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Accumulation of advanced glycation end-products in human dentine

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ABSTRACT

Cross-linking of collagen by Advanced Glycation End-products (AGEs) occurs by non-enzymatic glycation (Maillard reaction). The purpose of this study was to examine whether AGEs are formed in human dentinal collagen, and to consider any possible influence of AGEs on dentinal physiology. Mechanical characteristics, fluorescence spectra and immunohistochemical analyses of demineralized dentine sections from young subjects were compared with those of aged ones. The same investigations were performed with young dentine artificially glycated by incubation in 0.1 M ribose solution. Indentation measurement indicated that the sections from aged dentine were mechanically harder than those from young dentine. The hardness of young dentine increased after incubation in ribose solution. Fluorescence peak wavelength of the young dentine was shorter than that of the aged one, but shifted towards the peak wavelength of the aged one after incubation in ribose solution. These changes were considered to be due to accumulation of AGEs. Existence of AGEs in dentinal collagen was confirmed by immunohistochemical analysis. The obtained results suggest that AGEs accumulation occurs in dentinal collagen and is affected by both human age and physiological conditions such as glucose level in blood because dentinal collagen receives nourishment via dental pulp and tubules.

1. Introduction

Ageing is an irreversible and physiological phenomenon that cannot be avoided in human beings. In aged persons, the teeth usually appear more brown than those in young subjects. This phenomenon is characteristic of ageing. Glycation of body protein is one of the most interesting processes in human ageing. Several studies have reported that the nonenzymatic reaction of blood glucose with body proteins leads to cumulative chemical modifications of tissue proteins throughout the body.[1–4] This reaction, named Maillard reaction, is a biological process that occurs in tissues, finally resulting in formation of advanced glycation end-products (AGEs). Maillard first noted in 1912 that amino acids heated in the presence of reducing sugars turned brown. During the formation of AGE, first the aldehyde group of reducing sugars binds to ε-amino group of proteins without enzyme and forms Schiff base. The Schiff base becomes an Amadori product through the Amadori rearrangement. They are then converted non-reversibly into stable substances through oxidation, dehydration, and condensation. If oxidation accompanies glycation, the formed products are glycation products, for examples, pentosidine[5] and N-carboxymethyl-lysine (CML).[6–8] Thus, AGE is the collective term for the products of Maillard reaction. The free AGEs easily bind to collagen and act as a cross-link between collagen fibrils. Hence, accumulation

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of AGEs associated with cross-linking alters the mechanical characteristics of collagen-rich tissues.

We assume that the Maillard reaction also occurs in dentinal collagen and as a result, AGEs accumulate near dental canals during one's life. This phenomenon results in brownish discoloration and modification of the mechanical properties of dentine, because dentinal collagen receives nourishment through dentinal tubules via canals. However, there are few investigations concerning the Maillard reaction in vital tooth. The main purpose of this study was to determine whether AGEs produced by Maillard reaction accumulate in human dentine physiologically. In the present study, we performed mechanical indentation analysis, fluorescence spectroscopy, immunohistochemical staining and immuno-electron microscopy in demineralized dentine to detect AGEs formation.

2. Materials and methods

2.1. Sample preparation

Six caries-free third molars of young and aged patients (young 18–26 yrs, aged 68–76 yrs), extracted as part of routine treatment at Osaka University Dental Hospital, were prepared for the experiment. The teeth were collected with the patients' consent and preserved in Hank's balanced salt solution (HBSS) at 4 °C till the experiments. The experimental protocol was approved by the Ethics committee of the Faculty of Dentistry, Osaka University.

Samples were sectioned parallel to the tooth axis with an Isomet low-speed diamond wheel saw under water in order to obtain slabs of approximately 1.0 mm thickness. The sections were immersed in 4% paraformaldehyde and 0.1% glutaraldehyde (for immuno-electron microscopy), or 4% paraformaldehyde (for other measurements), then subsequently demineralized for four weeks with 10% EDTA in room temperature. For *in vitro* glycation, the demineralized sections were incubated in 0.1 M ribose solution in HBSS buffer at 37 °C for 6 weeks.[9,10]

2.2. Mechanical test

Hardness of the demineralized sections was evaluated using a mechanical indentation tester (Shimadzu EZ-S) equipped with an indentation probe of diameter 1 mm. The probe indented up to 50 μm in depth from the sample surface to measure force–displacement characteristics. Based on the resultant force–displacement curve, the slope corresponding to a spring constant was calculated (note: unit of the slope is N/mm, the slope becomes steeper as the sample becomes harder). More than 25 points were probed in demineralized dentine to examine the regional heterogeneity of the hardness. The glycated young dentine samples were also subjected to the same mechanical trials.

2.3. Fluorescence analysis

Autofluorescence of the sections from young and aged groups was measured. Because the measurement area of the dentinal collagen was a few mm square, we have employed a conventional fluorescence spectrometer for liquid sample (Shimadzu RF-5300, Kyoto, Japan) instead of a microfluorometer. The fluorescence excitation wavelength was set around 370 nm according to the previous report by Kleter et al.,[14] and fluorescence emission spectra between 390 nm and 700 nm were recorded. Because the spectrometer is designed for the measurement of a liquid sample in a quadratic prism-shaped glass cuvette, the sections were placed on the diagonal plane of the cuvette holder to examine the middle portion of the dentine layer. To eliminate unwanted stray light due to reflection and/or scattering on the sample surface, an additional UV cut filter was placed between the sample and the monochromator for fluorescence emission in the spectrometer.

2.4. Immunohistochemical staining

Demineralized dentine blocks were embedded in paraffin block after graded-ethanol dehydration, and sliced into 4 μm-thick section samples using a microtome (Leica Microsystems GmbH, Wetzlar, Germany). Immunohistochemical staining was performed with an LSAB2 kit, according to the manufacturer's instruction (DAKO, Glostrup, Denmark). The following primary antibody which recognizes the C-terminal region of CML was used: mouse anti-AGE monoclonal antibody (Clone No. 6D12; Transgenic Inc., Japan).[11,12] Sections were lightly counterstained with haematoxylin. As negative controls, mouse serum IgG (Dako) was used as the primary antibody, and these gave uniformly negative results.

2.5. Immuno-electron microscopy

Dentine blocks were embedded in LR Gold Resin System (Electron Microscopy Sciences, PA, USA) at −20 °C with an ultraviolet polymerizer system (DOSAKA EM CO. LTD., Kyoto Japan). After curing, samples were sectioned to 70 nm in thickness with a diamond knife (Nanotome Thick, Sakai Advanced Electron Microscope Research Center, Japan), and mounted on nickel grids (Nisshin EM Tokyo Japan) in a ultramicrotome (Ultrotome V, LKB, Sweden). The sections were immersed in Donkey serum (1:10) and incubated overnight at 4 °C with mouse monoclonal AGEs (1:100) primary antibody (Clone No. 6D12, Transgenic Inc., Japan). Secondary antibody, donkey anti-mouse IgG (18 nm colloidal gold; Jackson, PA, USA), was diluted (1:10) in 0.2% tween 20/TBS plus 5% albumin. Specimens were fixed with 2% glutaraldehyde and then stained with 2% uranylacetate. A transmission electron microscope (H800, Hitachi, Tokyo, Japan) at 200 keV was employed for detection of AGE-bound colloidal golds.

2.6. Statistical analysis

The values for the stiff were presented as mean ± SEM for statistical analysis. Obtained values were analyzed using one-way ANOVA and Tukey–Kramer tests with a significant level of $p < 0.05$.