HHS Public Access

Author manuscript

J Immunol Methods. Author manuscript; available in PMC 2017 November 01.

Published in final edited form as:

J Immunol Methods. 2016 November ; 438: 21–25. doi:10.1016/j.jim.2016.08.008.

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 ten minutes. 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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Keywords

Periodontal disease; gingival crevicular fluid; ligature-induced periodontal disease; mice

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 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 minutes.

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 anaesthetized, 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 (uL). Then, the concentrations of cytokines in GCF and Saliva were normalized based on the volume of GCF and saliva weighed, respectively, based on the readout from the ELISA kits. More specifically, the PBS-T containing GCF or saliva (See sections 2.2. and 2.3.) was directly applied to the ELISA plates. In particular, in order to save the samples, all incubation steps were carried out at the volume of 75 ul/well, and the same sample in PBS-T was transferred one by one to different ELISA kits. Finally, the level of each cytokine detected by respective ELISA kit was converted to the concentration at the order of pg/ml.

2.5. Determination of Alveolar Bone Loss Area in Ligature-induced Periodontitis

Alveolar bone loss induced in mice was evaluated by stereomicroscopy (Nikon dissecting microscope SMZ745T) and micro-CT (SCANCO micro–computed tomography (mCT)-40 scanner). Using a dissection microscope (Nikon, Tokyo, Japan), horizontal bone loss of buccal aspect was assessed by measuring the distance between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC) (Kawai et al., 2007). MicroCT (mCT20; SCANCO Medical AG, Bassersdorf, Switzerland) was performed on the maxillary bone of mice that received ligature for 3 and 7 days or control nontreated mice.

2.6. Histological Evaluation and tartrate-resistant acid phosphatase (TRAP) staining

Mice maxillae with or without ligature for 7 days were decalcified in 10% ethylene-diamine-tetraacetic acid (EDTA) for 2 weeks and embedded in OCT compound. Frozen sections 10-μm thick were cut in a medial-distal direction and stained with Hematoxylin and Eosin (H&E). TRAP staining was performed to detect osteoclasts, followed by nuclear counterstaining with methyl green as described (Kawai et al., 2007).

2.7. Statistical analysis

Student's *t*-test was used for comparison of two different outcomes of experiments performed. To assess possible differences in bone resorption, as well as the levels of cytokines produced in GCF during the course of periodontitis development in mice, ANOVA was performed followed by Tukey's test.

3. Results

3.1. Ligature-induced alveolar bone loss and histological analysis in PD

According to microscopic and microCT evaluations, mouse maxilla with ligature at second molar demonstrated remarkable bone loss (Suppl. Fig. 1A). While visible bone loss was observed as early as Day-3 after ligature placement (Suppl. Fig. 1A), statistical difference compared to control jaw without ligature was detected at Day-7, but not Day-3 (Suppl. Fig. 1B). Prominently elevated emergence of TRAP-positive osteoclast cells was found in the alveolar bone of tooth the received a ligature compared to that of control (Suppl. Fig. 1C, D). These observations indicated that ligature placement elicited local osteoclastogenesis which, in turn, induced periodontal bone loss.

3.2. Dynamic changes of GCF cytokines in the course of ligature-induced PD development

A ligature was placed on left second molar of the maxilla, while right second molar served as a control and did not receive ligature. At 24 h from ligature attachment, GCF was collected from the tooth that received a ligature and showed a significantly elevated volume compared to that of control group (Fig. 3).

Next, using this GCF sampling technique, we measured the levels of proinflammatory cytokines secreted in the GCF of mice induced of PD. At 24 h from placement of ligature, the highest levels of IL-1β, TNF-α, and IL-6 were detected in the GCF of inflammatory sites (left molar), gradually diminishing at Day 3 and Day 7 (Fig. 4A). In contrast to elevated levels of proinflammatory cytokines monitored in the GCF of ligated side, GCF collected from control side showed no elevation from Day-0 to Day-7 (Fig 3A). More specifically, the levels of these cytokines detected in the ligated site were all significantly higher than the control site (Fig. 4A). It is noteworthy that the sampling ligature soaked with saliva of mice did not show any detectable amount of IL-1β, TNF-α, or IL-6, as sampled from Day-0, 1, 3 and 7 (data not shown), suggesting that the detected pro-inflammatory cytokines are derived from GCF, not saliva.

