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Analysis of the salivary proteome in gingivitis patients

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Background and Objective: Gingivitis is a disease that is characterized by inflammation of the gingival tissue, which can progress to periodontitis and tooth loss. Although many studies have attempted to identify salivary proteins that are associated with the disease, this is the first study to use a proteomic approach to analyze and compare the proteomic profile of whole saliva from gingivitis patients and healthy controls.

Material and Method: To analyze the saliva proteome, two-dimensional gel electrophoresis and liquid chromatography were used, followed by mass spectrometry.

Results: The analyses showed that gingival inflammation was associated with increased amounts of blood proteins (serum albumin and hemoglobin), immunoglobulin peptides and keratins. In the control group, salivary cystatins, which were detected using capillary Liquid Chromatography on line to electrospray ionization Quadrupole Time-of-flight mass spectrometry, appeared to be more abundant.

Conclusion: This approach provides novel insight into profiles of the salivary proteome during gingival inflammation, which may contribute to improvements in diagnosis.

Periodontal diseases reflect interplay among a pathogenic bacterial biofilm that is present on the root surface/periodontal pocket, host-derived inflammatory cells and molecules from the periodontal tissue. Gingivitis, the mildest form of periodontal disease, is an inflammatory condition caused by a bacterial biofilm (dental plaque) that accumulates on teeth adjacent to the gingiva (gums), and it is commonly seen in individuals who stop brushing their teeth for 10–20 d. While gingivitis does not affect the underlying supporting structure of the teeth and is reversible, if not treated appropriately it can progress to periodontitis, which is a major cause of tooth loss in adults (1).

Saliva analysis may be useful to monitor a patient's current periodontal status. Many studies have suggested that determining the level of inflammatory mediators in biologic fluids indicates inflammatory activity (2). As a whole, saliva is a complex mixture of oral fluids that includes secretions from the major and minor salivary glands. In addition, saliva contains constituents of nonsalivary origin that are derived from gingival crevicular fluid, expectorated bronchial secretions, serum and blood cells from oral wounds, bacteria and bacterial products, desquamated epithelial cells and food debris. Accordingly, the composition of saliva is a complex, hypotonic aqueous solution that contains proteins, peptides, enzymes, hormones, sugars, lipids, growth factors and a variety of other compounds (3).

The proteome is the entire complement of proteins, including the

modifications made to a particular set of proteins, produced by an organism or system (4,5). Salivary proteome is the complete set of proteins existing at a given moment in whole saliva present in the oral cavity, independently of its origin (6).

Many salivary proteins have been studied, and proteomic analyses have shown that differences in the salivary protein profile are associated with systemic diseases, such as breast cancer (7), Sjögren's syndrome (8,9), diabetes mellitus (10), cystic fibrosis (11) and diffuse systemic sclerosis (12), as well as oral pathologies, such as oral lichen planus (13), oral cancer (14), dental caries (15), and chronic and aggressive periodontitis (16–18). However, no studies have examined gingival inflammation (gingivitis) alone.

In the present study, we analyzed differences in the protein profiles of whole saliva from gingivitis patients and from healthy controls. Our purpose was to detect changes in the level of expression of salivary proteins and the repression/induction of new proteins. The samples were analyzed using two-dimensional (2D) gel electrophoresis and liquid chromatography, followed by tandem mass spectrometry (MS/MS).

Material and methods

Patient selection

Patients seeking treatment at the School of Dentistry, Federal University of Espírito Santo (UFES), Brazil, were recruited during August 2008. To participate in this study, individuals signed an informed consent form for a research protocol that was reviewed and approved by the UFES Ethics Committee. All study subjects were otherwise healthy. Subjects were excluded from the study if they were nursing or pregnant, smoked, drank alcohol or had taken antibiotics in the previous year. The clinical parameters were measured using a North Carolina periodontal probe (UNC-15; Hu-Friedy, Chicago, IL, USA) and included the probing pocket depth, clinical attachment level and bleeding on probing. Patients without clinical attachment loss but with bleeding on probing and a probing pocket depth of > 3 mm in $> 50\%$ of sites were diagnosed with gingivitis. In the control subjects, $< 10\%$ of sites had bleeding on probing and a probing pocket depth of < 3 mm. Each group (control and gingivitis) consisted of 10 individuals (five women and five men), and the mean age $\pm$ standard deviation was 35.6 ± 9.5 for the control group and 32.4 ± 2.6 for the gingivitis group.

