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Analysis of proteins in human gingival crevicular fluid by mass spectrometry

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Background and Objective: Gingival crevicular fluid is a bodily fluid transuded from periodontal tissues into the gingival crevice and periodontal pocket, and contains many species of components. Proteins in gingival crevicular fluid have been studied as markers for periodontal diseases. Mass spectrometric analysis is used for the analyses of proteins, lipids, saccharides and metals, and expected as an approach for disease diagnosis. For better analysis of the protein components in gingival crevicular fluid, we investigated proteins in gingival crevicular fluid samples from the healthy gingival crevice and periodontal pocket using mass spectrometry.

Material and Methods: Gingival crevicular fluid samples were collected from subjects who gave their informed consent and were periodontally healthy or had diseased pockets. These samples were electrophoretically separated, and each fraction on the gels was analysed by nano liquid chromatography coupled with tandem mass spectrometry. Antimicrobial peptides detected in gingival crevicular fluid were confirmed by western blotting.

Results: One hundred and four proteins were detected in gingival crevicular fluid samples from both healthy sites and sites of periodontitis; 64 proteins were contained only in gingival crevicular fluid from healthy sites and 63 proteins were observed only in gingival crevicular fluid from periodontitis sites. These proteins were blood-, cytoskeleton-, immunity-, inflammation- and lipid-related proteins and enzymes. Some proteins, including ceruloplasmin, glycogen phosphorylase, glutathione *S*-transferase, phosphoglycerate mutase, psoriasin, S100A11 and resistin, were identified for the first time in gingival crevicular fluid. Antimicrobial peptides, such as lactoferrin, α1-antitrypsin, lipocalin, S100A7, S100A8, S100A9 and cathelicidin, were observed by mass spectrometry and western blotting.

Conclusion: Multiple protein components in gingival crevicular fluid were analysed at the same time using mass spectrometry, and this approach may be useful for the diagnosis of periodontal diseases.

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Gingival crevicular fluid is a transudate that exists in the healthy gingival crevice and in the periodontal pocket in cases of periodontal diseases, and contains proteins, saccharides, electrolytes, cellular components, degraded products of periodontal tissues and bacteria and their products (1–3). Gingival crevicular fluid contains multiple proteins, including serum proteins, immunoglobulins, enzymes, periodontal tissue-derived proteins, and inflammation-related proteins, cytokines and bacterial proteins (2–4). Albumin, macroglobulin and immunoglobulin are major proteins in gingival crevicular fluid (2,5). Enzymes, including lysozyme, alkaline phosphatase,

MMPs, aspartate aminotransferase, lactate dehydrogenase and cathepsins, as well as matrix proteins, such as fibronectin, proteoglycans and osteocalcin, were identified in gingival crevicular fluid (4–7). The levels of interleukin-1β (IL-1β) and interleukin-6 (IL-6), which are proinflammatory cytokines, are high in gingival crevicular fluid samples from periodontitis sites (8,9). Antimicrobial peptides (AMPs) have been identified in gingival crevicular fluid (8–12), and calprotectin, S100 calcium binding protein (S100), A8 (S100A8) and S100A9 complex, and lactoferrin in gingival crevicular fluid increase in cases of periodontal diseases (13–15).

The analysis of gingival crevicular fluid components is useful for diagnosis of periodontal disease because gingival crevicular fluid contains some inflammation-related proteins, tissue-degraded proteins and proteases. The gingival crevicular fluid volume is very small and the concentration of each protein is low; therefore, gingival crevicular fluid proteins have been separately detected and determined by immunoassay using respective specific antibodies or enzyme–substrate responses. However, to analyse multiple proteins in one gingival crevicular fluid sample at the same time is difficult. Liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) have been used for the analysis of chemical materials, drugs, hormones, metals and proteins (16,17). Liquid chromatography coupled with tandem mass spectrometry is expected to be a useful approach for clinical diagnosis by analysis of abundant protein biomarkers (17,18). By N-terminal amino acid sequencing or peptide mass fingerprinting and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS), some proteins, including S100A8 and S100A9, were identified in gingival crevicular fluid (19). Antimicrobial peptides, such as cathelicidin (LL-37), human neutrophil peptides and cystatin, were also detected in gingival crevicular fluid using surface-enhanced laser desorption/ionization (SELDI)-TOF-MS and ion-trap mass spectrometry through electrospray ionization (ESI-IT MS; 10,11). Ngo *et al.* (12) analysed gingival crevicular fluid samples collected from five periodontal pockets per subject among those with chronic periodontitis using nanoelectrospray and ESI-IT MS and MALDI-TOF-MS, and identified 33 peptides and 66 proteins in gingival crevicular fluid samples. Grant *et al.* (20) analysed gingival crevicular fluid samples from experimental gingivitis and periodontally healthy sites using LC-MS/MS, and reported the existence of 186 human proteins.

