple forms of gelatinolytic enzymes were detected, with gelatinase A (M_r 66 and 59 kDa -DTT) being predominant (58% of the samples). In addition, other gelatinolytic bands were detected in the 92 and 54 kDa M_r range of other MMPs known to have gelatinase activity (gelatinase B; stromelysin or collagenase, respectively). Other cleared bands were also found at 30 kDa, that may correspond to degradation products from gelatinase A (30).

Gelatinolytic activity in extracts obtained during EDTA demineralization of human dentin (E-extracts) was also analyzed, but proteolytic activity was not present in any of the 24 samples assayed. Because EDTA may have removed necessary divalent cations from the MMPs, assays were repeated by incubation in assay buffer containing 2 mM $ZnSO_4$. Nevertheless, gelatinolytic activity was still not detected (results not shown). Irreversible inactivation of MMPs resulting from EDTA treatment did not occur, as shown by the detection of gelatinolytic enzymes in the G2-extracts. Figure 2 shows the major bands of gelatinolytic activity detected in samples of individual G2 dentin extracts. The predominant cleared bands were identified as gelatinase A in 29% of the samples of the G2 dentin extracts. Low molecular weight cleared bands (30 kDa), also detected in G2-extracts, may have corresponded to gelatinase A degradation products. However, unlike the G1-extracts, no other gelatinolytic activity was observed at other M_rs in these guanidinium chloride extracts obtained after demineralization of dentin.

We also investigated the changes in gelatinase A in human dentin with aging. Table 1 shows the patterns of occurrence of

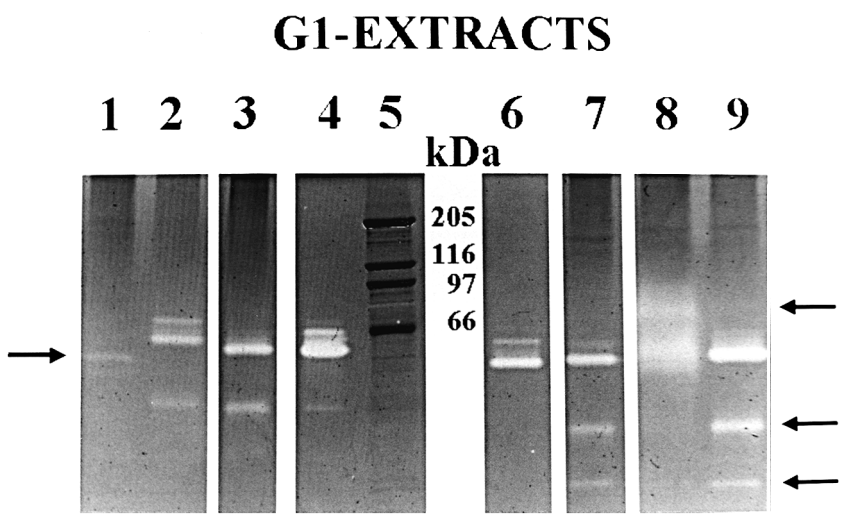

FIG. 1—*The presence of gelatinase A in human dentin prepared by guanidinium chloride extraction of pulverized samples (G1-extracts). Samples of dentin proteins from individual human molar were analyzed by zymography using gelatin as the substrate as described in Materials and Methods. Undigested substrate was stained with Coomasie Brilliant Blue R-250. G1-extracts of dentin samples from individuals representing different age groups were analyzed in lanes 1–3 and 7–9. Chronological age of the individual for each sample was as follows: lane 1, 73 years; lane 2, 64 years; lane 3, 56 years; lane 7, 19 years; lane 8, 20 years and lane 9, 36 years. A single arrow indicates the presence of other proteinases with gelatinolytic activity different from gelatinase A. Lanes 4 and 6 show gelatinase A standard (M_r 66 and 59 kDa). Lane 5, molecular mass markers: rabbit muscle myosin (205 kDa),* Escherichia coli *β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97 kDa) and bovine albumin (66 kDa).*

