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Research paper

A novel method of sampling gingival crevicular fluid from a mouse model of periodontitis

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ABSTRACT

Using a mouse model of silk ligature-induced periodontal disease (PD), we report a novel method of sampling mouse gingival crevicular fluid (GCF) to evaluate the time-dependent secretion patterns of bone resorption-related cytokines. GCF is a serum transudate containing host-derived biomarkers which can represent cellular response in the periodontium. As such, human clinical evaluations of PD status rely on sampling this critical secretion. At the same time, a method of sampling GCF from mice is absent, hindering the translational value of mouse models of PD. Therefore, we herein report a novel method of sampling GCF from a mouse model of periodontitis, involving a series of easy steps. First, the original ligature used for induction of PD was removed, and a fresh ligature for sampling GCF was placed in the gingival crevice for 10 min. Immediately afterwards, the volume of GCF collected in the sampling ligature was measured using a high precision weighing balance. The sampling ligature containing GCF was then immersed in a solution of PBS-Tween 20 and subjected to ELISA. This enabled us to monitor the volume of GCF and detect time-dependent changes in the expression of such cytokines as IL-1b, TNF-α, IL-6, RANKL, and OPG associated with the levels of alveolar bone loss, as reflected in GCF collected from a mouse model of PD. Therefore, this novel GCF sampling method can be used to measure various cytokines in GCF relative to the dynamic changes in periodontal bone loss induced in a mouse model of PD.

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1. Introduction

Periodontal diseases (PD) are inflammatory bone lytic diseases caused by polymicrobial infection, indicating that several opportunistic pathogens act on host cells to produce inflammatory cytokines and enzymes that destroy periodontal soft tissue and alveolar bone (Taubman and Kawai, 2001). Importantly, host-destructive inflammatory biomarkers can be monitored in gingival crevicular fluid (GCF) in a noninvasive manner (Giannobile, 1997; Champagne et al., 2003; Armitage, 2004). Among a variety of host biomarkers in GCF thus far assessed (Loos, 2005), interleukin (IL)-1β, IL-6, and TNF-α appear to be signature biomarkers that reflect the level inflammation in the periodontal lesion (Buduneli and Kinane, 2011). In addition to these proinflammatory cytokines, it was previously demonstrated that an osteoclastogenic cytokine, receptor activator of NF-kB ligand (RANKL), is produced by activated adaptive immune cells in the bone resorption lesion of PD (Kawai et al., 2006). Since osteoclastogenic activity of RANKL is counter-regulated by its soluble decoy receptor osteoprotegerin (OPG) (Kajiya et al., 2010), measurement of RANKL/OPG ratio in GCF can indicate the activity of osteoclast precursors involved in pathological bone resorption.

It is well known that animal studies complement *in vitro* experiments prior to testing new treatments or diagnostic modalities. To reproduce human periodontal disease in a non-human laboratory *in vivo* system, numerous animal models of PD have been developed using small and large mammals (Struillou et al., 2010). Among these PD models, the mouse model of ligature-induced PD is one of the most

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frequently employed animal models to understand the molecular mechanism(s) underlying the onset and progression of PD, as well as to elevate the efficacy of novel interventions against pathogenic outcomes of PD (Abe and Hajishengallis, 2013). Especially, the availability of a large panel of mouse gene-knockout strains offers an advantage over other animal models in our efforts to elucidate pathogenic engagement of the gene of interest. On the other hand, this model is essentially flawed by the lack of suitable methodology to collect GCF, which is typically sampled by paper point in human or large mammals, such as dogs or monkeys (Chambers et al., 1991; Ha et al., 2011). However, it is nearly impossible to use such method to sample GCF from mice (Fig. 1A & B). Thus, it would be desirable to develop a simple method to sample GCF in a mouse model of ligature-induced PD.

The present study established a method to collect GCF from mice. The basic protocol for this technique is summarized as follows (Fig. 2):

I. Induce experimental periodontitis by placing a ligature at the second maxillary molar as described (Abe and Hajishengallis, 2013).
II. Remove the old silk ligature and mount a fresh ligature at the sampling site for 10 min.
III. Measure the volume of GCF collected in the sampling ligature using a high precision weighing balance.
IV. Submerge the sampling ligature in PBS containing 0.05% Tween 20 and shake with a vortex mixer.
V. PBS solution containing sampled GCF can be subjected to measurement of biomarkers using ELISA or similar methods.