Investigation of protein components in gingival crevicular fluid by mass spectrometry seems to be useful for a search of biomarkers of periodontal diseases. In the present study, we comprehensively analysed proteins in a gingival crevicular fluid sample collected from one gingival crevice or periodontal pocket using LC-MS/MS and compared protein components between gingival crevicular fluid samples from the healthy gingival crevice and the inflamed periodontal pocket. Furthermore, AMPs detected in gingival crevicular fluid samples were confirmed by western blotting.

Material and methods

Gingival crevicular fluid samples

Gingival crevicular fluid samples were obtained from nine subjects who gave written informed consent to participate in this study. Gingival crevicular fluid sampling and periodontal examination were approved by the Ethics Committee of Tokushima University Hospital. The subjects were eight patients with periodontitis, who included four men (43, 59, 60 and 72 years old) and four women (46, 53, 60 and 65 years old), and a healthy man (54 years old) without periodontitis, who had not taken antibiotics for 1 month. Gingival crevicular fluid was collected using Periopaper® (Oraflow Inc., Plainview, NY, USA) from periodontal pockets with more than 6 mm pocket depth and gingival index of two and from healthy gingival crevices with 2 mm pocket depth and gingival index of zero, in accordance with a previously reported method (13,14). Gingival crevicular fluid was extracted from paper strips into 10 mM Tris–HCl (pH 7.4) with 200 μM phenylmethylsulfonyl fluoride.

Polyacrylamide gel electrophoresis (PAGE) and in-gel digestion

Gingival crevicular fluid samples for mass spectrometry were individually collected from each site of three subjects, who included one subject without periodontitis and two periodontitis patients; these were mostly used for electrophoresis and partly for protein determination. Briefly, the amount of protein in gingival crevicular fluid samples was determined using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Inc., Hercules, CA, USA) and was 24.9 μg/lane of healthy sample (H) and 153.3 μg/lane of periodontitis sample (P) in Fig. 1A, and 22.4 μg/lane for Fig. 1B.

Gingival crevicular fluid samples were mixed with a sample buffer containing 4% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol, and boiled for 5 min. Proteins in gingival crevicular fluid samples were electrophoretically separated on 10, 12 or 15% SDS-polyacrylamide gel at 25 mA (constant) for 2 h. After electrophoresis, gels were fixed with 50% methanol–10% acetic acid solution, stained with Quick CBB® (Wako, Osaka, Japan) and then destained with 7% acetic acid solution and distilled water. In-gel digestion was performed in accordance with a modified version of the method of Shevchenko *et al.* (21). Briefly, the stained gel fractions were cut out from whole gel and washed with distilled water and 50% acetonitrile–20 mM Tris–HCl buffer (pH 8.0), digested with 4 μg/mL trypsin in 20 mM Tris–HCl buffer (pH 8.0) at 37°C for 20 h and suspended in 0.1% formic acid.

