Detection of Biomarkers for Periodontal Diseases Using Salivary Proteomics

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Because of the irreversible nature of periodontal disease, early diagnosis is an important aspect of management of patients with periodontal disease. Human saliva is an attractive medium for disease diagnosis because its collection is noninvasive and simple. Analysis of saliva may be especially beneficial in the determination of current periodontal status and serve as means for the screening of periodontal disease. In the present study, we investigated potential biochemical markers in whole saliva samples for the screening of periodontal disease using proteomics technique. We enrolled five subjects each from four different groups on the basis of measures of periodontal health (healthy group, gingivitis group, chronic periodontitis group and aggressive periodontitis group). Eleven proteins in whole saliva samples were identified as differentially expressed proteins between the healthy and periodontal disease groups using 2–dimensional electrophoresis and matrix–assisted laser desorption/ionization time–of–flight / time–of–flight mass spectrophotometry (MADLI–TOF/TOF MS) approaches. Although the diagnostic value of oral fluid has been recognized for some time and potential biomarkers of periodontal disease have been identified in saliva, this, to our knowledge, is one of the first studies to examine large–scale proteomic profiling to identify the extent of periodontal destruction. Thus, this work provides an important framework for future efforts aimed at understanding salivary responses to periodontal destruction and predicting the future disease progression.

**Key words**: Periodontal disease, Saliva, Biomarker, Proteomics

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\(^*\) I. INTRODUCTION

Periodontitis is an inflammatory disease characterized by destruction of connective tissue attachment and bone around the teeth resulting in the formation of periodontal...
pockets due to the apical migration of the junctional epithelium. The pathogenesis of periodontal disease involves the participation of numerous inflammatory and immune mediators released from the inflammatory and immune cells in response to bacteria and bacterial products.

Periodontal diseases are a group of disorders with different clinical appearance. Gingivitis, the mildest form of periodontal disease, is characterized by gingiva that bleed easily. Chronic periodontitis is a common destructive periodontal disease and shows a slow progression. Aggressive periodontitis is characterized by the early onset of the severe and rapid destruction of periodontal attachment and alveolar bone.

In many countries throughout the world the periodontal diseases have been seen at an increased rate. Despite the availability of several new therapeutic methods and agents, the high prevalence of periodontal disease has been associated with the increase in tooth loss. Because of the irreversible nature of this disease, early diagnosis is an important aspect of management of patients with periodontal disease. Conventionally, diagnosis of periodontal disease has been primarily based upon radiographic and clinical examination including the plaque index, gingival inflammation, pocket depth, and attachment level. Clinical measurements are often of only limited value in that they are indications of past destruction rather than the present disease activity. Furthermore, when screening for periodontal disease in a large number of subjects, considerable time and effort are needed to evaluate the attachment level and probing depth of one index tooth each of the sextants for each individual.

Analysis of saliva and gingival crevicular fluid (GCF) may be especially beneficial in the determination of current periodontal status, because of the determination of inflammatory mediator levels in biologic fluids is a good indicator of inflammatory activity. In addition, saliva testing provides several advantages including low invasiveness, low cost, and easy sample collection. GCF may serve as means for the screening of periodontal disease. A specific relationship between periodontal disease and parameters related to GCF has been reported. However, the sampling technique to GCF collection is not easy and a long time is needed. Thus, GCF is not feasible for mass screening.

In the present study, the author investigated whole saliva samples for the screening of candidate biochemical markers of periodontal disease using proteomics technique. With the significant advances in mass spectrophotometry and proteomics technologies, protein biomarker discovery has become one of the central applications of proteomics.

II. MATERIALS AND METHODS

1. Study population

The Ethics Committee of Chonbuk National University School of Dentistry approved this study. All subjects provided written informed consent and received incentives (monetary compensation as well as a clinical examination) as part of the study protocol.

In this study, 20 subjects (9 male, 11 female) were evaluated ranging in age from 26 to 47 years. All consecutive subjects were recruited from the Department of Periodontics, Chonbuk National University Hospital. We enrolled five subjects each from four different groups on the basis of measures of periodontal health (healthy group, gingivitis group, chronic periodontitis group and aggressive periodontitis group).

