Immunoassay analysis of proteins in gingival crevicular fluid samples from resorbing teeth

Wellington J. Rody Jr.; Manjula Wijegunasinghe; L. Shannon Holliday; Kevin P. McHugh; Shannon M. Wallet

ABSTRACT

Objective: To carry out an immunoassay analysis of biomarkers expressed in gingival crevicular fluid (GCF) with the main goal of finding a useful diagnostic pattern to distinguish between resorbing deciduous teeth and nonresorbing controls.

Materials and Methods: A split-mouth design was used in this study with a total of 22 GCF samples collected from 11 patients in the mixed dentition. For each child, one deciduous molar with radiographic evidence of root resorption was used as the test tooth whereas the contralateral first permanent molar with formed roots was used as the control tooth. Samples were processed with immunoassays using a panel of selected biomarkers including interleukin-1 beta (IL-1b), interleukin-1 receptor antagonist (IL-1RA), nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG), matrix metalloproteinase-9 (MMP-9), and dentin sialoprotein (DSP).

Results: There were no statistically significant differences in levels of IL-1b, OPG, and MMP-9 between test and control sites \((P > .05)\). IL-1RA was the only biomarker to show a significant down-regulation \((P < .04)\) in GCF samples collected from resorbing teeth. RANKL data showed a heavily skewed distribution and was deemed unreliable. Only one deciduous GCF sample had detectable levels of DSP; therefore, no further statistical calculation was applicable because of the limited amount of data for this biomarker.

Conclusions: This study indicated that IL1-RA is down-regulated in GCF from resorbing primary molars, thus suggesting this cytokine as a potential analyte to be included in a panel that can discriminate between resorbing and nonresorbing teeth. (Angle Orthod. 2016;86:187–192.)

KEY WORDS: GCF; Microarray; Root resorption; Biomarkers

INTRODUCTION

The ability to diagnose diseases and to monitor ongoing biological processes through fluid-based diagnostic tests has been a desirable goal in health care. At present, immunoassays are the primary tool for determining protein concentrations in biological samples for many reasons, including high sensitivity and specificity. An immunoassay is a biochemical test that can detect a substance in a complex solution via the binding between antigens and antibodies and detectable labels that signal this binding. Over the past few years, the development of advanced proteomic platforms has given clinicians the ability to measure multiple biomarkers in just one experiment. Microarrays, for instance, are multiplex enzyme-linked immunosorbent assay (ELISA) platforms that use miniaturization in chip format to quantify large numbers of proteins in a small sample volume and therefore provide an attractive approach for gingival crevicular fluid (GCF) research.

GCF biomarkers can be categorized into broad categories on the basis of their biological significance. The inflammatory cytokines are classified as pro-inflammatory or anti-inflammatory. Pro-inflammatory ones are tumor necrosis factor, interleukin-1 (IL-1),...
IL-2, IL-6, and IL-8. Anti-inflammatory cytokines are interleukin-1 receptor antagonist (IL-1RA), IL-4, IL-10, and IL-13. Cytokines act synergistically or antagonistically on each other; thus, the relative activities of inflammatory mediators may be useful to predict underlying biological changes. Osteoclastogenesis-related factors, such as receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) form the second group of biomarkers that has received considerable attention in the past several years. Osteoclast differentiation requires the binding of RANKL, a cell membrane protein found on osteoblasts, to receptor activator of nuclear factor kappa (RANK), also a cell membrane protein but found on osteoclast and osteoclast-precursor cells. OPG, on the other hand, acts as a decoy receptor that binds to RANKL and blocks osteoclastogenesis. Abnormalities of these biomarkers in body fluids have been implicated in the pathogenesis of periodontitis and tooth movement. A third group encompasses molecules associated with extracellular matrix destruction. Turnover of extracellular matrix is governed mainly by matrix metalloproteinases (MMPs), which are a family of secreted and membrane-bound enzymes. Matrix metalloproteinase-9 (MMP-9), a gelatinase derived predominantly from monocytes and macrophages, is thought to play a critical role in periodontal tissue degradation and its levels in GCF may be used as a marker for collagen turnover. Since larger areas of dentin resorption do not repair, root-derived proteins in GCF may also be useful markers for root resorption and tooth exfoliation. Of the various dentin noncollagenous proteins, dentin sialoprotein (DSP) and dentin phosphoprotein are the most abundant proteins present within dentin and their presence in GCF of resorbing teeth has been reported by previous human studies.