Mass spectrometric analysis

The digested gingival crevicular fluid protein fraction was used for mass spectrometric analysis. Briefly, the gingival crevicular fluid sample was injected into a nanoLC system (Waters, Milford, MA, USA) with Atlantis

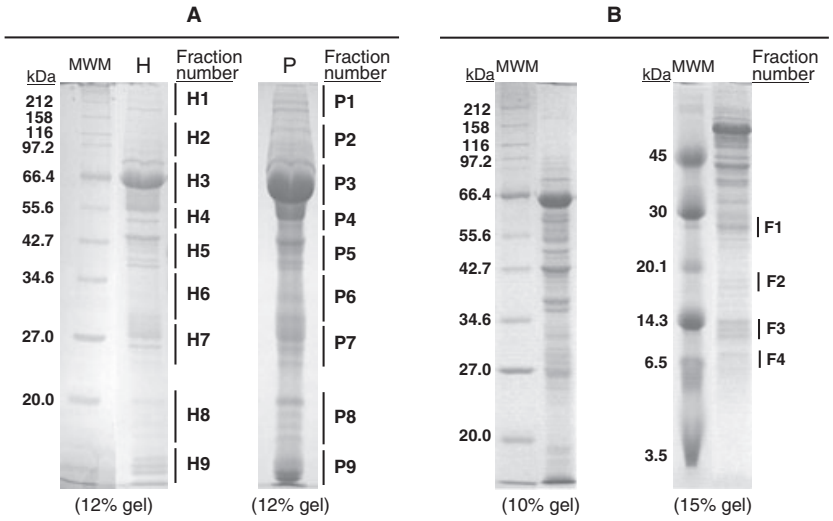

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of gingival crevicular fluid samples. Gingival crevicular fluid samples were collected from one gingival crevice of a healthy subject and two periodontal pockets of two periodontitis patients. After determination of protein in gingival crevicular fluid, the extracts of gingival crevicular fluid from the healthy site (H; 24.9 μg of protein per lane) and one periodontitis site (P; 153.3 μg of protein per lane) were subjected to SDS-PAGE (12% gel) and stained with CBB (A; H and P, respectively). The gingival crevicular fluid sample from the other periodontitis patient was divided into two (each 22.4 μg of protein per lane), electrophoretically separated on 10 and 15% gels and stained by CBB (B). Bars and numbers by the side of gel lanes show the fractions analysed by mass spectrometry. MWM, molecular weight marker.

dC18 column (0.075 mm × 150 mm) for nano-scale LC (Waters). The mobile phase consisted of solvent A (5% acetonitrile–0.1% formic acid solution) and solvent B (95% acetonitrile–0.1% formic acid solution). Peptides were separated under a gradient condition of 5–50% solvent B for 45 min at a flow rate of 200 nL/min. The peptides were continuously analysed using Q-Tof Ultima API (Waters), and the MS scan was performed between m/z 350–1950 (MS) and 50–1950 (MS/MS). The obtained MS and MS/MS data were searched for *Homo sapiens* origin (227,560 sequences) and analysed using the NCBI nr database (10,141,316 sequences and 3,459,383,033 residues) using MASCOT server program (version 2.0.05; Matrix Science Ltd, London, UK). Proteins were identified by only one peptide with a score higher than 38. The search results of each fraction of three gingival crevicular fluid samples in Fig. 1 were assessed using decoy methods (22) and showed a false discovery rate of $< 5\%$.

Western blotting

Antimicrobial peptides, including lactoferrin, α1-antitrypsin, lipocalin, S100A7 (psoriasin), S100A8, S100A9 and cathelicidin, in gingival crevicular fluid were confirmed by western blotting. Briefly, the gingival crevicular fluid samples (25–30 μg of protein) derived from six subjects with periodontitis were separated by SDS-PAGE with 10, 12.5 or 15% gel. The proteins on the gel were transferred to Hybond-P (GE Healthcare Life Sciences, Chalfont, UK) at 50 V (constant) for 3 h. The membrane was incubated in Starting Block™ Blocking Buffer (Thermo Scientific, Rockford, IL, USA)–Tween 20 solution for 1–2 h, washed in 10 mM Tris-HCl buffer (pH 7.4)–Tween 20 (0.05%) and then incubated with anti-lactoferrin antibody (1:1000 dilution; AbD Serotec, Kidlington, UK), anti-α1-antitrypsin antibody (1:1000 dilution; Novus Biologicals, LLC, Littleton, CO, USA), anti-lipocalin antibody (1:1000 dilution; R&D Systems, Minneapolis, MN, USA), anti-S100A8/S100A9 (calprotectin) antibody (1:1000 dilution; Hycult Biotech, Uden, The Netherlands), anti-S100A7 antibody (1:500 dilution; Acris Antibodies GmbH, Herford, Germany) or anti-cathelicidin antibody (1:200 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 3 h at room temperature. After washing, the membranes were reacted with each horseradish peroxidase-labeled secondary antibody (each at 1:2000 dilution; ECL-anti-mouse IgG peroxidase-linked species-specific whole antibody from GE Healthcare Life Sciences; polyclonal rabbit anti-goat immunoglobulin/horseradish peroxidase from Dako Cytomation, Carpinteria, CA, USA; or donkey anti-rabbit IgG horseradish peroxidase-linked species-specific whole antibody from GE Healthcare Life Sciences) for 2 h at room temperature, and were developed with ECL™ Western Blotting Detection reagents (GE Healthcare Life Sciences) for 1 min and exposed to Hyperfilm™ ECL (GE Healthcare Life Sciences).