Subjects over 18 years of age who were in good general health and had at least 20 erupted teeth were eligible to participate. The subjects were selected according to the clinical and radiographic criteria proposed by the 1999
Healthy group: Healthy group consisted of three females and two males with no evidence of clinical attachment loss, clinical inflammation, sulcular bleeding and radiographic evidence of bone loss. These patients ranged in age from 31 to 39 (mean age 34.8 ± 2.95 years).

Gingivitis group: This group included three females and two males (age 27–47 years, mean age 34.6 ± 7.8 years). Clinical evidence of gingivitis was based on the presence of bleeding on probing at any sites, but no radiographic evidence of bone loss was observed.

Chronic periodontitis (CP) group: The CP group consisted of three females and two males ranged in age from 26 to 43 (mean age 34.6 ± 7.8 years). They had moderate to severe alveolar bone loss (clinical attachment loss of 5 mm) and probing pocket depth of = 6 mm in multiple sites of all four quadrants of the mouth, but with no evidence of rapid progression.

Aggressive (AgP) periodontitis: The AgP group consisted of three females and two males between the ages of 31 and 41 years old (mean 34.0 ± 4.0 years). These patients demonstrated a generalized pattern of severe alveolar bone destruction (clinical attachment loss of 5 mm) on eight or more teeth: at least three of those were other than central incisors or first molars.

2. Determination of periodontal status

To determine the clinical periodontal status, all subjects were evaluated clinically and radiographically at the first visit. Clinical parameters of pocket depth, supragingival plaque accumulation, clinical attachment loss and bleeding on probing were assessed. Measurements were performed at six sites per tooth for whole mouth.

3. Samples collection and preparation

Unstimulated whole saliva samples were collected early in the morning (between 9 and 11 a.m.) and all the subjects were asked to be on an empty stomach, without having had any drink or eaten any kind of food (including gum or candies). After tooth brushing for 3 min by using a tooth brush without toothpaste, the whole saliva samples were collected by spitting, without chemical stimulation. In order to minimize the degradation of the proteins, the samples were kept on ice during the collection procedure and Complete™ Protease Inhibitor Cocktail (Roche, Mannheim, Germany, 40 µL/mL whole saliva) and 0.5 mM of N-Ethylmaleimide were added immediately after sample collection. The samples were centrifuged at 14,000 × g for 20 min in a microcentrifuge at 4°C to remove undissolved materials. The supernatant was then dialyzed against 12 mM ammonium bicarbonate buffer (pH 7.1) overnight using 12,000–14,000 M_r cut off MEMBRA-CEL™ Dialysis Tubing (Viskase Co., Inc. IL). The protein amount of the dialysate was estimated using an RC-DC protein Assay (Bio-Rad Laboratories, Inc. CA) and the sample dried by vacuum centrifugation.

4. Two-Dimensional Electrophoresis (2DE)

Saliva were pooled from five subjects each from four different groups, Aliquots containing 300 µg protein were diluted to 450 µL with rehydration solution [8M urea, 2% Chaps, 1% DTT, 0.5% immobilized pH gradient (IPG) buffer (Amersham Biosciences, Stockholm, Sweden), and trace bromophenol blue] and applied to IPG strips (pH 3–10, 24cm, Amersham Biosciences) by 12 h rehydration at room temperature. Then proteins were focused successively for 1 h at 500 V, 1 h at 1,000 V and 8.3 h at 8,000 V to give a total of 59 kVh on an IPGphor (Amersham Biosciences). Focused IPG strips were equili-
brated for 15 min in a solution (6 M urea, 2% SDS, 40% glycerol, 50 mM Tris-HCl, pH 8.8 and 1% DTT), and then for an additional 15 min in the same solution except that DTT was replaced by 2.5% iodoacetamide (Sigma–Aldrich, St. Louis, MO). After equilibration, second-dimension 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on Ettan DALT II system (Amersham Biosciences).