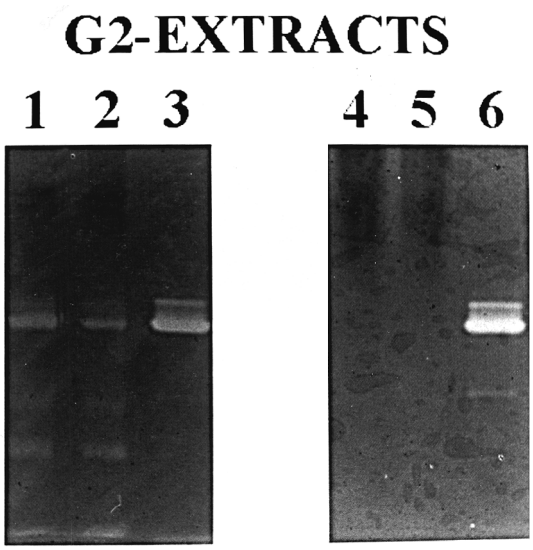

FIG. 2—*The presence of gelatinolytic proteinases in human dentin prepared by guanidinium chloride extraction of pulverized samples after EDTA demineralization (G2-extracts). Samples of dentin from individuals representing different age groups were analyzed as described in the legend of Fig. 1 and in Materials and Methods. Lanes 1, 2, 4, and 5 correspond to samples from individuals aged 19, 17, 57, and 56 years respectively. Lanes 3 and 6 show gelatinase A standards.*

gelatinase A in G1- and G2-extracts from human dentin in different age groups. Differences in gelatinase A patterns were detected between age groups: in age Groups 1 and 2 (individuals younger than 40 years old) gelatinase A was present in 67% of G1-extracts, whereas this percentage decreased to 50% in individuals older than 41 years. Because the first guanidinium treatment extracts only proteins that were associated with hydroxyapatite crystals and not those embedded in mineral, this enzyme fraction represents protein synthesized after mineralization has been completed. Differences between age groups were also evident for the changes in patterns of gelatinase A expression in the G2-extracts. In age Group 1, gelatinase A was present in all cases (100%); in Group 2 this decreased to 17% of samples containing gelatinase A; and the enzyme was not detected at all in samples from Groups 3 and 4 (subjects from 41 to 73 years old). Significant differences in the presence of gelatinase A in G2-extracts were found between individuals younger than 20 years old and the rest of the sample ($\chi^2_{exp} = 19.429$; 1 d.f.; $p \leq 0.001$). It is interesting to note that the second guanidinium treatment dissociates proteins from the collagenous dentin matrix remaining after mineral dissolution. Thus, our data indicate that gelatinase A is incorporated into the mineralized matrix of the dentin during mineralization, and is most likely bound to the collagen but not to the hydroxyapatite (32,33). The presence of gelatinase A in the G2 fraction of dentin extracts from young people is interesting for two reasons. First, the presence of active enzyme is strong evidence for in vivo activity of gelatinase A during dentin matrix remodeling prior to and during mineralization. Second, the presence of gelatinase A in the G2-extract shows that gelatinase A was deposited in the dentin matrix during tooth development prior to or during mineralization. Therefore, these findings highlight the extreme stability of the enzyme when embedded in mineral.

Statistical studies of the incidence of true and false positive and negative values were performed, and sensitivities and specificities were calculated for the presence of gelatinase A in G2 human dentin extracts as a possible discriminatory criterion in establishing the age of an individual. Accordingly, sensitivity and specifity were calculated using 20 years old as the best possible cut-off point of the test (maximizing sensitivity and specificity). Therefore, the presence of gelatinase A in G2-extracts from human dentin is suggestive (100% sensitivity and 94% specificity) of an individual younger than 20 years old. This result is interesting because of its

TABLE 1—*Characteristics of the samples and presence (+) or absence (−) of gelatinase A in human dentin G1- and G2-extracts.*

	Sample No.	Age (Years)	Sex*	Molar†	Gelatinase A	
					G1-extracts	G2-extracts
Group 1	1	15	F	46	+	+
	2	17	F	46	+	+
	3	17	F	48	−	+
	4	17	M	48	−	+
	5	19	F	28	+	+
	6	20	F	48	+	+
Group 2	7	27	M	18	+	−
	8	31	M	18	+	+
	9	36	M	46	−	−
	10	36	F	48	+	−
	11	37	F	38	+	−
	12	40	F	27	−	−
Group 3	13	41	M	27	−	−
	14	50	M	17	+	−
	15	52	M	16	+	−
	16	52	M	17	−	−
	17	56	F	36	+	−
	18	57	M	16	−	−
Group 4	19	61	M	46	−	−
	20	61	M	36	+	−
	21	61	F	36	−	−
	22	64	F	47	+	−
	23	69	F	47	+	−
	24	73	F	27	−	−

* F = Female; M = Male.
† According to FDI notation.

potential application in forensic dentistry, especially in cases of fragmented or isolated teeth when other morphological methods are of limited usefulness.

In summary, we conclude that determination of gelatinase A in human dentin may be a useful tool, providing forensic odontologists with an alternative method to estimate the age of an individual, especially when used in combination with, complementary, morphological or biochemical dental methods. Further research is still needed to provide more information on the postmortem establishment of human age.

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