Results

CBB staining of proteins in gingival crevicular fluid

The total amount of protein in gingival crevicular fluid samples collected from periodontitis sites was approximately sixfold that of healthy site samples (total proteins: 24.9 and 153.3 μg;

Fig. 1A; H and P). There were 23–28 visible stained bands on the gels of gingival crevicular fluid samples, and their patterns were almost the same (Fig. 1A; H and P). Gingival crevicular fluid samples contained several bands of proteins with a high molecular weight (more than 158 kDa), major bands with a molecular weight between 55.6 and 97.2 kDa, and about eight bands between 34.6 and 55.6 kDa. Seven to eight bands were present in the region reflecting a low molecular weight (< 27 kDa). For further analysis of proteins with a low molecular weight, a gingival crevicular fluid sample from a subject with periodontitis was separated using 10 and 15% polyacrylamide gels (Fig. 1B). Four visible groups, including several bands, were observed in the region reflecting a weight of < 27 kDa.

Identification of proteins in gingival crevicular fluid by mass spectrometric analysis

When gingival crevicular fluid samples were separated into nine fractions on an SDS-PAGE gel and each fraction was analysed by mass spectrometry, 168 proteins were identified in gingival crevicular fluid samples from a healthy site and 167 proteins were detected in gingival crevicular fluid samples from a periodontitis site (Table 1 and Table S1). One hundred and four proteins were contained in both healthy and periodontitis samples; 64 proteins were contained in only the healthy sample and 63 proteins were detected in only the periodontitis sample. Major proteins identified in gingival crevicular fluid samples from both healthy and periodontitis sites were α2-macroglobulin and its precursor, myosin heavy polypeptide nine, serum albumin and its precursor/preproprotein, gelsolin isoform, a precursor, lactotransferrin precursor, chain C solution structure of human immunoglobulin M, transferrin precursor, chain A α1-antitrypsin, enolase 1, anti-rabies SOJA immunoglobulin heavy chain, β-actin, chain A crystal structure of lipid-free human apolipoprotein A-1, Rho GDP dissociation inhibitor (GDI) β, peptidylprolyl isomerase A (cyclophilin A), cofilin 1 and S100 calcium-binding protein (S100) A9 (S100A9). Main proteins contained in only the healthy gingival crevicular fluid sample were tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β polypeptide and its ζ polypeptide, stratifin, chain A Ggermline V-genes sculpt, the binding site of a family of antibodies neutralizing human cytomegalovirus, transthyretin precursor, thioredoxin peroxidase PMP20 and α- and β-globin. In the periodontitis sample alone, chain L comparison of the three-dimensional structures of a humanized and a chimeric Fab of an anti-γ interferon antibody, tyrosine 3/tryptophan 5-monooxygenase activation protein ζ polypeptide, chain A crystal structure of the Fab fragment of a human monoclonal Igm cold agglutinin, protein NIG 64 λ Bence-Jones, haptoglobin isoform 1 preproprotein, chain D neutron structure analysis of deoxy-human hemoglobin and chain A hemoglobin (α V1m) mutant were mainly detected. Complement component C4A, immunoglobulin heavy chain, heat shock protein (HSP) 70-1, hemopexin precursor, ADP-ribosylation factor 1, transgelin 2, cathelicidin antimicrobial peptide (LL-37) and thioredoxin were detected in both healthy and periodontitis gingival crevicular fluid samples, and MMP-8 preproprotein was contained in the periodontitis sample at a low level (Table S1).