5. Gel scanning and image analysis

Following electrophoresis, gels for analysis were fixed and stained using the SeePico™ CBB stain kit (Benebiosis, Seoul Korea). The stained 2–DE gels were scanned with MagicScan 4.5 software on Image scanner (Bio–Rad) and PDQuest Advanced 8.0.1 2D Gel Analysis Software (Bio–Rad) was used for spot–intensity calibration, spot detection, background abstraction, matching, 1-D calibration, and the establishment of average-gel. Intensity of each spot was quantified by calculation of spot volume after normalization of the image using the total spot volume normalization method multiplied by the total area of all the spots. Proteins were classified as being differentially expressed when spot intensity was showed a difference >2-fold or <2-fold variation in periodontal disease group in comparison to healthy group.

Fig. 1 shows the two-dimensional gel electrophoresis (2–DGE) separation of proteins extracted from whole saliva of healthy group. Fig. 2, 3 and 4 show 2–DGE separations of proteins extracted from whole saliva of gingivitis group, chronic periodontitis group, and aggressive periodontitis group, respectively.

Most of the proteins/peptides were distributed between pI 5-8 and between 25 and 80 kDa. Spots of interest were excised, in-gel-digested with trypsin, and analyzed via matrix Assisted Laser Desorption/Ionization Time-of–Flight (MALDI–TOF) mass spectrophotometry (MS) for protein identification.

6. In-gel digestion

The stained protein spots were excised from gels and digested with trypsin (Promega, Madison, WI, USA). The gels were destained with a solution of 10 mM ammonium bicarbonate in 50% acetonitrile (ACN), and the gels were then rinsed three times with distilled water to stop the destaining reaction. After drying using a speed vacuum concentrator, swelling the gels with 30 μl 50 mM ammonium bicarbonate, 7–8 μl trypsin solution (0.1 μg/mL) was added prior to incubation at 37°C for 12–16 h for protein digestion. The peptides were recovered by two extraction steps using 50 mM ammonium bicarbonate and 50% ACN containing 5% ACN containing 5% trifluoroacetic acid (TFA). The resulting peptide extracts were pooled and lyophilized in a vacuum concentrator and stored at -20°C. Tryptic peptides were dissolved with 0.5% TFA prior to further analysis.

7. MS analysis using MADLI–TOF/TOF MS

Tryptic peptides were dissolved in 0.5% TFA, and then desalted using ZipTipC18 (Millipore, Bedford, MA, USA). Peptides were eluted directly onto a MALDI plate using a α-cyanohydroxycinnimic acid (CHCA) matrix solution (10 mg/mL CHCA in 0.5% TFA/ 50% ACN, 1:1). All mass spectra were acquired in the reflection mode using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). External calibration was performed using a standard peptide mixture of des-Arg bradykinin, angiotensin I, Glu–fibrino–peptide B, adrenocorticotropic hormone (ACTH) clip 1,17, ACTH clip 18,39, and ACTH clip 7,38. Internal calibration was also performed using two autolysis peaks of trypsin ([M+H]+ = 842,5099 and 2211,1046). When the protein spots were not identified by peptide mass fingerprint (PMF), fragmentation patterns of the tryptic peptide molecular ions ([M+H]+) were analyzed by MS/MS methods to obtain their partial se–
quences using the matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MADLI-TOF/TOF MS) technique. All samples were irradiated with UV light (355 nm) by an Nd:YAG laser with a repetition rate of 200 Hz; 1000 and 3000 laser shots were used on average for normal mass spectra and MS/MS spectra, respectively. The samples were analyzed at 25 kV source acceleration voltage with two-stage reflection in MS mode. In the MS/MS experiment, collision energy, which was defined by the potential difference between the source acceleration voltage (8 kV) and the floating collision cell (7 kV), was set to 1 kV.