2. Materials and methods

2.1. Mouse model of ligature-induced periodontitis

To induce experimental periodontitis, wild-type C57BL/6j mice (6- to 8-week-old) were used. The animals were anesthetized by intraperitoneal injection of a cocktail of ketamine (80 mg/kg) and xylazine (10 mg/kg). This cocktail allows us to anesthetize animals for at least 30 min. After mice were anesthetized, a silk ligature (5-0 silk threads, Johnson & Johnson, New Brunswick, NJ, USA) was placed on the upper left second molar and left for 24 h, 3, and 7 days as described (Abe and Hajishengallis, 2013). Briefly, a ligature was placed through the proximal contacts of the upper left second molar until reached the gingival margin. The procedure was performed using two Castroviejo micro needle holder (Fine Science Tools, CA, USA) under the stereomicroscope assistance (Seiler Evolution xR6, Seiler Microscope, MO, USA). Suture was tied firmly with a double-knot on the buccal side. Altogether, 10–15 min was required for successful ligature placement. The excess suture was cut using Vannas spring scissors (Fine Science Tools, CA, USA). The upper right second molar without ligature was used as a control.

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the experimental procedures were approved by the Forsyth Institutional Animal Care and Use Committee (IACUC).

2.2. Sampling and measurement of GCF volume

After induction of PD, the ligature was removed from the left side, followed by placement of a new, fresh weighed silk thread (2 cm in length) around the second molar at the left side (inflammatory site) and right side (healthy, non-ligature site). After 10 min, the ligatures from the both sides were collected and then weighed using a Mettler Analytical Balance to a sensitivity of 0.05 μg (Mettler Instrument Corporation, Hightstown, NJ, USA). In order to avoid the bleeding, the primary ligature, that had been used for induction of periodontitis, was removed as gently as possible which barely caused the bleeding. However, if we found the bleeding from gingiva at the removal of primary ligature or at the placement of fresh sampling ligature, we excluded the GCF samples from the subsequent measurements of cytokines. Finally, the collected sampling ligatures were submerged in 100 μL of phosphate buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T) and shaken with a vortex mixer for 30 min at +4 °C.

2.3. Saliva collection

In order to rule out the possibility that cytokines in the saliva may be contaminated at the GCF sampling which may, in turn, affect the measurements of GCF cytokines, saliva of mouse was also collected and the amounts of cytokines in saliva were measured using ELISA. We used the same silk threads (5-0, Johnson & Johnson) as that used for the GCF collection. Briefly, the silk threads (2 cm length) were weighed first and then were placed into the mouse mouth for 10 min just before GCF collection.

2.4. Enzyme-linked immunosorbent assay (ELISA)

IL-1 β, IL-6, TNF-α, RANKL and OPG levels in GCF and saliva were determined using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). The weight of GCF and saliva (ng) that was collected using a ligature was converted to volume (μL). Then, the concentrations of cytokines in GCF and Saliva were normalized based on the

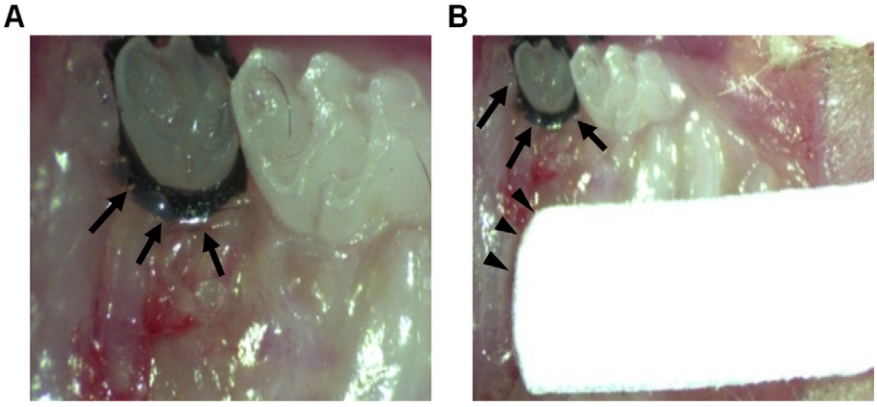

Fig. 1. The size of mouse gingival crevice as compared to the PerioPaper strip used for GCF sampling from human patients. **A)** To induce a periodontal lesion in mice, a silk ligature was placed around the second molar. **B)** By comparing PerioPaper strip (Oraflow Inc. Hewlett, New York, USA), which is typically used to sample human GCF, and the anatomy of mouse gingival crevice, the difficulty of collecting GCF for the ligature-induced lesion is readily apparent. Arrows indicate ligature attached to induce PD in mouse. Arrowheads indicate the PerioPaper strip used for human GCF sampling.

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