Analyses of GCF collected from human deciduous teeth are very few in the literature in spite of the fact that a deeper understanding of the GCF proteome profile in resorbing teeth may be of paramount importance for the management of important clinical challenges. On one hand, proteins that are differentially expressed in the GCF of resorbing teeth may be useful markers of root resorption. On the other hand, a different expression pattern between resorbing and nonresorbing teeth may be of clinical use as prognostic markers for tooth exfoliation. Prolonged retention of primary teeth is a common cause of malocclusion and tooth impaction. Thus, the GCF protein content of resorbing primary molars was examined in great detail by our group using cutting-edge proteomic techniques including mass spectrometry and immunoassays. The result of the mass spectrometry analysis was published in another article, and readers are referred to Rody et al. for further information. In this paper, we report an ELISA-microarray analysis of proteins expressed in GCF with the main goal of finding a useful diagnostic pattern to distinguish between resorbing primary molars and nonresorbing controls.

**MATERIALS AND METHODS**

**Study Population**

Eleven children (4 boys, 7 girls) who sought treatment in the department of Orthodontics at the University of Manitoba were enrolled in this study. The patients met the following inclusion criteria: (1) Good health, (2) no evidence of caries or periodontal problems, and (3) mixed dentition with mandibular permanent first molars fully erupted and lower primary second molars with radiographic evidence of half of the roots resorbed. Assent and consent forms were obtained from all participating children and their parents. The research protocol was approved by the Health Research Ethics Board at the University of Manitoba (H2011:289).

**Study Design**

A split-mouth clinical design was used in this study with a total of 22 GCF samples collected from 11 patients. For each patient, one mandibular primary second molar with radiographic evidence of root resorption was used as the test tooth, whereas the first permanent molar with fully formed roots on the contralateral quadrant was used as the control tooth.

**GCF Collection**

GCF sampling was performed with paper strips (Periopaper, Oraflow Inc, Plainview, NY). Before GCF collection, supragingival plaque was removed with cotton pellets and the area gently dried for 5 seconds with an air stream. Cotton rolls were used to keep the area free of saliva, and the paper strip was inserted for 40 seconds into the gingival sulcus on the lingual side of each selected tooth until mild resistance was felt. Strips contaminated with saliva or blood were discarded. The selected paper strips were then placed into sealed tubes and frozen at −80°C.

**Protein Elution and Immunoassay Analysis**

Proteins were eluted from paper strips by centrifugation. Each strip was eluted separately by adding 100 μL of phosphate-buffered saline (PBS, Invitrogen, Camarillo, Calif) and shaken at room temperature for 15 minutes. Subsequently, the strips were carefully removed from the Eppendorf tube and the eluate was centrifuged at 3000 g for 5 minutes at 4°C to remove
bacterial biofilms and cellular elements. A set of target proteins was chosen based on their relevance to periodontal tissue remodeling as outlined in the Introduction. Thus, a customized protein microarray slide containing IL-1b, IL1-RA, MMP-9, RANKL, and OPG (Quantibody, RayBiotech, Norcross, Ga) was used to analyze the GCF samples. In summary, small droplets of antibodies were fixed in an orderly pattern onto a glass surface and used as capture molecules to detect the selected biomarkers. A schematic of the chip and images of control (permanent tooth) and test (resorbing primary tooth) arrays are shown in Figure 1.

**Figure 1.** Microarray chip spotted with 16 wells of identical biomarker antibody arrays (left). The array was customized to identify the biomarkers IL-1b, MMP-9, IL1-RA, RANKL, and OPG. Notice that each antibody droplet, together with the positive (Pos) and negative (Neg) controls, is printed in quadruplicate. Array images representative of a control tooth (top) and a resorbing tooth (bottom) from the same patient demonstrate the lower intensity of the fluorescent signal that comes from the line that represents IL-1RA (*) in the resorbing tooth.

