

Application of Proteomic Technologies to Discover and Identify Biomarkers for Periodontal Diseases: Moesin is a Potential Mediator and Biomarker for Periodontal Disease

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Abstract

As a fluid in close proximity to periodontal tissue, the Gingival Crevicular Fluid (GCF) is the principal target in the search for biomarkers of periodontal disease, because its protein composition may reflect disease pathophysiology.

For the identification of novel GCF biomarkers of periodontal disease, GCF samples from five patients with severe periodontal disease were analyzed by liquid chromatography-mass spectrometry/mass spectrometry. Two hundred twenty-five proteins were identified as possible candidates for GCF biomarkers. Of these marker candidates, we focused on a protein not well studied in periodontal disease, i.e., moesin, a member of the ezrin-radixin-moesin family. Western blotting analysis revealed that moesin expression in GCF was higher in patients with severe periodontal disease than in controls. We further examined moesin protein expression levels in human gingival fibroblasts (HGFs) following stimulation with lipopolysaccharide (LPS) from *Porphyromonas gingivalis*. Moesin expression in HGFs was found to be increased by LPS treatment in a dose-dependent manner. Our findings suggest that moesin may be involved in progression of periodontal disease and is a potential biomarker of periodontal disease.

Keywords: Moesin; Periodontal disease; Proteomics

Abbreviations: LC-MS/MS: Liquid Chromatography and Tandem Mass Spectrometry; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; 2-DE: Two-Dimensional Electrophoresis; AU: Arbitrary Units; WB: Western Blotting

Introduction

Periodontal diseases are primarily caused by bacterial infections that damage the gingiva and the surrounding tissues of the oral cavity. In addition, some systemic diseases have been shown to affect periodontal health [1].

Gingival crevicular fluid (GCF) is a fluid located in the gingival crevices and periodontal pockets. It contains both host-derived and bacteria-derived proteins [2], which play a central role in periodontal tissue turnover [3-5]. Protein profiles of GCFs obtained from healthy individuals have not been well characterized. To identify periodontal disease biomarkers, a comparative study of protein profiles of GCFs obtained from individuals with and without periodontal disease is required. In our previous study, we characterized the proteomic patterns of GCFs obtained from healthy subjects as a first step toward identifying periodontal disease biomarkers [6]. In this study, we sought to characterize the protein profiles of GCFs obtained from individuals with periodontal disease.

Proteomic analysis of complex protein mixtures, such as GCFs, is commonly performed using one-dimensional polyacrylamide gel electrophoresis (1-DE) or two-dimensional polyacrylamide gel electrophoresis (2-DE), followed by the identification of resolved proteins by using MS or MS/MS. These techniques have been previously used to monitor quantitative changes of GCF proteins [7].

A comparative analysis of protein expression of GCFs obtained from healthy individuals and individuals with periodontal diseases would help to identify proteins involved in periodontal disease progression. In our previous study, we used the aforementioned methods for identifying proteins expressed in GCFs of healthy individuals [6]. Among the identified proteins, moesin [a disease-associated protein that belong to the Ezrin-Radixin-Moesin (ERM) protein family] was of particular interest because of its role in cell-cell recognition, signaling, and cell movement [8]. In addition, another ERM family protein, ezrin, was detected in GCF from 2 patients with severe periodontal disease [9]. Recently, Amar et al. [10] demonstrated that moesin may play a role in lipopolysaccharide (LPS) signaling via CD14 and toll-like receptor (TLR) pathways. Previous studies have demonstrated that LPS from periodontal pathogens stimulate host cells to secrete pro-inflammatory mediators such as interleukin (IL)-1, IL-6, tumor necrosis factor-alpha, and prostaglandin E2 [11,12]. The role of inflammatory cytokines in the development of periodontal disease is also well documented [13]. Thus,

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moesin and other ERM family proteins may play an important role in the recognition of oral bacteria and in the consequent development of inflammatory immune responses involved in periodontal disease development.

In this study, proteomic studies were performed and several biomarker candidates were identified. Moesin was characterized as a potential mediator and biomarker of periodontal disease.