8. Protein identification

Resulting data were analyzed by GPS Explorer™ 3.5 (Applied Biosystems) software. The proteins were identified by searching rodent subset of the National Center for Biotechnology Information (NCBI) protein databases (using MASCOT 2.0 search algorithm (Matrix Science, London, UK). The general parameters for PMF searching were considered to allow maximum one missed cleavage, ±50 ppm of peptide mass tolerance, and the modifications of N-terminal Gln to pyroGlu, oxidation of methionine, acetylation of protein N-terminus, carbamidomethylation of cysteine, and acrylamide modified cysteine. A peptide charge state of +1 and fragment mass tolerance of ±0.5 Da were used for the MS/MS ion search. Probability based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as \(-10 \times \log_{10}(p)\) where p is the absolute probability. Individual MS/MS ions scores >37 indicated extensive homology (p<0.05) for MS/MS ion search. All the protein identifications were in expected molecular weight and pI obtained from image analysis. For MASCOT MS/MS ion search, the identified proteins had to be in the top hit with more than two peptide sequences matched in NCBI BLAST search. A matched protein was accepted if it ranked as the hit with a single–peptide match.

III. RESULTS

1. Identification of differentially expressed proteins by MALDI–TOF–MS

To identify proteins that were changed in abundance in a way that correlates with periodontal disease status or progression compared to healthy status, we carried out 2–DE using whole saliva. Fig. 1–4 show four representative 2–DE maps from healthy group, gingivitis group, chronic periodontitis group and aggressive periodontitis group. Among the spots investigated, almost 30 proteins were differentially expressed. But some of them were unknown proteins and some of them with different pI and molecular weight (MW) finally proved to be the same protein, which suggest different post–translational modification. Finally, 11 spots were identified as differentially expressed proteins between the healthy and periodontal disease groups. The spots found by the statistical analyses were re–searched in the match–set created by the PDQuest software.

The spots are marked in enlarged sections of representative gels showing the respective spots in the healthy and periodontal disease groups (Fig. 5–8).

The 11 differentially expressed protein spots were analyzed by MADLI–TOF/TOF MS and identified by Mascot search. The identity of all peptides has been confirmed at least by three fragment ions in the MS/MS spectrum. As shown in Table 1, the following three proteins were up–regulated in gingivitis group: i) lipocalin–1 (LCN1) precursor, ii) human serum albumin and iii) zinc–alpha2–glycoprotein (ZAG), whereas cystatin D was down–regulated (Fig. 5). In chronic periodontitis group, the following proteins were all up–regulated: i) human
Fig. 1. Two-dimensional gel electrophoresis, using isoelectric focusing with pH range 3–10 in the horizontal dimension and SDS–PAGE (12%) in the vertical dimension. Total protein (50 μg) extracted from pooled whole saliva of healthy group (n=5) was loaded and stained with Coomassie Blue. Labeled gel spots indicate differentially expressed proteins (compared to healthy group, Fig. 1) that were cut for in–gel trypsin digestion and subsequently were analyzed by MALDI–TOF/TOF MS. The protein assignments are listed in Table 1.

Fig. 2. Two-dimensional gel electrophoresis, using isoelectric focusing with pH range 3–10 in the horizontal dimension and SDS–PAGE (12%) in the vertical dimension. Total protein (50 μg) extracted from pooled whole saliva of gingivitis group (n=5) was loaded and stained with Coomassie Blue. Labeled gel spots indicate differentially expressed proteins (compared to healthy group, Fig. 1) that were cut for in–gel trypsin digestion and subsequently were analyzed by MALDI–TOF/TOF MS. The protein assignments are listed in Table 1.

Fig. 3. Two-dimensional gel electrophoresis, using isoelectric focusing with pH range 3–10 in the horizontal dimension and SDS–PAGE (12%) in the vertical dimension. Total protein (50 μg) extracted from pooled whole saliva of chronic periodontitis group (n=5) was loaded and stained with Coomassie Blue. Labeled gel spots indicate differentially expressed proteins (compared to healthy group, Fig. 1) that were cut for in–gel trypsin digestion and subsequently were analyzed by MALDI–TOF/TOF MS. The protein assignments are listed in Table 1.

Fig. 4. Two-dimensional gel electrophoresis, using isoelectric focusing with pH range 3–10 in the horizontal dimension and SDS–PAGE (12%) in the vertical dimension. Total protein (50 μg) extracted from pooled whole saliva of aggressive periodontitis group (n=5) was loaded and stained with Coomassie Blue. Labeled gel spots indicate differentially expressed proteins (compared to healthy group, Fig. 1) that were cut for in–gel trypsin digestion and subsequently were analyzed by MALDI–TOF/TOF MS. The protein assignments are listed in Table 1.