We also conducted a single-plex ELISA in an attempt to measure the levels of DSP in GCF samples with the Human DSP Elisa Kit (USCN LIFE; Wuhan EIAab Science Co, Wuhan, China). Briefly, standards and 100 μL of diluted GCF samples were added to the precoated microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for DSP. Next, avidin conjugated to horseradish peroxidase was added to each microplate well and incubated. This was followed by adding 100 μL of the color reagent solution tetramethylbenzidine peroxidase substrate to each well. Only those wells containing DSP, biotin-conjugated antibody, and enzyme-conjugated avidin exhibit a color change. The enzyme-substrate reaction was terminated by adding stop solution to each well and the color change measured spectrophotometrically at a wavelength of 450 nm using a microplate reader. The concentration of DSP (ng/mL) was determined by comparing the optical density of the samples with the standard curves.

**Statistical Analysis**

Paired *t* tests were used to determine whether the within-pair differences for each measure were significantly greater than zero. The paired analysis was necessitated by the study design, which collected data from two teeth (test vs control) in the same mouth. In addition, the results were confirmed with nonparametric tests. All the measures were analyzed on the original scale. Significance was set at a *P* < .05 and the SAS statistical software package (version 9.22; SAS Institute, Cary, NC) was used for all analyses.

**RESULTS**

In total, four of the six evaluated biomarkers were detectable within the range of the assays and were present in both resorbing (test) and nonresorbing (control) sites. The results are shown in Table 1. Figure 2 displays the microarray data in box-and-whisker plots, which permit better visual summarization of skewed distributions with outliers. The diamond shape inside the boxes is the mean, and the middle line inside the boxes is the median. Whiskers range from lowest observation at the bottom to largest observation at the top. The edges of the boxes are the lower and upper quartiles. In our study, there were no statistically significant differences for IL-1b, OPG, or MMP-9 between resorbing deciduous teeth and permanent teeth control sites (*P* > .05). RANKL data were deemed unreliable so were excluded from the final analysis due to heavy skewness and the large number of zeros. IL-1RA was the only cytokine to show a statistically significant down-regulation (*P* = .04) in GCF samples collected from test sites (resorbing deciduous teeth). There was a lower concentration of IL-1RA in the resorbing site than in the control site (3552 vs 7827 pg/mL). ELISA revealed that only one
decidual molar GCF sample (patient 6) had detectable levels of DSP (1.1 ng/mL). The other 21 samples from primary and permanent teeth had levels of DSP at or below the detection limit of the assay (0.312 ng/mL) and were set as zero; therefore, no further statistical calculation was applicable because of the limited amount of data for this biomarker.

**DISCUSSION**

At present, the diagnosis of abnormal tooth root resorption is based on a radiograph or a computed tomography scan. Nevertheless, the risks associated with ionizing radiation have caused many health care professions to rethink their use at preset intervals as an attempt to screen for asymptomatic diseases. Therefore, diagnosis of root resorption can be challenging due to the absence of a reliable noninvasive screening method. Fluid biomarkers are becoming increasingly important in diagnosis because they allow less invasive and more precise measurement of a disease process. In a previous study, we carried out a proteomic analysis of GCF from root resorption sites that have yielded a list of novel biomarker candidates to evaluate periodontal tissue remodeling. Studies are underway to test these candidates with regard to their ability to distinguish resorbing from nonresorbing sites. The goal of the present work was to evaluate the levels of well-studied GCF proteins that show promise in improving the monitoring of the tissue remodeling processes associated with root resorption and exfoliation.

Primary tooth exfoliation and permanent tooth eruption are tightly regulated processes in which not only bone resorption but also dentin resorption are required. The dental follicle that lies above the erupting permanent tooth plays a key role in tooth transposition through the mineralized tissue crypt since the follicular cells secrete important chemicals to replenish osteoclasts and odontoclasts in the area. In this cascade of molecular signals that regulates tooth eruption within the follicle, Que et al. demonstrated that the IL-1 family plays a major role. The IL-1 family is comprised of 3 ligands, IL-1a, IL-1b, and IL-1RA, all of which bind the IL-1 receptor. IL-1RA is a naturally occurring anti-inflammatory protein, which acts by limiting IL-1-mediated inflammatory conditions and bone resorption. Our study found expression-level variations between resorbing and nonresorbing sites for most biomarkers; however, the differences reached statistical significance only for IL-1RA (Table 1, Figure 2). This cytokine is present in high levels in GCF, and previous studies demonstrate that IL-1RA can be reliably quantitated in human GCF using immunoassays. The role of IL-1RA as an important inhibitor of bone remodeling is well documented in the literature, and some studies show a positive correlation between decreased levels of IL-1RA in GCF and faster bone resorption in periodontitis and orthodontic tooth movement. Our finding seems to support this association: The down-regulation of IL-1RA in the periodontal tissues during tooth eruption could allow for greater mineralized tissue resorption and may help explain the low IL-1RA concentration in the GCF of resorbing teeth.