For further investigation of proteins in CBB-stained bands with low molecular weight of the periodontitis sample, another gingival crevicular fluid sample was separated on 10 and 15% PAGE gels and four fractions of narrow ranges of molecular weight were analysed by mass spectrometry (Table 2 and Table S2). Eighteen proteins with low molecular weight were identified in a gingival crevicular fluid sample from another periodontitis patient. Major proteins were peptidylprolyl isomerase A, hemoglobin β chain, hemoglobin α2, S100A8, S100A11 and MMP-9 precursor. Interleukin-1 family member 9, psoriasin (S100A7), TMSB4X protein, resistin and myotrophin were detected at low levels in the fractions with low molecular weight (Table 2). In contrast, the three analysed gingival crevicular fluid samples contained seven AMPs, including lactoferrin (lactotransferrin), α1-antitrypsin, neutrophil lipocalin, S100A7, S100A8, S100A9 and cathelicidin (Tables 1 and 2 and Tables S1 and S2).

Verification of AMPs in gingival crevicular fluid

To verify AMPs that were detected using mass spectrometry, six gingival crevicular fluid samples from periodontitis sites were analysed by western blotting (Fig. 2). The proteins of lactoferrin (molecular weight 78 kDa) and α1-antitrypsin (52 kDa) were detected in two gingival crevicular fluid samples. The bands of lipocalin (25 kDa), S100A9 (14 kDa), S100A8 (8 kDa) and S100A7 (11.4 kDa) were observed in two or three gingival crevicular fluid samples. Cathelicidin was detected in one gingival crevicular fluid sample. Seven AMPs identified by mass spectrometric analysis were confirmed in other gingival crevicular fluid samples from the inflamed periodontal pockets.

Discussion

Gingival crevicular fluid contains many proteins derived from physiological and inflammatory bodily fluid, and these proteins have been studied as markers of periodontal diseases (2,5,23). However, the entirety of protein components in gingival crevicular fluid remains poorly understood. Ngo *et al.* (12) reported 33 peptides and 66 proteins in gingival crevicular fluid samples collected from multiple periodontal pockets, and Grant *et al.* (20) identified 186 human proteins in gingival crevicular fluid samples from periodontally healthy and experimental gingivitis sites using LC-MS/MS. In contrast, in the present study, 231 proteins were identified in two gingival crevicular fluid samples from a healthy gingival crevice and an inflamed periodontal pocket using the LC-MS/MS system. These proteins included circulating blood proteins, enzymes, cytoskeleton-related proteins, immunity-related proteins, AMPs, inflammation-

Table 1. Gingival crevicular fluid proteins* detected by liquid chromatography coupled with tandem mass spectrometry