Sample collection and processing

The volunteers were asked to rest for 15 min before saliva collection (which was carried out in the morning, 2 h after they had brushed their teeth); they sat in an upright position and were asked not to speak until the saliva collection was finished. Unstimulated whole-saliva samples were immediately put on ice and were subsequently centrifuged at 14,000 *g* for 15 min at 4°C to remove insoluble materials, cell debris and food remnants. The supernatant of each sample was collected and 1 mM EDTA (Sigma, St Louis, MO, USA) and 1 mM phenylmethanesulfonyl fluoride (Sigma) was added to inhibit protease activity. The total protein concentration was determined via the Lowry method using a Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA). The supernatants were frozen at −70°C until analysis.

Sample preparation and 2D gel electrophoresis

Protein samples (100 μg) from each subject ($n = 10$) were pooled, giving a total of 1 mg of protein. This pooled protein sample was precipitated with 1 mL of cold acetone/0.2% dithiothreitol (DTT) (w/v) overnight and centrifuged at 14,000 *g* for 30 min at 4°C. The pellets were washed three times with cold acetone (90% v/v) containing 0.02% DTT. The pellets were solubilized in 340 μL of a rehydration solution [8 M urea, 2% 3-[3-Cholamidopropyl dimethylammonio]-1-propanesulfonate, 0.5% ampholyte-containing buffer for isoelectric focusing (IPG) buffer, 18 mM DTT and 0.002% Bromophenol Blue (BPB)]. Isoelectric focusing was carried out in the first dimension on precast 18-cm Immobiline DryStrips, pH 3–10 L (GE Healthcare, Uppsala, Sweden) in an Ettan IPG Phor unit, as described by the manufacturer (GE Healthcare). The gel was focused in five steps (60 V, 720 Vh; 200 V, 200 Vh; 500 V, 500 Vh; 1000 V, 1000 Vh; and 8000 V, 32,000 Vh) at a constant temperature of 20°C. For the second dimension, each strip was incubated for 15 min in 10 mL of 50 mM Tris–HCl buffer, pH 8.8, containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 0.002% BPB and 65 mM DTT, and then in the same buffer solution supplemented with 135 mM iodoacetamide instead of DTT. The strips were then rinsed in Tris–glycine buffer and transferred to a homogeneous 12% SDS–polyacrylamide gel, and 5 μL of Bench Mark protein ladder (Invitrogen, Carlsbad, CA, USA) was added to the top right position of the gel. Finally, the gel strips were overlaid with 0.5% (w/v) agarose in running buffer that contained BPB. The gels were run on an Ettan DALTsix system (GE Healthcare) at 2.5 W/gel for 30 min and 100 W for six gels until the end of the run. The gels were fixed and stained with Coomassie Brilliant Blue G-250 (Sigma, St. Louis, MO, USA). All experiments were performed in triplicate and all sample gels were electrophoresed under identical conditions for the first and second dimensions.

Gel imaging, processing and statistical analyses

All gels were digitized at 300 dpi (dots per inch) and 16-bit depth resolution by an ImageScanner (GE Healthcare) using the LabScan software (GE Healthcare). The ImageMaster 2D Platinum software (GE Healthcare) was used for image analysis. The protein sample replicates were normalized to partially quantify the spot intensity and to minimize analytical variation among the gels. To analyze the protein intensity, triplicate 2D gels of the gingivitis group were compared with gels

of the control group, as well as with each other. At least five well-defined landmarks were used to match the gels. Spots that exhibited a change of more than two-fold in their normalized volume between the gingivitis group and control group, and were present in at least five of the six replicate gels for each group, were submitted for statistical analysis. Statistical significance was evaluated with the Student's *t*-test using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA) and $p < 0.05$ was considered significant. Protein spots that exhibited a statistically significant difference between the two study groups were further analyzed and identified using mass spectrometry.