Since RANKL/OPG ratio is an important indicator of osteoclastogenesis-mediated periodontal bone loss, we evaluated the fluctuation dynamics of OPG and RANKL secreted in GCF. The protein levels of RANKL in GCF increased at 3 and 7 days after ligature placement, while a significantly higher level of OPG was only detected in the ligated site at Day-7 compared to control site. Comparing RANKL/OPG ratio between ligated site and control site, a slight increase was detected at Day-1, reaching a maximum level at Day-3 and then decreasing by Day-7. Importantly, RANKL/OPG ratios were significantly higher in ligated site compared to control site throughout the experimental period (Fig. 4B).

Altogether, the results from this study suggested that the established method of sampling mouse GCF allows us to monitor various cytokines in GCF relative to the dynamic changes that occur during the progression of periodontal bone resorption.

4. Discussion

In the present study, we established a novel noninvasive method of sampling gingival crevicular fluid (GCF) from a silk ligature-induced mouse model of periodontitis. GCF is an exudate that seeps into gingival crevices or periodontal pockets around teeth with inflamed gingiva (Cimasoni, 1983). GCF can be obtained in a noninvasive fashion, making it an accessible source of biomarkers to evaluate ongoing pathophysiological events in periodontal tissues (Armitage, 1996; Kennett et al., 1997; Eley and Cox, 1998). As noted in the introduction, it is nearly impossible to use regular paper points to sample GCF from mice (Fig. 1B). In the present study, we could successfully sample mouse GCF using a silk ligature. We showed that GCF volume was significantly increased in the inflammatory ligature-induced site compared to the control non-ligature placed site (Fig. 3). This is, indeed, a significant technical breakthrough in utilizing a mouse model of PD in that it allows the measurement of real-time production of biomarkers in affected periodontal tissues without sacrificing mice.

The signature proinflammatory cytokines associated with human PD, including IL-1β, TNF-α, and IL-6, were detectable in GCF of the ligature-induced mouse model of PD and showed peak expression as early as Day-1 (Fig. 4A). A previous study induced PD by attachment of a silk ligature soaked with live *Porphyromonas gingivalis* suspension to a molar in a non-human primate model. Results showed that inhibition of IL-1 β and TNF-α could suppress periodontal bone destruction (Graves and Cochran, 2003), indicating the pathogenic roles of IL-1 β and TNF-α in the onset of PD. However, the kinetics of IL-1 β and TNF-α expressions in relation to the expressions of other bone resorption regulatory molecules have been unclear. As demonstrated in this study, detection of IL-1β and TNF-α in GCF which peaked at Day-1 and followed by elevation of RANKL in the later days corresponded to the progression of periodontal bone resorption. Since IL-1β and TNF-α are known to promote RANKL production of bone marrow stromal cells and upregulate RANKL-induced osteoclastogenesis (Romas et al., 2002; Kitaura et al., 2013), it is plausible that peak expressions of IL-1β and TNF-α precede peak expressions of RANKL, as well as bone resorption. Indeed, the peak RANKL/OPG ratio was detected later on Day-3 compared to the maximum level of IL-1 β and TNF-α on Day-1 (Fig. 4A & B). These results indicated that the established GCF sampling method can detect the smallest differences of biomarker expression in GCF.

In conclusion, this novel method of sampling mouse GCF can measure the time-dependent secretion patterns of bone resorption-related cytokines relative to changes in periodontal bone loss, but without sacrificing experimental mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by grants DE-03420, DE-18499, DE-19917, and T32 DE 7327-12 from the National Institute of Dental and Craniofacial Research (NIDCR), Brain Circulation Program to Develop New Leaders for

International Dental Education Course through International Collaborative Dental Research and a research fund from King Abdulaziz University.

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HIGHLIGHTS

1. No suitable methodology to collect GCF from mice;
2. An assay to collect GCF from a mouse model of periodontal disease was developed;
3. The assay could detect various biomarkers in GCF using ELISA.