Materials and Methods

Collection of gingival crevicular fluid (GCF)

GCF samples were collected from 17 volunteers (10 males, 7 females, the mean age of 43.0 years). Prior to sample collection, the subjects rinsed their mouths. All volunteers provided informed consent to participate in the procedures. All were in good general health with no history of antimicrobial or anti-inflammatory therapy or periodontal treatment for 6 months before the start of the study. Since smoking is a risk factor for periodontal disease, the current study did not include smokers. Clinical parameters such as probing depth, clinical attachment level, gingival bleeding index, full-mouth plaque scores and bleeding scores of each group were recorded. The probing depth at six sites for each tooth was measured using the PCP-UNC15 probe (Hu-Friedy, Chicago, IL, USA). For recording the loss of clinical attachment level, the cemento-enamel junction was used as a reference. Participants were first examined for periodontal disease. Full-mouth Löe-Silness gingival index and clinical attachment loss were measured at six sites (mesio-, mid-, disto-buccal/palatal or lingual) per tooth using the PCP-UNC15 probe with a diameter of 0.5 mm at the tip [14,15]. The periodontal disease subjects were assigned to three groups (Moderate, Mild and Severe) on the basis of overall clinical diagnostic criteria and the classification of periodontal diseases by the American Academy of Periodontology (AAP) 1999 [16] (Table 1). The study sample included in this study was as follows: healthy; n=50, mild periodontal disease; n=30, moderate periodontal disease; n=30, severe periodontal disease; n=110.

All GCF samples were collected and processed as described previously [6]. Briefly, GCF was collected from the labial side of maxillary incisors without crown and restoration. Supra-gingival plaque was carefully removed from the tooth by a curette, the teeth were rinsed with saline, and the sampling sites were isolated with cotton rolls and dried. Each sample site was carefully isolated using cotton rolls to avoid saliva contamination. Absorbent paper points (ZIPPERER®, Munich, Germany) were gently inserted into the gingival crevice and left in place for 30 sec. Mechanical irritation was avoided and the absorbent paper points contaminated with blood were discarded. These paper points were stored at -80°C for further processing. GCF obtained from the 17 patients was randomly divided into two groups; a test set for proteomic analysis, and a validation set (Table 1). The test set included GCF from five patients. The validation set included GCF from 12 patients and GCF from 5 healthy volunteers.

In-solution digestion of proteins

Protein extraction, reduction, alkylation and enzymatic in-solution digestion of proteins were performed as described previously [6].

Four molar urea and 100 mM ammonium bicarbonate were added to the paper point containing GCF. The paper point was vortexed for 10 min and centrifuged at 20000 × g for 15 min. Then, 2 µl of 200 mM DTT was added and incubated at 57°C for 30 min. After incubation, 2 µl of 600 mM iodacetamide was added and incubated at room

temperature for 30 min in dark. Five microliter of trypsin with 26 µl of distilled water was incubated at 37°C for 6 h. The resulting peptides were added to 5 µl of 5% TFA or 5% formic acid.

In-solution digested peptides were injected into a trap column (C₁₈, 0.3×5 mm, DIONEX, CA, USA), and an analytical column (C₁₈, 0.075×120 mm, Nikkyo Technos, Tokyo, Japan), which was attached to the Ultimate 3000 (DIONEX, CA, USA). The flow rate of the mobile phase was 300 nL/min. The solvent composition of the mobile phase was programmed to change in 120-min cycles with varying mixing ratios of solvent A (2% v/v acetonitrile and 0.1% v/v formic acid) to solvent B (90% v/v acetonitrile and 0.1% v/v formic acid) : 5-10% B 5 min, 10-13.5% B 35 min, 13.5-35 % B 65 min, 35-90% B 4 min, 90% B 0.5 min, 90-5% B 0.5 min, 5% B 10 min. Purified peptides were introduced from HPLC to LTQ-Orbitrap XL (Thermo Scientific, San Jose, CA, USA), a hybrid ion-trap Fourier transform mass spectrometer. The MASCOT search engine (version 2.2.6, Matrixscience, London, UK) was used to identify proteins from the mass and tandem mass spectra of peptides. Peptide mass data were matched by searching the UniProtKB database (SwissProt 2010x, November 2010, 9590 entries). Database search parameters were: peptide mass tolerance, 1.2 Da; fragment tolerance, 0.6 Da; enzyme was set to trypsin, allowing up to one missed cleavage; variable modifications, methionine oxidation. The minimum criteria of protein identification were set as false discovery rate (FDR) <1%. The FDR was estimated by searching against a randomized decoy database created by the Mascot Perl program supplied by Matrix Science.