In-gel digestion

The protein spots were excised from the gels and washed with 25 mM ammonium bicarbonate in 50% acetonitrile overnight at room temperature to destain the proteins. The excised samples were subsequently dehydrated in 100% acetonitrile for 10 min and dried completely in a Speed-Vac centrifuge (Thermo Fisher, San Jose, CA, USA). The gel fragments were rehydrated in 10 μL of digestion buffer containing trypsin (modified sequencing grade; Promega, WI, USA) at a final concentration of 10 ng/μL in 25 mM ammonium bicarbonate. The gel fragments were digested with trypsin for 20 h at 37°C. The resulting tryptic peptides were extracted from the gel by incubation with 50 μL of 50% acetonitrile in 5% trifluoroacetic acid, twice, for 15 min, first with agitation and then with sonication. The supernatants were transferred, pooled and concentrated to near dryness in a Speed-Vac centrifuge. Each sample was then diluted with 10 μL of Milli-Q water in 0.1% trifluoroacetic acid.

Matrix-assisted laser desorption ionization-tandem mass spectrometry (MALDI-Tof/Tof) analysis

Approximately 0.4 μL of the extracted peptide solution was mixed with an equal volume of CHCA matrix solution [10 mg/mL of α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI, USA) in 50% acetonitrile/0.1% trifluoroacetic acid] and dried. The data for protein identification were obtained using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). Both mass spectrometry (MS) and MS/MS data were acquired using a neodymium-doped yttrium aluminum garnet (Nd:YAG) laser with a 200-Hz repetition rate. Typically, 1600 shots were recorded for spectra in the MS mode, while 2400 shots were recorded for spectra in the MS/MS mode. The MS and MS/MS mass spectra were acquired in reflector mode and internally calibrated with trypsin autolysis peptides. Up to eight of the most intense ion signals, with a signal to noise ratio of > 30, were selected as precursors for MS/MS acquisition. External calibration in the MS mode was performed with a mixture of four peptides: des-Arg1-bradykinin [mass-to-charge ratio (m/z) = 904.468], angiotensin I (m/z = 1296.685), Glu1-fibrinopeptide B (m/z = 1570.677) and Adrenocorticotropic Hormone (peptides 18–39) (m/z = 2465.199). The MS/MS spectra were externally calibrated with known fragment ion masses that were observed in the MS/MS spectrum of angiotensin I.

Enzymatic digestion for Liquid Chromatography-Electrospray Ionization-Mass Spectrometry experiments and whole-saliva peptide ultrafiltrate preparations

An aliquot of pooled whole saliva (1 mg/mL) from each group was ultrafiltered in two types of Microcon concentrators (YM-10K and YM-3K; Millipore, Billerica, MA, USA) to enrich the whole-saliva sample for three different fractions (fraction 1, salivary peptides of < 3 kDa; fraction 2, proteins and peptides of 3–10 kDa; and fraction 3, proteins of > 10 kDa). From 1 mL of the whole-saliva sample, approximately 500 μL of fraction 1, 100 μL of fraction 2 and 100 μL of fraction 3 were obtained. All fractions were treated with 10 mM DTT for 30 min, then with 50 mM iodoacetamide for 30 min in the dark. Trypsin (1 μg/50 μg of protein) was added to the sample for overnight digestion at 37°C. All samples were concentrated in a Speed-Vac centrifuge to produce 20 μL of a digested ultrafiltrate sample (DUF).

Analysis of DUFs by capillary Liquid Chromatography on line to electrospray ionization Quadrupole Time-of-flight mass spectrometry (nLC-Q-Tof)

The DUFs were loaded on a Waters® nanoACQUITY UPLC® System (Waters, Milford, MA, USA) with a Waters Opti-Pak C18 trap column coupled to Q-Tof Ultima® (Waters). The digested protein was fractionated and desalted by liquid chromatography (LC) on line to electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF). The sample (3.0 μL) was injected onto a nanoEase C18 150 mm × 75 μm column (Waters) at a flow rate of 0.6 μL/min, and the peptides were eluted in 60 min gradient from 10 to 50% of acetonitrile (containing 0.1% formic acid). The ESI voltage was 3.5 kV using a metal needle, the source temperature was 80°C and the cone voltage was 100 V. Instrument control and data acquisition were performed using a MassLynx data system (Version 4.0; Waters). The experiments were performed by scanning from an m/z of 200 to 2000 using a scan time of 1 s, which was applied during the whole chromatographic process. A maximum of three ions, with charge states of 2, 3, or 4, were selected for MS/MS from a single MS survey. Adduct masses of Na^+ and K^+ were automatically excluded. Collision-induced dissociation MS/MS spectra were obtained with argon as the collision gas at a pressure of 13 psi, and the collision voltage was varied between 18 and 45 V, depending on the mass of the precursor. The reference ion used was the mono-charged ion m/z 588.8692 of phosphoric acid. Mass spectra that corresponded to each signal from the total ion current chromatogram were averaged, allowing an accurate determination of molecular mass. The exact mass MS/

