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Analysis of contribution of collagen fibre component in viscoelastic behaviour of periodontal ligament using enzyme probe

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Abstract

The aims of this study are to observe microscopic changes in the periodontal ligament (PDL) collagen fibres after collagenase treatment, to analyse stress–relaxation behaviour of PDL specimens treated with collagenase, and to elucidate the contribution of the collagen component to the viscoelastic behaviour of the PDL. Transverse sections of rat mandibular first molars ($n = 24$) were treated *in vitro* with 0, 8, 16, or 24 units of bacterial collagenase for 4 h at 37 °C. Histological specimens were then prepared, and image analyses were done for polarised light microscopic appearances of collagen fibres. Further, stress–relaxation tests were performed for PDL specimens treated with 8 units of collagenase ($n = 7$) and control specimens ($n = 7$). Image analysis showed that higher concentrations of collagenase reduced greater area occupied by the PDL collagen fibres and birefringent retardation of the fibres. The amount of stress–relaxation during 600 s was 1.37 times greater in the collagenase-treated specimens than in the controls. The observed values of the stress–relaxation process were well described by a function with three exponential decay terms. The relaxation parameters of the first and second terms did not show significant differences, but those of the third term did so between the collagenase-treated and control specimens. The ratio and relaxation time of the third term for the collagenase-treated specimens were significantly less than those for the controls. These findings suggest that in the long-term relaxation component of the stress–relaxation process of the PDL the viscoelastic properties of the collagen fibres may play an important role.

Keywords: Periodontal ligament; Viscoelastic behaviour; Collagen fibres; Collagenase

1. Introduction

The periodontal ligament (PDL) is a dense fibrous connective tissue between the root of a tooth and its bony socket. The PDL binds the tooth to alveolar bone, resists displacing forces impinging upon the tooth, and supports the tooth in the jaw (Berkovitz et al., 1995). The PDL consists of collagen fibres embedded in ground substances containing cells, blood vessels, and nerves (Sloan and Carter, 1995). If connective tissues are viewed as composite materials, one must recognise that their mechanical behaviour is optimised in nature by an effective sharing of stress between the different phases (Hoffman and Daly, 1980). It is of considerable interest to separate and understand the contributions of the different components to the overall biomechanical behaviour of the PDL. Such knowledge is required for effective replacement or repair of damaged structures and functional tissue engineering (Robinson et al., 2004).

Various techniques have been utilized to remove various components from tissues so that any changes in the biomechanical properties may be ascribed to the missing components (Hoffman and Daly, 1980; Schmidt et al., 1990; Millesi et al., 1995). The *in vitro* treatment of specimens with enzymes is a more specific technique for removing individual components from specimens. We have previously shown that *in vitro* treatment with collagenase effectively degrades collagen fibres from the rat PDL and reduced the mechanical strength (Kawada and Komatsu, 2000). However, the effect of collagenase treatment on viscoelastic property of the PDL has not yet been investigated.

When a tooth is subjected to an excessive force, the PDL may dissipate the strain energy stored in the tissues.

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Viscoelastic responses are principal causes of energy dissipation. This would reduce the risk of injury to the PDL in the case of prolonged static deformation (Fung, 1981; Kwan et al., 1993; Tanaka et al., 1999; Provenzano et al., 2001; Sanctuary et al., 2005; Shibata et al., 2006). The stress–relaxation, one of viscoelastic responses, of the rat PDL during 10 min reaches about 50% (Komatsu et al., 2004a), which is greater than those of other soft connective tissues. Such viscoelastic response should play a role in the tooth support mechanism of the PDL. Methods of stress–relaxation test of the PDL and the analysis have previously been established (Komatsu et al., 1997, 2002a, 2004a, b; Toms et al., 2002; Sanctuary, 2003; Natali et al., 2004).

The aims of this study are to analyse changes in histologic appearances of the collagen fibres and those in the stress–relaxation behaviour of PDL specimens after collagenase treatment, and to elucidate the contribution of the collagen component to the viscoelastic behaviour.

2. Materials and methods

The experiment was approved by the Institutional Animal Care Committee of Tsurumi University School of Dental Medicine.

2.1. Experiment 1

Twenty-four male rats of the Wistar strain, aged 5 weeks, were purchased from Clea Japan (Tokyo, Japan). They were kept in metal cages (3 or 4 rats/cage), and given a powdered diet (CE-2, Clea Japan, Tokyo, Japan) and water ad libitum. The animals were killed with an overdose of ether at 6 weeks of age.

2.1.1. Preparation of PDL specimens

Immediately after death, the left mandibles were dissected free and the adhering soft tissues were removed. For each mandible, a transverse section (about 0.45 mm in thickness) of the first molar with surrounding PDL and alveolar bone were cut at the middle level of the mesial root through an axis perpendicular to the long axis of the root (Komatsu and Chiba, 1993) using a bone saw (Isomet, Buehler, IL, USA) with special care to avoid tissue denaturation (Watanabe and Komatsu, 1997).