SDS-PAGE and western blot analysis

SDS-PAGE and Western blotting was performed as described previously [6]. The protein concentration of the extract was estimated using the Bradford method, with bovine serum albumin as a standard. We separated 10 µg/µl GCF protein using SDS-PAGE on a 10–20% polyacrylamide gradient gel and transferred to PVDF membranes (Millipore, Bedford, MA) in a Bio-Rad Trans-Blot apparatus (Bio-Rad, Hercules, CA). The membranes were blocked at room temperature in PBS with pH 7.5, containing 5% skim milk and 0.05% Tween 20. Anti-Moesin mouse polyclonal antibody (A01: Abnova, Taipei, Taiwan) diluted 1:500 in blocking buffer were used as primary antibodies diluted 1:1000 with the same buffer, and the membranes were incubated in it overnight at 4°C. Subsequently, the membranes were washed three times in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and incubated for 1 h with goat anti-mouse IgG HRP (Bio-Rad Laboratories, Hercules, CA) diluted 1:2000 in blocking buffer used as secondary antibodies. The bands were visualized by use of ECL (GE Healthcare UK Ltd., Buckinghamshire, UK) Band intensities were quantified using TotalLab TL12 imaging analysis software (Shimadzu Co., Ltd. Kyoto, Japan) and were represented by arbitrary units (AU).

Human gingival fibroblasts culture

Primary human gingival fibroblasts (HGFs) were isolated by the outgrowth method using gingival tissues. HGFs were obtained from a sample of healthy tissue from patients presenting at the periodontics clinic of the Tokyo Medical and Dental University after providing informed consent. The ethical committee of the Tokyo Medical and Dental University approved the study protocol. HGFs were cultured in 35-mm tissue culture dishes with Dulbecco's Modified Eagle's Medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Bioserum, Melbourne, Victoria, Australia) and 1% antibiotic-antimycotic mixture (Invitrogen Corporation, Carlsbad, CA), and incubated at 37°C under humidified atmosphere of 95% air and 5% CO₂. HGFs were stimulated with different concentration of

LPS from *Porphyromonas gingivalis* (PG-LPS; InvivoGen, San Diego, CA) for 24 h.

Immunoprecipitation

HGF cell lysate (200 µl) was added to anti-Toll-like receptor 4 (TLR4) rabbit polyclonal antibody (H-80; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and incubated with gentle rocking overnight at 4°C. Protein L-Sepharose (BioVision, Milpitas, CA, USA) (20 µl of 50% bead slurry) was also added. The resulting lysate was incubated with gentle rocking for 1–3 h at 4°C and microcentrifuged for 30 s at 4°C. The pellet was washed five times with 500 µl of 1× cell lysis buffer, kept on ice during washes, re-suspended with 20 µl of 3× SDS sample buffer, vortexed, then microcentrifuged for 30 s.

The sample was then heated to 95–100°C for 2–5 min, microcentrifuged for 1 min at 14,000×g, then and loaded (15–30 µl) on an SDS-PAGE gel (12–15%). The sample was then analyzed by silver staining.

Statistical analysis

The differences in values between periodontal and healthy subjects were analyzed by Mann-Whitney U-test and Kruskal-Wallis test with the Dwass-Steel-Christchlow-Flinger method. Differences at $p < 0.05$ were considered statistically significant.

Results

LC-MS/MS analysis for the identification of expressed proteins in GCF obtained from individuals with severe periodontal disease

In our previous study, GCF samples from five healthy individuals were pooled and subjected to trypsin digestion. The samples were then used for protein identification using LC-MS/MS [6]. Proteins, including moesin, were identified in GCF by using LC-MS/MS analysis [6].