MS was automatically determined using the Q-Tof LockSpray∠ software (Waters).

Database searching

The data were processed using the Data Explorer® software 4.0 (Applied Biosystems) for MALDI-TOF/TOF analysis and with the ProteinLynx Global server (version 2.0; Waters) for ESI-Q-TOF analysis. The proteins were identified by correlation of the tandem mass spectra to the National Center for Biotechnology Information proteins and Mass Spectrometry protein sequence database using the mascot online software (http://www.matrixscience.com). The first analysis considered all taxonomies, and the second analysis was restricted to *Homo sapiens* to remove possible protein redundancy. One missed cleavage per peptide was allowed. An initial peptide mass tolerance of ± 0.3 Da was used for MALDI-TOF/TOF analysis and ± 0.1 Da was used for ESI-Q-TOF analysis. The cysteines were assumed to be carbamidomethylated, and variable modification of methionine (oxidation) was allowed. For MALDI-TOF/TOF identifications, at least two peptides were required to match for each protein.

Results

2D gels of whole-saliva proteins from gingivitis patients and healthy subjects

Proteins from three whole-saliva pools of the healthy control subjects and gingivitis patients were precipitated with cold acetone/0.2% (w/v) DTT and then separated by 2D SDS–polyacrylamide gel electrophoresis (Fig. 1). A typical 2D gradient gel (pH 3–10) showing separated proteins from the whole saliva of healthy controls is presented in Fig. 1A. Proteins of interest were chosen for identification. The typical salivary protein spots identified by MALDI-TOF/TOF mass spectrometry are numbered 1–4 (alpha-amylase, serum albumin, cystatin-1 and protein S100-A9, respectively). This finding is in accordance with previous publications (3,12,19–21).

Analyses of the images of triplicate 2D gel experiments for each saliva pool detected 236 ± 10 proteins for the control group (Fig. 1A) and 256 ± 16 proteins for the gingivitis group (Fig. 1B). Comparisons between the samples from healthy controls and gingivitis patients identified four unique spots (Fig. 1B and Table 1) in