Fig. 5. Close-ups of four–matched spots from 2–DE gel images of whole saliva proteins between the healthy (left side) and gingivitis groups (right side). Labeled gel spots that show a prominent difference were chosen for further analysis by MALDI–TOF/TOF MS. Four protein spots (153, 218, 325, and 350) were identified as serum albumin, Zn–alpha2–glycoprotein, lipocalin–1 precursor, and cystatin D. Cystatin D (spot 350) was down–regulated in the gingivitis group compared to healthy group.

serum albumin, ii) profilin I and iii) salivary amylase (Fig. 6). In aggressive periodontitis group, the following three proteins were up–regulated: i) LCN1 precursor, ii) human serum albumin and iii) S100 calcium–binding protein A9 (S100A9), whereas cystatin SN was down–regulated (Fig. 7).
Fig. 6. Close-ups of matching regions from 2-DE gel images of whole saliva proteins between the healthy (left side) and chronic periodontitis groups (right side). Labeled gel spots that show a prominent difference were chosen for further analysis by MALDI-TOF/TOF MS. Four protein spots (549, 1024, and 1498) were identified as alpha amylase, profilin I, and serum albumin. All three matched proteins were up-regulated in the chronic periodontitis group.

Fig. 7. Close-ups of matching regions from 2-DE gel images of whole saliva proteins between the healthy (left side) and aggressive periodontitis groups (right side). Labeled gel spots that show a prominent difference were chosen for further analysis by MALDI-TOF/TOF MS. Four protein spots (429, 761, 1339, and 1354) were identified as serum albumin, S100 calcium-binding protein A9, cystatin SN precursor, and profilin I. Cystatin SN precursor (spot 1339) was down-regulated in the aggressive periodontitis group compared to healthy group.

Fig. 8. Peptide mass fingerprints of S100 calcium-binding protein A9. S100 calcium-binding protein A9 was up-regulated in aggressive periodontitis group. Fingerprint mass spectra was generated via MALDI-TOF/TOF MS analysis.

Table 1. Identification of differentially expressed salivary proteins in gingivitis group compared to healthy group

<table>
<thead>
<tr>
<th>2-D gel spot no.</th>
<th>Protein namea</th>
<th>Relative volumeb (healthy vs. disease group)</th>
<th>Computed Mr/pl 2-DGEC</th>
<th>Score</th>
<th>Matched peptide</th>
<th>NCBI accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>325</td>
<td>lipocalin-1 precursor</td>
<td>1:8.2 ± 2.4</td>
<td>18,381/4.9</td>
<td>302</td>
<td>4</td>
<td>gi</td>
</tr>
<tr>
<td>153</td>
<td>Chain A, serum albumin</td>
<td>1:3.9 ± 1.1</td>
<td>50,921/6.4</td>
<td>346</td>
<td>5</td>
<td>gi</td>
</tr>
<tr>
<td>218</td>
<td>Zn-alpha2-glycoprotein</td>
<td>1:9.6 ± 3.7</td>
<td>35,557/5.9</td>
<td>187</td>
<td>3</td>
<td>gi</td>
</tr>
<tr>
<td>350</td>
<td>cystatin D</td>
<td>1:−4.1 ± 1.5</td>
<td>11,764/7.9</td>
<td>233</td>
<td>3</td>
<td>gi</td>
</tr>
</tbody>
</table>

aThe proteins correspond to spots whose volume showed ± 2-fold difference compared to healthy group. Spots (numbered in Fig. 5) were cut from 2-DGE, digested with trypsin, and subjected to MALDI-TOF/TOF MS for analysis. bRelative volumes of matched spots were determined by PDQuest 8.0.1 (Bio-Rad). cRelative mass and pI determined from 2-DEG.
Table 2. Identification of differentially expressed salivary proteins in chronic periodontitis group compared to healthy group