2.1.2. Treatment with collagenase

Twenty-four transverse sections were divided into four groups of 6 specimens each. Specimens of each group were treated *in vitro* with 0, 8, 16, or 24 units of highly purified collagenase (from Clostridium histolyticum, type III; activity of collagen digestion, 1600 units/mg; Sigma, USA)/ml of phosphate-buffered saline (PBS). Each specimen was placed in 1 ml of a test solution and shaken (150 times/min) using an incubator (Bio-shaker 40BR-LF, Taitec Co., Japan) for 4 h at 37 °C. The concentrations of the enzyme were based on our previous study (Kawada and Komatsu, 2000). After treatment, the specimens were washed 3 times with cold PBS.

2.1.3. Histological analysis

The specimens were placed in neutral buffered 3.7% formaldehyde solution, decalcified in 10% EDTA solution, and embedded in glycol methacrylate resin. Seven μm-thick serial sections of the mesial root were cut longitudinally in a bucco–lingual direction using an electrically driven rotary microtome (2050 Supercut, Leica Instruments, Germany). Unstained sections were mounted in Diatex® (refractive index 1.50; AB Wilch. Becker, Sweden), and examined with a polarised light microscope (Laborlux 12 pol S, Leica Microscopie, Germany) (Komatsu and Viidik, 1996; Komatsu et al., 2002b): we observed the sections while crossed analyser and polariser were rotated around the optical axis of the microscope from 0° to 90°. The rotation angle was determined as zero when the cementum surface was parallel to the transmission direction of the polariser. As the mean angle of the PDL collagen fibres at the middle root level of rat mandibular first molars is about 60° (Komatsu and Chiba, 1993), the birefringent fibres could be seen with maximum brightness when the rotation angle was set at about 15°.

(1) *Estimation of area occupied by collagen fibres:* In each section, the polarised light microscopic image of birefringent fibres with maximum brightness at such angle on the buccal side of the PDL was taken on 35-mm colour positive film. The film was scanned and the area occupied by birefringent collagen fibres was estimated in 6 successive rectangular regions (30 μm in depth, 240 μm in width; Fig. 1) using NIH Image (Version 1.61, Bethesda, MD, USA)(Kawada and Komatsu, 2000) to examine changes in the amount of collagen fibres in the PDL of the specimen by collagenase treatment.
(2) *Measurement of birefringent retardation:* To examine changes in the molecular organisation of collagen fibres in the PDL specimens after collagnase treatment, we measured birefringent retardation of collagen in tissue sections (Whittaker et al., 1989; Nollie et al., 1996; Komatsu et al., 2002a, b) by the method of de Sénarmont–Friedel (Bennett, 1961; See the details in the Supplementary website material 1). Measurements were made for 6 successive rectangular regions (Fig. 1) on each unstained section.

Regression analysis was used to examine the relation between the concentration of collagenase and the area occupied by birefringent collagen fibres and between the concentration of collagenase and the birefringent retardation in each region. Significance of correlation coefficients (r) was examined by Student's t-test. The difference of the mean values between any two groups was examined with Scheffé's test.

2.2. Experiment 2

2.2.1. Preparation of mechanical specimens

Another 14 rats of the same strain and age were kept and killed in the same way as in experiment 1. Immediately after death, the left and right mandibles were dissected free and the adhering soft tissues were removed. From the left and right mandibles in each rat, a pair of transverse sections [455±12 (SD)μm in thickness] of the first molar with surrounding PDL and alveolar bone were cut at the middle level of the mesial root in the same method as in the experiment 1.

2.2.2. Measurement of area and width of the PDL

Radiographs of the transverse sections were taken in a soft X-ray apparatus (type EMB, Softex, Tokyo, Japan). The radiographic images were processed in an image analyser (Luzex 3U, Nikon, Japan). We measured the perimeters of the root cementum and socket inner wall of the mesial root and the sectional area of the PDL (Fig. 2). The area of the undeformed PDL facing the root cementum (SA) and the average width of the undeformed PDL (W) were calculated as previously described (Komatsu and Chiba, 1993).

2.2.3. Treatment with collagenase

Fourteen paired transverse sections were divided into two groups. The sections were placed in 8 units of collagenase/ml of PBS (collagenase group) or PBS only (PBS group), and shaken in the same method as in experiment 1. After treatment, the specimens were washed 3 times with cold PBS and kept at 4 °C until mechanical testing.

2.2.4. Mechanical tests

The method of stress–relaxation test was described previously in detail (Komatsu et al., 2004b). In brief, a transverse section was mounted on a micro-testing apparatus (Chiba and Komatsu, 1993). Then, by loading the