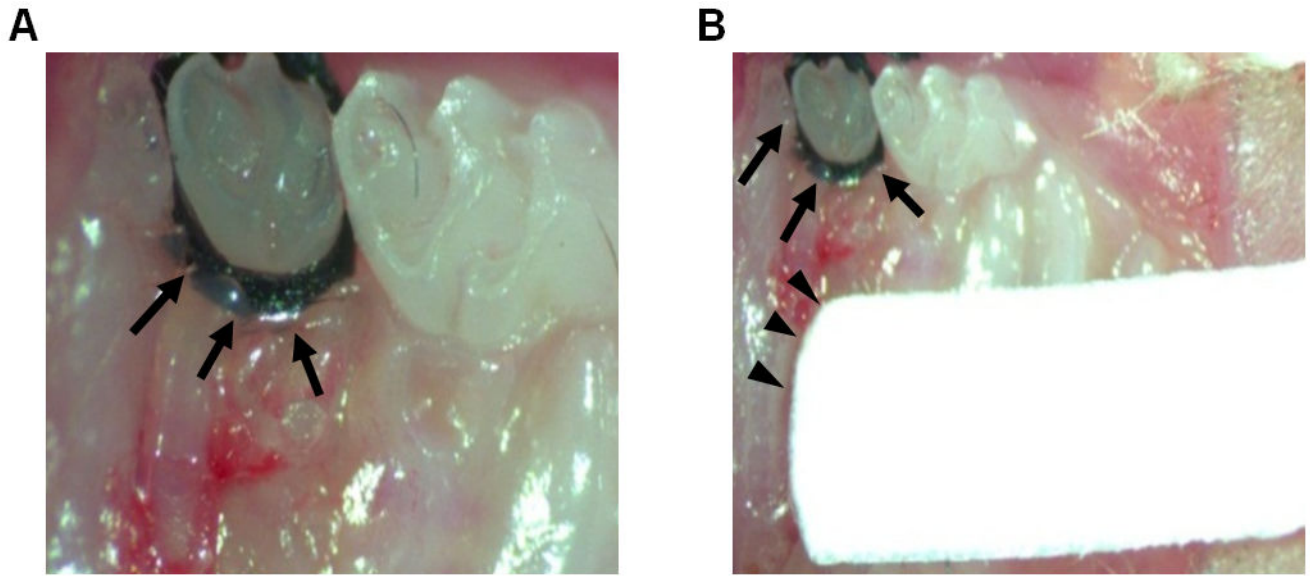

Figure 1. The size of mouse gingival crevice as compared to the PerioPaper strip used for GCF sampling from human patients

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.

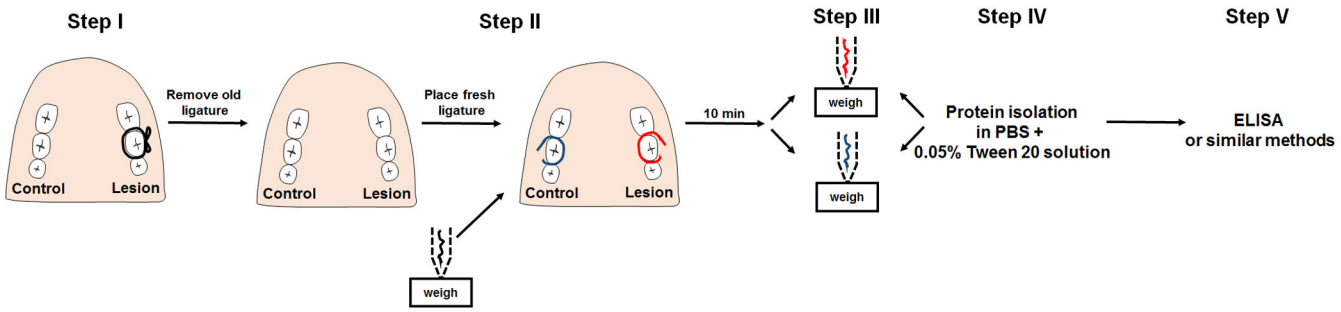

Figure 2. Schematic presentation of the developed GCF collection protocol

Step I: Induce experimental periodontitis by placing a ligature at the second maxillary molar; Step II: Replace the old silk ligature by a fresh one with known weigh; Step III: Collect the ligatures after 10 minutes and weighed; Step IV: Protein isolation from the collected ligatures in 100 μl PBS solution containing 0.05% Tween 20; Step V: Biomarkers measurement by ELISA or similar methods.