<table>
<thead>
<tr>
<th>2-D gel spot no.</th>
<th>Protein namea</th>
<th>Relative volumeb (healthy vs. disease group)</th>
<th>Computed Mr/pI 2-DGEC</th>
<th>Score</th>
<th>Matched peptide</th>
<th>NCBI accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1498</td>
<td>Chain A, serum albumin</td>
<td>1:11.9 ± 5.7</td>
<td>18,381/6.4</td>
<td>302 4</td>
<td>gi</td>
<td>4389275</td>
</tr>
<tr>
<td>549</td>
<td>alpha amylase</td>
<td>1:6.1 ± 1.4</td>
<td>32,586/7.5</td>
<td>346 5</td>
<td>gi</td>
<td>3212456</td>
</tr>
<tr>
<td>1024</td>
<td>Profilin I</td>
<td>1:2.7 ± 0.4</td>
<td>12,583/9.3</td>
<td>185 3</td>
<td>gi</td>
<td>999511</td>
</tr>
</tbody>
</table>

aThe proteins correspond to spots whose volume showed ± 2-fold difference compared to healthy group. Spots (numbered in Fig. 5) were cut from 2-DGE, digested with trypsin, and subjected to MALDI-TOF/TOF MS for analysis. bRelative volumes of matched spots were determined by PD Quest 8.0.1 (Bio-Rad). cApparent mass and pI determined from 2-DGE.

Table 3. Identification of differentially expressed salivary proteins in aggressive periodontitis group compared to healthy group

<table>
<thead>
<tr>
<th>2-D gel spot no.</th>
<th>Protein namea</th>
<th>Relative volumeb (healthy vs. disease group)</th>
<th>Computed Mr/pI 2-DGEC</th>
<th>Score</th>
<th>Matched peptide</th>
<th>NCBI accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>761</td>
<td>S100 calcium-binding protein A9</td>
<td>1:7.5 ± 2.5</td>
<td>14,065/6.1</td>
<td>346 5</td>
<td>gi</td>
<td>3212456</td>
</tr>
<tr>
<td>429</td>
<td>Chain A, serum albumin</td>
<td>1:4.3 ± 1.5</td>
<td>49,285/6.1</td>
<td>302 4</td>
<td>gi</td>
<td>4389275</td>
</tr>
<tr>
<td>1339</td>
<td>Cystatin–SN precursor</td>
<td>1:-8.9 ± 3.2</td>
<td>14,946/8.4</td>
<td>187 3</td>
<td>gi</td>
<td>118188</td>
</tr>
<tr>
<td>1354</td>
<td>Profilin I</td>
<td>1:2.9 ± 0.7</td>
<td>13,746/9.3</td>
<td>233 3</td>
<td>gi</td>
<td>999511</td>
</tr>
</tbody>
</table>

aThe proteins correspond to spots whose volume showed ± 2-fold difference compared to healthy group. Spots (numbered in Fig. 5) were cut from 2-DGE, digested with trypsin, and subjected to MALDI-TOF/TOF MS for analysis. bRelative volumes of matched spots were determined by PD Quest 8.0.1. cApparent mass and pI determined from 2-DGE.

IV. DISCUSSION

With the significant advances in mass spectrometry and proteomics technologies, protein biomarker discovery has become one of the central applications of proteomics. Human saliva is an attractive medium for disease diagnosis because its collection is noninvasive and simple. Moreover, compared to blood, saliva may express more sensitive and specific markers for certain local oral diseases such as oral cancer, Sjögren syndrome, periodontitis, etc.5

Periodontal disease is a bacteria-induced chronic inflammatory disease which attacks the soft and hard structures supporting the teeth. Early diagnosis, treatment and prevention of progressive periodontitis is of critical importance because of the irreversible nature of this disease. Conventional periodontal diagnostic methods include assessment of clinical parameters such as pocket depth and attachment level, as well as radiographs. A complete periodontal examination is a skilled and time-consuming procedure and is primarily based on the skill and judgment of the dentist. Furthermore, these conventional techniques are inherently limited in that only a historical perspective, not current disease status or prediction of future disease can be determined.