GCF samples obtained from five individuals with severe periodontal disease were pooled together and then subjected to trypsin digestion. The samples were then used for protein identification by using LC-MS/MS. As shown in supplemental table 1, 225 proteins, including previously identified GCF proteins (apolipoprotein A-I, superoxide dismutase 1, dermcidin, matrix metalloproteinase-8, matrix metalloproteinase-9, and moesin), were identified. However, ezrin was not detected in GCF from individuals with severe periodontal disease.

Moesin expression in GCF from healthy individuals and individuals with periodontal disease

After identifying moesin as a candidate biomarker, we compared the levels of moesin protein expression in GCF from both healthy individuals and individuals with periodontal disease. Western blot analysis was performed and the data indicated that moesin expression was significantly higher in GCF from individuals with severe periodontal disease than in GCF from healthy individuals ($p = 0.00106$; Figure 1).

Expression levels of moesin in LPS-stimulated HGFs

The abundance of gingival fibroblasts in patients with chronic adult periodontitis varies in response to the changing levels of bacterial LPS [17]. As mentioned earlier, moesin was shown to be involved in LPS induced intracellular signaling via TLR pathways. We therefore sought to characterize the effect of LPS stimulation on moesin expression in

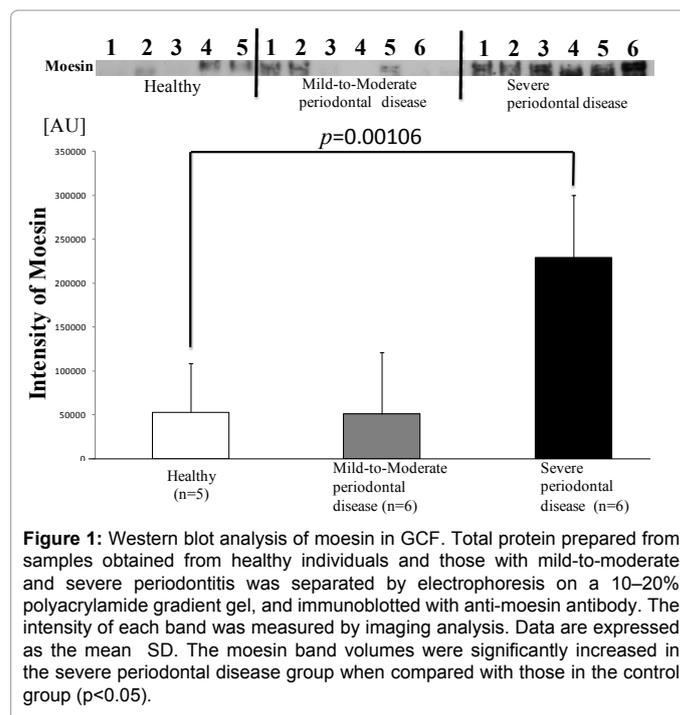


Figure 1: Western blot analysis of moesin in GCF. Total protein prepared from samples obtained from healthy individuals and those with mild-to-moderate and severe periodontitis was separated by electrophoresis on a 10–20% polyacrylamide gradient gel, and immunoblotted with anti-moesin antibody. The intensity of each band was measured by imaging analysis. Data are expressed as the mean SD. The moesin band volumes were significantly increased in the severe periodontal disease group when compared with those in the control group ($p < 0.05$).