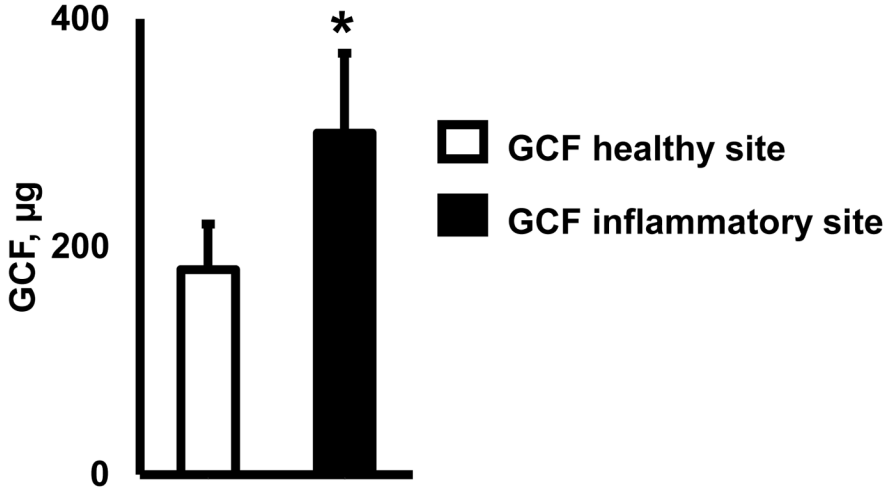

Figure 3. The volume of GCF was elevated in ligated site compared to non-ligated site using GCF sampling ligature

Volume of GCF sampled from healthy control sites (right) and inflammatory sites (left) of mice that received ligature to the left side, but not right side, of second molar in maxilla for 24 hours (n=5 mice/group). *$p<0.05$

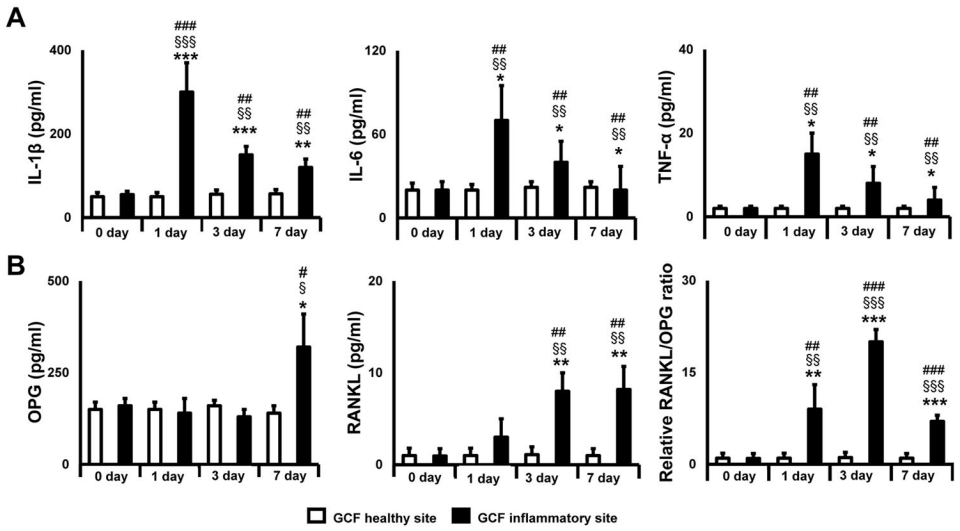

Figure 4. Detecting a battery of cytokines in the GCF using a new silk ligature

A) Dynamic changes of some pro-inflammatory cytokines in murine GCF of healthy and inflammatory ligature-induced sites; **B)** Dynamic changes of RANKL and OPG ratio in in murine GCF of healthy and inflammatory ligature-induced sites. GCF was sampled from the healthy and inflamed sites of PD-induction ligature at the indicated time points. The levels IL-1β, TNF-α, and IL-6 were evaluated by ELISA (n=5 mice/group) and the concentrations of cytokines in GCF were normalized based on the volume of GCF weighed (See sections 2.2.). *p<0.05, **p<0.01, ***p<0.001.