When we looked at the results, the first unexpected finding was the lack of difference in DSP concentrations between test and control teeth. Numerous studies in recent years suggest that root resorption can be evaluated in GCF using fragments of noncollagenous dentin proteins released in the periodontium. In our study, DSP, a promising biomarker of root resorption, was not detected with ELISA in most of the test samples. This can be explained by the fact that dentin proteins are so highly phosphorylated and shielded by carbohydrates that they are not particularly antigenic, which makes the development of antibodies for immunoassays particularly challenging. Moreover, it is possible that our samples were either too diluted for subsequent analysis or the traditional ELISA method was not sufficiently sensitive to detect DSP in GCF. Recently, Sha et al. recommended a novel method that combines ELISA with electrochemical analysis of GCF samples for the detection of dentin proteins. The authors state that the high sensitivity of this method extends the detection limit of the assay.

**Table 1.** Concentration of Biomarkers and Statistical Analysis Results

<table>
<thead>
<tr>
<th></th>
<th>IL-1b (pg/mL)</th>
<th>IL1-RA (pg/mL)</th>
<th>RANKL (pg/mL)</th>
<th>OPG (pg/mL)</th>
<th>MMP-9 (pg/mL)</th>
<th>DSP (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>36.669</td>
<td>30.827</td>
<td>3552</td>
<td>7827</td>
<td>145.46</td>
<td>290.67</td>
</tr>
<tr>
<td>SD</td>
<td>86.5</td>
<td>47.4</td>
<td>1763</td>
<td>5770</td>
<td>169.67</td>
<td>319.58</td>
</tr>
<tr>
<td>Median</td>
<td>6.189</td>
<td>10.863</td>
<td>2777</td>
<td>8760</td>
<td>101.86</td>
<td>169.67</td>
</tr>
</tbody>
</table>

Paired t test NS S (P < .05); S, significant difference; NA, not analyzed.
and could be a new way to measure dentin proteins in minute samples such as GCF. This issue will be taken into account by our group in planning future studies.

Despite the recent advancement in diagnostic techniques, root resorption remains the most distressing side effect of orthodontic treatment. The main challenge in the clinics is to detect root resorption at early stages; thus, it is of great interest to confirm that GCF analysis remains a feasible aim. Compared with saliva, the analysis of GCF offers important opportunities. It is less complex and more site-specific. In addition, GCF seems not to require any purification or depletion of highly abundant proteins (eg, albumin), which in turn may remove low concentration biomarkers from the sample. Because of the high dynamic range of proteins in most human body fluids, it is very unlikely that one standalone GCF biomarker for detecting root resorption will be available in the near future. On the other hand, we hypothesize that a panel of multiple biomarkers reflecting inflammation, periodontal tissue injury, and destruction of mineralized tissue matrices would have better sensitivity and specificity for diagnosis of root resorption than any single biomarker.

Some limitations of this study need to be addressed. First, GCF was collected from resorbing primary teeth.

Figure 2. Box plots displaying biomarker concentrations in GCF samples obtained from resorbing deciduous teeth and permanent teeth control sites. A significant difference (* \( P < .05 \)) between resorbing and nonresorbing sites was observed only for IL-1RA.
Physiologic root resorption may differ from orthodontic root resorption; thus, the results from our study may not fully apply to the latter case. Second, although GCF was collected from the most distal site at the permanent first molar to prevent contamination, proteins released during resorption of the primary molar may have influenced the GCF composition of the control tooth. Finally, this study did not include a method error quantitation analysis to further validate the reproducibility of results as suggested by Perinetti et al.\textsuperscript{22}

**CONCLUSION**

The results of this pilot study suggest that IL1-RA is down-regulated in GCF from resorbing primary molars, thus indicating this cytokine as a potential analyte to be included in a biomarker panel to monitor tooth eruption or diagnose root resorption.

**ACKNOWLEDGMENT**

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**REFERENCES**