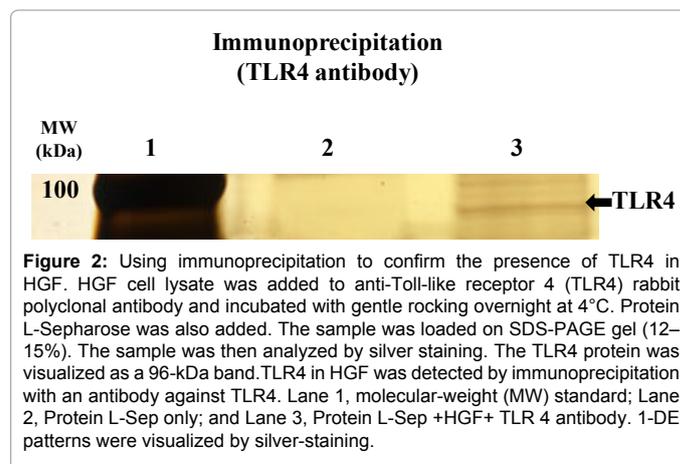


Figure 2: Using immunoprecipitation to confirm the presence of TLR4 in HGF. HGF cell lysate was added to anti-Toll-like receptor 4 (TLR4) rabbit polyclonal antibody and incubated with gentle rocking overnight at 4°C. Protein L-Sepharose was also added. The sample was loaded on SDS-PAGE gel (12–15%). The sample was then analyzed by silver staining. The TLR4 protein was visualized as a 96-kDa band. TLR4 in HGF was detected by immunoprecipitation with an antibody against TLR4. Lane 1, molecular-weight (MW) standard; Lane 2, Protein L-Sep only; and Lane 3, Protein L-Sep + HGF + TLR 4 antibody. 1-DE patterns were visualized by silver-staining.

HGF. We first demonstrated that TLR4 is expressed on HGF cells by performing immunoprecipitation and 1D-PAGE analysis (Figure 2). The TLR4 protein was visualized as a 96-kDa band (Figure 2).

The effect of LPS stimulation on moesin expression was assessed using HGFs stimulated with LPS from *P. gingivalis*. Moesin expression in HGFs increased after LPS treatment in a dose-dependent manner (Figure 3). Furthermore, expression levels of moesin were higher in LPS-stimulated HGFs than in control HGFs ($p = 0.02$; Figure 3).

Discussion

Previously, Tsuchida et al developed a novel protocol for effective protein extraction from GCF and then conducted gel-based and gel-free proteome analyses of GCF in comparison with supragingival saliva from five healthy subjects [6]. Analysis of biological fluids is only the first step in the identification of a biomarker. Tsuchida et al. [6] performed proteomic profiling of GCF samples from healthy individuals. Proteins, including moesin, were identified in GCF using LC-MS/MS analysis

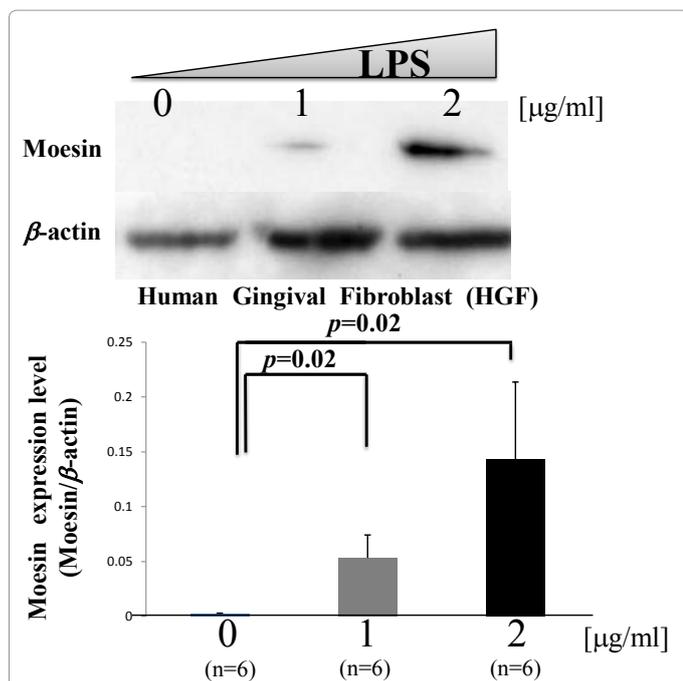


Figure 3: Expression of LPS-stimulated moesin in HGF. HGF was cultured in 6-well dishes and stimulated with LPS from *P. gingivalis* treatment for 24 hrs. Total cell lysates were prepared and separated by electrophoresis on a 10–20% polyacrylamide gradient gel and immunoblotted with the anti-moesin antibody. The intensity of each band was measured and the moesin protein levels normalized with β -actin were calculated. The intensity of each band was measured by imaging analysis. Data are expressed as the mean \pm SD of the number of replicates (n). Results shown are from three independent experiments. The moesin band volumes were significantly increased in the severe periodontal disease group when compared with those in the control group ($p < 0.05$).