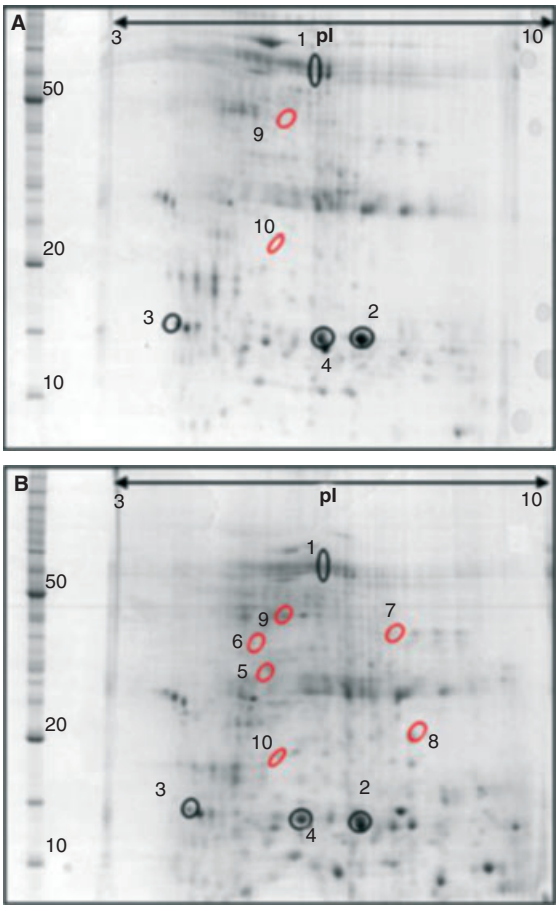

Fig. 1. Reference two-dimensional (2D) gel maps of human salivary proteins from control (A) and gingivitis (B) groups. Proteins were separated in the first dimension by isoelectric focusing (pH 3–10 L, 18 cm) and in the second dimension on the orthogonal 15% sodium dodecyl sulfate polyacrylamide gel under reducing conditions. The spots were visualized by staining with Coomassie Brilliant Blue R-250. The circled spots represent typical salivary proteins (spots 1–4) and proteins with significant differences in abundance (spots 5 and 6), as reported in Table 1.

Table 1. Statistical data of proteins that showed a difference in abundance between the two groups and were analyzed using Matrix-assisted laser desorption ionization-tandem mass spectrometry

Spot	Protein name	NCBI number	Sequence coverage (%)	Score	Matched peptides	*p*-value*	Folds in
Gingivitis group vs. healthy group							
9	Serum albumin	gi|28592	16	292	7	$p < 0.05$	↑4.66
10	Alpha-amylase	gi|178585	9	175	4	$p < 0.05$	↑4.72

**p*-values, related to gingivitis group, were calculated according to the Student's *t*-test. The peptide score was calculated as 10*Log(P), where P is the probability that the observed match is a random event.
NCBI, National Center for Biotechnology Information.

the gel of the sample from gingivitis patients: different fragments of serum albumin (spots 5 and 6), a fragment of alpha-amylase (spot 7) and IgG1 (spot 8).

Identification of high- and low-abundant proteins in the gels

To identify proteins in whole saliva that are present at higher or lower abundance in the two groups, a comparative analysis was performed using the ImageMaster 2D Elite software (see the Material and methods). Two protein spots that correspond to two different proteins with statistically significant differences in intensity between the groups were identified (summarized in Table 1 and are illustrated in Fig. 1). Compared with the healthy control group, the gingivitis group had two proteins that were present at higher concentrations and were differently expressed: serum albumin (spot 9) and alpha-amylase (spot 10).

nLC-Q-TOF analysis

The peptides that resulted from tryptic digestion were separated into three fractions, as described in Material and methods, and analyzed using capillary Liquid Chromatography on line to electrospray ionization Quadrupole Time-of-flight mass spectrometry (nLC-MS/MS). Because fraction 1 (< 3 kDa) of all of the groups contained peptides of the most abundant proteins, our subsequent analyses focused on the other two fractions. Approximately 932 and 755 MS/MS peptide spectra were collected for fractions 2 and 3, respectively, from the healthy controls, while 752 and 779 MS/MS spectra were collected for fractions 2 and 3, respectively from the gingivitis group. Database searching identified 32 and 14 proteins from fractions 2 and 3, respectively, of the healthy controls, and identified 11 and 23 proteins from fractions 2 and 3, respectively, of the gingivitis group.

Table 2 lists the proteins that were identified with a score of > 50. The majority of the peptides detected in all fractions corresponded to alpha-amylase or serum albumin. Additionally, prolactin and transferrin/serotransferrin were present in all of the groups. However, the gingivitis group had a higher number of MS/MS spectra for hemoglobin, while the control group had more cystatin peptides. In addition, zymogen granule protein 16 homolog B, carbonic anhydrase 6 and several keratins were detected in the gingivitis group only.