In the recent years, several research groups have attempted to assess biochemical disease indicators, such as matrix metalloproteinases6-8, type 1 collagen degradation fragment9, inflammatory mediators10,11, in the GCF to determine disease status of the periodontal tissues. However, the sampling technique to GCF col—
lection is not easy and a long time is needed. Thus, GCF is not feasible for mass screening. In contrast, whole saliva sampling is much easier, noninvasive, and economical than GCF collection. Although the diagnostic value of oral fluid has been recognized for some time and potential biomarkers of periodontal disease have been identified in saliva, this, to our knowledge, is one of the first studies to examine large-scale proteomic profiling to identify the extent of periodontal destruction.

Analysis of the saliva proteome, however, has been hindered by the great variability of the specimens, and the presence of various proteins in low abundance or modified forms. To alleviate some of these problems saliva samples from five different individuals were pooled, concentrated and the proteome characterized by a combination of preparative electrophoresis and 2-DE, followed by PMF. In addition, proteomic analysis was analyzed in triplicates and the reproducibility was confirmed.

Using two-dimensional electrophoresis and mass spectrometry, a proteome profile of human whole saliva from gingivitis group, chronic periodontitis group and aggressive periodontitis group comprising 11 differentially expressed proteins was established.

As shown in Table 1, in gingivitis group, LCN1 precursor, human serum albumin and ZAG were up-regulated, whereas cystatin D was down-regulated. Lipocalins are a group of heterogeneous superfamily of extracellular proteins, first described by Pervaiz and Brew, that are able to bind lipophiles by enclosure within their structures, minimizing solvent contact. Ghafouri et al. reported that LCN1 was identified in the human saliva together with many small proteins. LCN1 is known as a potent inhibitors of cysteine proteases similar to cystatins and therefore can play a role in the control of inflammatory processes in oral tissues. Although the expression of LCN1 has not been reported as participating in periodontal disease, the higher quantity of LCN was found in the saliva from caries–free subjects. The observations suggest that inhibition of proteolytic events may indirectly provide tooth protection. In the present study, oppositely, LCN1 was markedly increased in the gingivitis group compared to the healthy group. Thus, this finding suggests that LCN1 is induced in infection or inflammation and supports the idea that this LCN functions as a physiologic protection factor of epithelia in vivo. The opposite expression pattern of cystatin and LCN1 in the gingivitis group was difficult to explain since Van’t Hof et al. reported that LCN1 is belong to the cystatin class and play a role in the control of inflammatory processes, together with cystatins. Whole saliva concentration of serum albumin was markedly increased in all periodontal disease group (e.g., gingivitis, chronic periodontitis and aggressive periodontitis) compared to healthy group in this study. Even though some albumin can pass through the gingival crevice, higher level of serum albumin in whole saliva has been used as an indicator of periodontal inflammation–mediated serum leakage.

ZAG is a glycoprotein assigned to the major histocompatibility complex (MHC) class I family of proteins. Burgi and Schmid purified ZAG from plasma for the first time and was so named from its electrophoretic mobility in the alpha–2 region and from its ability to bind zinc ions. To date, ZAG appears inherently in most body fluids, such as sweat, seminal fluid, cerebrospinal fluid, urine, and saliva. Biochemically, ZAG was suggested to be involved in the regulation of melanin production, prostate and bladder cancer, cachexia, obesity and inhibition of cell proliferation. Whole saliva concentration of ZAG was markedly increased in gingivitis group compared to healthy group in the present study. Although the expression of ZAG has not been reported in periodontal
disease, it recently has been suggested as a biomarker which is related to inflammation. Thus, this finding suggests that ZAG has a potential as a diagnostic marker for early periodontal disease. Since it is unclear the role of ZAG concerning inflammatory processes to date, further studies are required to determine the relationship between salivary ZAG level and progression of periodontal disease.