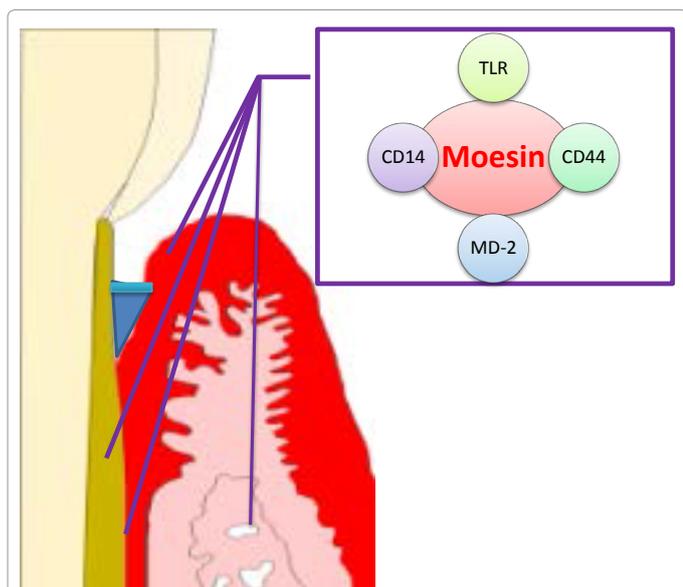


Figure 4: Hypothesis of the protein-protein interaction among Moesin, CD44 and several other key co-regulated factors such as CD14 and TLR. We have presented a hypothesis that the protein–protein interaction among Moesin, CD44, and several other key coregulated factors, such as members of the CD14 and TLR pathways, contribute to periodontal disease.

[6]. However, to discover and identify biomarkers for periodontal disease, proteomic analysis for the identification of expressed proteins in GCF obtained from individuals with periodontal disease is required. Therefore, we focused on GCF from subjects with severe periodontal disease, for which we conducted the gel-free proteome analysis. In this study, proteins, including moesin, were identified in GCF obtained from individuals with periodontal disease. Therefore, moesin may be a highly reliable candidate for a periodontal disease marker. To assist researchers during the different stages of analysis and to improve our understanding of biological system, the development of specific bioinformatics tools for proteomics applications is required.

Moesin, a membrane-organizing extension spike protein, is involved in connections of major cytoskeletal structures to the plasma membrane [18]. Moesin has been implicated in the development of several human diseases, including cancer and liver injury [19,20]. However, the role of moesin in periodontal disease has not been explored thus far.

Our western blot studies indicated that moesin protein expression was significantly increased in GCF of individuals with periodontal disease (Figure 1). Thus, moesin may either be a byproduct or a mediator of the pathophysiology of periodontal disease. The elevated moesin levels also indicate that moesin could be potentially used as a biomarker for periodontal disease. Furthermore, our cell culture stimulation studies demonstrated that moesin expression was increased by LPS stimulation in a dose-dependent manner (Figure 3). Thus, moesin may be involved in the TLR4 mediated responses that induce the production of inflammatory responses, which can exacerbate periodontal disease progression.

Toll-like receptor (TLR) 2 and TLR4 have been identified as signaling receptors for bacterial cell wall components [21]. Cell wall components of the major periodontal pathogenic bacteria (*P. gingivalis*, *A. actinomycetemcomitans*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Tannerella forsythensis*) stimulate TLR2 and TLR4. Following the receptor-ligand interaction, downstream signaling events lead to the production of pro-inflammatory cytokines such as interleukin-1 β and tumor necrosis factor [22-24]. These cytokines recognize and specifically bind to pathogenic agonists such as lipopeptide (via TLR2) and lipopolysaccharide (LPS; via TLR4) by molecular pattern recognition. For LPS recognition, the LPS-binding protein, CD 14, and MD-2 are required. Moesin has roles in LPS signaling in the CD14 and TLR pathways [10]. Zawai et al. [25] reported that moesin appears to be increasingly expressed in LPS-stimulated macrophages. Among the periodontal tissues, HGFs are the most abundant in the gingival [26]. Since HGFs express LPS-recognition membrane proteins (CD14, TLR4, and MD-2) that can mediate pro-inflammatory cytokine production upon LPS stimulation, the HGFs may have an important role in modulating host defense against putative gram-negative periodontopathogens [27] (Figure 2). We showed that moesin expression in HGFs increased with increasing LPS concentrations (Figure 3). Moreover, we demonstrated that moesin protein levels increased significantly in severe periodontal disease (Figure 1). Analysis of our data indicates that moesin may be involved in the development and progression of periodontal disease (Figure 4).