Table 2. Proteins analyzed using nLC-MS/MS

		Number of peptides (queries matched/unique peptides)			
		Healthy control		Gingivitis	
Protein	NCBI	3–10 kDa	> 10 kDa	3–10 kDa	> 10 kDa
Alpha-amylase	gi|178585	40/13	85/10	55/11	15/7
Alpha-2-macroglobulin	gi|112911	–	1/1[a]	1/1[a]	2/1[a]
Apolipoprotein APO-A1	gi|119587681	–	2/2	–	–
Carbonic anhydrase 6	gi|179732	–	2/2	10/3	1/1[a]
Cystatin-S	gi|30366	12/4	33/4	2/1[a]	–
Cystatin-SA	gi|235948	5/3	17/3	–	–
Cystatin-SN	gi|118188	17/5	33/5	14/2	3/2
Hemoglobin subunit alpha	gi|122440	–	1/1[a]	12/3	11/3
Hemoglobin subunit beta	gi|122698	–	–	20/9	16/6
Ig alpha-1 chain C region	gi|113584	2/1[a]	1/1[a]	1/1[a]	3/2
Ig kappa chain region	gi|306958	–	2/2	11/2	1/1[a]
Keratin, type II cytoskeletal 1	gi|1346343	–	–	–	10/6
Keratin, type II cytoskeletal 2	gi|547754	–	–	–	5/3
Keratin, type II cytoskeletal 6A	gi|1346344	–	–	–	3/2
Keratin, type I cytoskeletal 9	gi|453155	–	–	–	2/1[a]
Keratin, type I cytoskeletal 10	gi|251757513	–	–	–	2/1[a]
Mucin 5-B	gi|11275568	–	4/3	–	–
Prolactin-induced protein	gi|4505821	10/4	42/1	17/2	1/1[a]
Proline-rich-protein 3	gi|21264497	9/2	–	–	–
Protein S100-A9	gi|4506773	–	1/1[a]	1/1[a]	–
Serum albumin	gi|28592	48/14	20/9	40/10	59/12
Transferrin/serotransferrin	gi|553788	2/1[a]	4/3	8/1[a]	4/1
Zymogen granule protein 16 homolog B	gi|94536866	–	–	1/1[a]	8/4

[a]Ion score > 50.
NCBI, National Center for Biotechnology Information.
nLC-MS/MS, capillary Liquid Chromatography on line to electrospray ionization Quadrupole Time-of-flight mass spectrometry.

Discussion

Gingivitis is a biofilm-induced infection that is caused by components of pathogenic oral microbiota. Inflammation of the gingiva can cause a transient deterioration of the connective tissue, which can be reversed if the inflammation is treated. In the absence of treatment, gingivitis may progress to periodontitis, which is a destructive disease that can ultimately result in tooth loss. Saliva contains locally derived and systemically derived markers of periodontal disease and can be easily collected for analysis. While some previous studies have examined possible changes in saliva proteins that are associated with gingival inflammation, the study presented here represents the first proteomic approach. We compared the protein profiles of unstimulated whole saliva obtained from healthy controls and from gingivitis patients using two complementary

experimental approaches: 2D gel electrophoresis and liquid chromatography.

In order to utilize proteomic techniques properly to solve a clinically relevant question, a number of items should be considered, such as the choice of technology. Bidimensional electrophoresis is a powerful technique for protein fractionation (22), but has some limitations, including poor recovery of hydrophobic proteins and limited resolving power for proteins with a low/high relative molecular mass or a basic isoeletric point. Advances in liquid chromatography instrumentation and the development of MS/MS with peptide-sequencing and database-searching facilities provide a higher ability to identify protein from extremely complex mixtures of proteins (23). In shotgun peptide sequencing, proteins are digested to peptides and therefore the hydrophobicity, the isoeletric point and the molecular mass are not limiting factors (24). The nLC-Q-TOF analysis allowed the identification of high-molecular-weight proteins, such as the 590-kDa polypeptide chain of MUC5, and of low-molecular-weight proteins, such as proline-rich protein 3.

In order to minimize the effects of inherited polymorphisms in human saliva (25), samples were pooled (saliva samples were pooled from 10 patients and from 10 control individuals). However, some spot positions may have changed. A better alternative would be to increase the number of biological replicates and produce gels from individual samples of each patient/control rather than to use pooled samples.

It is important to consider that our goal was not to study the total proteome, but to identify proteins that appeared to be differentially expressed.

Gingivitis, the mildest form of periodontal disease, is an inflammatory condition restricted to gingival tissue and does not involve the loss of connective tissue and tooth bone support. Compared with our previous study that included subjects with chronic periodontitis, salivary proteomic analysis showed that the level of protein regulation does not change significantly between these inflammatory conditions, except for the alpha-amylase proteolysis found in the periodontitis group (18). The results of the present study also showed a higher prevalence of alpha-amylase fragments in the gingivitis group, but more studies are necessary to correlate this finding to a proteolytic activity.