Cystatins comprise four superfamilies of cysteine–containing proteins that are widespread among tissues and body fluids of mammalian origin. This family comprises cystatins S, SA, SN, C, and D, which are encoded by CST1, 2, 3, 4, and 5, respectively. There are, however, as many as 11 different cystatin isoforms that has been identified to date. The total amount of secreted cystatins is much more pronounced in submandibular/sublingual secretion than in parotid secretion, Cystatin C level was significantly higher in whole and parotid saliva of subjects with periodontitis than in that of healthy controls. Cystatin S, the major salivary cystatin, however was higher in the whole saliva of the healthy group compared to the subjects with periodontitis. Cystatins are known physiologic inhibitors of cysteine proteases and as such have been suggested to play a vital role in inhibiting the proteolytic events on other salivary proteins. In the present study, cystatin D level in the gingivitis group and cystatin SN level in the aggressive periodontitis group were markedly lower than in the healthy group. These observations were consistent with the previous reports. This raised the possibility that cystatins may provide periodontal protection via inhibition of proteolytic events. In addition, the amount of cystatins in saliva may be a potential indicator of the risk for periodontitis.

As shown in Table 2, in chronic periodontitis group, human serum albumin, profilin I and salivary amylase were remarkably up-regulated. Profilins play important roles in the regulation of actin dynamics through polymerization for cell mobility and other actin–linked processes and in various cellular processes such as membrane trafficking, small-GTPase signaling and nuclear activities. Profilin 1, the most ubiquitously expressed isoform, is essential for cytokinesis during embryogenesis, although the signaling pathway remains poorly defined. In addition, the recent studies have suggested that profilin 1 had a tumor suppressor function and atheroprotection. Even though the expression of profilin 1 has not been reported in periodontal disease, this finding suggests that profilin 1 has a potential as a diagnostic marker for advanced periodontal disease. Since the role of profilin concerning inflammatory processes is unclear, further investigations are needed to determine the relationship between profilin I and progression of periodontal disease. Amylase, one of the most prevalent enzymes in the saliva, is mainly synthesized in the parotid salivary gland. In addition to enzyme activity, amylase has other functions such as coating of the oral mucosa and antimicrobial activity on Streptococci. Amylase concentration of whole saliva was markedly increased in chronic periodontitis group compared to healthy group in the present study. Similar result was found by Henskens et al, from saliva samples of healthy and periodontitis patients. These results suggest that the salivary amylase has considerable potential as a diagnostic marker for periodontal diseases, particularly chronic periodontitis.

As shown in Table 3, in aggressive periodontitis group, LCN1 precursor, human serum albumin and S100A9 were up-regulated, whereas cystatin SN was down-regulated, S100A9 is subunit of a heterocomplex commonly called calprotectin, and belong to the S100 family of calcium–binding proteins. The function of calprotectin has been proposed to be involved in
cell-cycle progression, cell-type differentiation, signal transduction, and morphological differentiation. S100A9 has been suggested as possible markers of human inflammatory neurological and neoplastic diseases. In the oral area, there are few observations concerning S100 proteins. Topoll et al. and Schlegal Gomez et al. showed that the number of S100A9-positive gingival macrophages and keratinocytes increases according to the degree of inflammation. In the present study, whole saliva concentration of S100A9 was markedly increased in aggressive periodontitis. As has recently been shown by others, they could be utilized as a periodontal marker for acute inflammation.

Whole saliva proteomic analysis is undoubtedly a valuable approach to identification of salivary protein markers for clinical diagnosis of periodontal diseases. However, several issues remain to be addressed. First, we need to standardize the sample collection and handling procedures, which are extremely important for subsequent saliva biomarker studies. Secondly, human saliva proteome contains a large number of proteins whose concentration differs by an extraordinary dynamic range. Immunoaffinity depletion may be effective to unmask low-abundance proteins in saliva by removing highly abundant proteins, such as amylase and proline-rich proteins. In addition, a limitation was associated with the small sample size due to the strict guidelines for recruiting patients. An analysis with more patients would have greater statistical power and precision. Despite these challenges, saliva proteomics remains one of the most promising approaches to human periodontal disease biomarker identification.

Although the diagnostic value of oral fluid has been recognized for some time and potential biomarkers of periodontal disease have been identified in saliva, this, to our knowledge, is one of the first studies to examine large-scale proteomic profiling to identify the extent of periodontal destruction. This work provides an important framework for future efforts aimed at understanding salivary responses to periodontal destruction and predicting the future disease progression.

V. REFERENCES


34. Kojima T, Andersen E, Sanchez JC, Wilkins MR,