Previously, ERM proteins have been implicated in aiding the spread of invasive bacteria via rearrangement of the cytoskeletal actin [28]. Duterme et al. [29] speculated that hyaluronidase-2 separates CD44 molecules at the cell surface and the cleavage leads to loss of pericellular coat and inhibition of ERM activation. Hyaluronidases of

pathogenic bacteria have been implicated in the current literature as virulence factors facilitating the bacterial invasion of tissues [30,31]. Bacterial hyaluronidases directly facilitate the spread of infection by degrading HA [32]. Lokeshwar et al. [33] reported that low molecular weight HA is associated with inflammation and tumor progression. CD44 is involved in cell migration and cell adhesion in a variety of physiological and pathological processes. Moreover, activity of CD44 requires binding to ERM protein [34]. To examine the differences in expression of CD44 in various periodontal tissues, Hirano et al. [35] established *in vitro* cell cultures of HGFs, human periodontal ligament cells, and human gingival epithelial cells. The study indicated that HGFs expressed CD44 protein in all the tissues that were analyzed [35]. It is tempting to speculate that moesin in conjunction with CD44 and bacterial hyaluronidases may be involved in the progression of periodontal diseases. However, further studies are required to elucidate the underlying molecular mechanisms.

Our data also indicated that GCF from individuals with severe periodontal disease contained bactericidal/permeability-increasing protein (BPI) (Supplemental Table 1). Weiss et al. [36] reported that BPI was an antibacterial protein isolated from granules of blood-derived polymorphonuclear leukocytes [36] and that BPI was specifically active against gram-negative bacteria [37,38]. Moreover, for decades, BPI has been known to bind LPS with high affinity [39]. In future studies, we plan to investigate the association of the identified candidate biomarkers, including BPI, with periodontal diseases. On the other hand, they included exportin-2, vinculin, eukaryotic translation initiation factor 6, probable UDP-sugar transporter protein SLC35A5, bromodomain adjacent to zinc finger domain protein 2A, beta-catenin 1, and glycogenin-1, which are associated with systemic disease in human (Supplemental Table 1). Further studies are required to determine whether the identified GCF proteins can be used as biomarkers for various periodontal diseases.

Giannobile et al. [40] and Ramseier et al. [41] focused on salivary biomarkers correlated with periodontal disease. Saliva is considered useful for screening for periodontal disease and for monitoring responses to treatment. Saliva has been reported to be diagnostic biomarkers of periodontal disease and mainly include proteins of host origin, host cells, bacteria, and bacterial products [42]. Saliva contains both host- and microbial-derived factors, including several enzymes that degrade proteins, proteoglycans, lipids, and carbohydrates [42]. We have strongly focused on GCF. The components of GCF may include breakdown products of host epithelial and connective tissues, products of host cells in the periodontium, and products derived from subgingival microbial plaque. These factors represent possible candidate predictors of disease activity. A variety of proteins detected in GCF play a central part in periodontal tissue turnover [3-5]. Consequently, analysis of a biochemical marker in GCF may help in diagnosis, because this marker also predicts the progression of periodontal disease [2]. In contrast, salivary biomarkers may provide an overall assessment of disease status as opposed to the site-specific assessment offered by the analysis of GCF.

In summary, we used LC-MS/MS analysis to identify proteins expressed in GCF samples that were obtained from five individuals with severe periodontal disease. A total of 225 proteins, including GCF proteins that we identified in our previous study, were identified in this study. Of these marker candidates, we focused on moesin, a disease-associated protein that has not been studied in relation to periodontal disease. Western blotting analysis revealed that moesin expression in GCF was significantly higher in individuals with severe periodontal

disease than in healthy individuals. We also showed that moesin expression in HGFs increased after LPS treatment in a dose-dependent manner. Overall our data indicated that moesin may contribute to the progression of periodontal diseases on cellular levels. Our findings indicate that moesin may be involved in the progression of periodontal disease and is a potential biomarker of periodontal disease. Further studies using a larger sample size are required to verify the findings of this study.

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