For simplicity, the major differences in protein abundance, found in this study, between whole saliva from patients with gingivitis and from healthy subjects are discussed in the following sections.

Immunoglobulin

Analysis of fractions 2 and 3 indicated that peptides from this class of protein were more abundant in the gingivitis group compared with the control group. This result agrees with a previous study which showed that there was a statistically significant increase in the IgA secretion rate in saliva from subjects presenting with experimental gingivitis (26).

Hemoglobin and albumin

There was an increased level of serum albumin in the gingivitis group compared with the control group (spot 10; 2D analysis). DUF analysis using nLC-Q-TOF also indicated that there was an abundance of hemoglobin peptides in the gingivitis group (Table 2), which is consistent with the increased number of red blood cells (spontaneous bleeding) that is often seen with gingival inflammation (27).

Cystatin

Cystatins are natural inhibitors of cysteine proteinases that may play protective and regulatory roles during inflammation. Therefore, several studies have investigated the possible relationship between salivary cystatins and periodontal disease. In previous studies, no significant differences were observed in the salivary concentrations of cystatins C, S, SA and SN among gingivitis patients, periodontal patients and healthy controls (28,29). However, patients with gingivitis or periodontitis were shown to have a higher level of cystatin activity and an increased concentration of cystatin C in whole and parotid saliva, compared with periodontally healthy individuals (30,31). Finally, other studies that used a proteomic approach found a significant decrease in the salivary cystatin levels in patients with oral cavity bleeding or periodontal disease (18,21). In our study, nLC-Q-TOF analysis showed an abundance of cystatin peptides in the control group.

Keratins

DUF analysis using nLC-Q-TOF detected a variety of keratins in the saliva of the gingivitis group. It has been previously suggested (27) that phenotypic markers for junctional and oral sulcular epithelia might serve as indicators of periodontal disease; however, no studies demonstrate a clear association between specific types of keratins in the saliva and the progression of gingivitis and periodontitis. McLaughlin *et al.* (32) stated that the keratin concentration in gingival crevicular fluid might serve as a marker of gingival damage, although similar findings were not observed in saliva. Considering the various types of keratins, further studies of saliva from gingivitis patients are needed.

Prolactin-induced protein

Prolactin-induced protein presented a decreased number of MS/MS spectra (nLC-Q-TOF analysis) in saliva from gingivitis patients. Prolactin-inducible protein, which is also known as extraparotid glycoprotein, has been associated with secretory cell differentiation and used in diagnostic evaluation of salivary glands (29). Huang (21) also found decreased levels of this protein by 2D electrophoresis analysis of saliva from a bleeding oral cavity, but no other studies have correlated this alteration in saliva from gingivitis subjects.

Zymogen granule protein 16 homolog B

Zymogen granule protein 16 homolog B was detected in the gingivitis group only. This is probably because of its low abundance or an unknown proteolysis.

Carbonic anhydrase 6

Despite carbonic anhydrase being a 40-kDa protein, a higher number of MS/MS spectra were observed between 3 and 10 kDa, suggesting the presence of carbonic anhydrase fragments in the saliva of the gingivitis group. This preliminary finding was first described in our report. More studies are necessary to investigate this finding in greater detail.

Mucin, histatin, proline-rich-protein 3, lipocalin 1 precursor and protein S100-A9

Mucin, histatin, proline-rich-protein 3, lipocalin 1 precursor and protein S100-A9 are commonly found in saliva proteome analysis and could not be associated with a specific group.

The proteomic profiles obtained from 2D electrophoresis and complementary nL-Q-TOF analysis showed some differences in protein abundance between whole saliva from the gingivitis patients compared with whole saliva from the healthy controls. Increased levels of some blood proteins (probably associated with spontaneous bleeding), peptides associated with inflammation (immunoglobulins) and keratins were observed in the gingivitis group. In contrast, cystatin peptides were more abundant in the control group compared with the gingivitis group. The results suggest that proteomic analysis is a good tool for using to study changes in the protein composition of saliva that are associated with gingival alterations/disease. These studies may aid in the identification of a disease biomarker and can therefore improve periodontal diagnosis.

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