Protein name	Relative molecular mass	Accession number	Number of detected peptides (Mascot score)		Classification	Fraction number†
			H	P		
Alpha-2-Macroglobulin	163175	gi | 112911	53 (1837)	50 (1769)	Blood	1
Myosin heavy polypeptide 9, nonmuscle	226392	gi | 12667788	10 (531) ND***	35 (1777)	Cytoskeleton	1
				6 (248)		2
Serum albumin precursor	69180	gi | 6013427	9 (267)	17(699)	Blood	1
			27 (979)	24 (847)		2
			28 (967)	43 (1116)		6
			5 (171)	ND		8
			7 (269)	ND		9
Apolipoprotein B-100 precursor	515236	gi | 178730	9 (420)	10 (482)	Lipid	1
			ND	5 (250)		2
Immunoglobulin γ2 heavy chain constant region	35791	gi | 25987831	10 (290)	7 (267)	Immunity	1
Talin	269661	gi | 4235275	7 (254)	4 (125)	Cytoskeleton	1
Fibronectin precursor	256529	gi | 31397	5 (200)	4 (130)	Cytoskeleton	1
Alpha-2-Macroglobulin precursor	163189	gi | 66932947	29 (1030)	37 (1472)	Blood	2
Gelsolin isoform a precursor	85644	gi | 4504165	21 (926)	15 (886)	Cytoskeleton	2
Complement component C3	187046	gi | 179665	7 (335)	ND	Immunity	2
			2 (62)	7 (292)		5
			2 (195)	14 (734)		6
Lactotransferrin precursor	78132	gi | 54607120	13 (623)	12 (549)	Immunity	2
Actinin, α1 isoform a	105502	gi | 194097350	13 (640)	5 (235)	Cytoskeleton	2
Chain C, Solution structure of human immunoglobulin M	56666	gi | 166007160	10 (412)	9 (422)	Immunity	2
			8 (430)	26 (512)		6
92 kDa type IV collagenase	78377	gi | 177205	7 (386)	12 (602)	Enzyme	2
Glycogen phosphorylase	97062	gi | 3170407	7 (301)	11 (389)	Enzyme	2
Ceruloplasmin precursor	122128	gi | 4557485	9 (419)	19 (905)	Enzyme	2
Actinin, α4	104788	gi | 12025678	8 (379)	ND	Cytoskeleton	2
Ig A1 Bur	73331	gi | 229585	7 (365)	6 (345)	Immunity	2
Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	55151	gi | 15029894	6 (223)	5 (297)	Others	2
Ubiquitin-activating enzyme E1	117774	gi | 23510338	5 (276)	3 (176)	Enzyme	2
Complement factor B	85450	gi | 291922	5 (114)	2 (87)	Immunity	2
Inter-α (globulin) inhibitor H4 isoform 2 precursor	99795	gi | 262050538	5 (193)	ND	Others	2
Chain A, crystal structure of human full- lengthvinculin (residues 1–1066)	115928	gi | 83753119	ND	5 (167)	Cytoskeleton	2
Albumin preproprotein	69321	gi | 4502027	93 (1857)	102 (1561)	Blood	3
			31 (1183)	54 (1150)		4
			29 (1010)	30 (996)		5
Transferrin precursor	77000	gi | 4557871	13 (617)	12 (562)	Blood	3
Complement component 3 precursor	187030	gi | 115298678	1 (62)	5 (296)	Immunity	3
Chain A, Active site distortion is sufficient for proteinase inhibit second crystal structure of covalent serpin–proteinase complex	40041	gi | 83754916	ND	8 (350)	Enzyme	3
Ig α1 chain C region	37631	gi | 113584	ND	5 (257)	Immunity	3
			9 (417)	ND		4
Pyruvate kinase	57841	gi | 35505	ND	5 (310)	Enzyme	3
Chain A, α1-antitrypsin	44223	gi | 157831596	22 (850)	27 (864)	Immunity	4
Enolase 1	47139	gi | 4503571	15 (847)	16 (707)	Enzyme	4
Pyruvate kinase 3 isoform 1 variant	57947	gi | 62897413	15 (616)	ND	Enzyme	4
Anti-rabies SOJA immunoglobulin heavy chain	51752	gi | 27728681	15 (525)	15 (332)	Immunity	4
Adenylyl cyclase-associated protein	51641	gi | 5453595	7 (308)	ND	Enzyme	4
			ND	6 (209)		8
α-Amylase	57714	gi | 178585	5 (290)	ND	Enzyme	4

Table 1. (Continued)

Protein name	Relative molecular mass	Accession number	Number of detected peptides (Mascot score)		Classification	Fraction number†
			H	P		
Coronin, Actin binding protein, 1A	50994	gi | 5902134	5 (108)	ND	Cytoskeleton	4
L-plastin	70244	gi | 167614506	3 (173)	6 (361)	Blood	4
			ND	5 (223)		6
Phosphogluconate dehydrogenase	53118	gi | 984325	2 (135)	6 (249)	Enzyme	4
Coronin-like protein	51038	gi | 1002923	ND	6 (203)	Cytoskeleton	4
Vitamin D-binding protein precursor	52883	gi | 32483410	ND	5 (193)	Blood	4
Glucose phosphate isomerase	63107	gi | 18201905	ND	5 (254)	Enzyme	4
β-Actin	41710	gi | 4501885	34 (967)	47 (882)	Cytoskeleton	5
			5 (233)	23 (757)		6
			ND	11 (281)		7
			6 (223)	8 (370)		8
Glyceraldehyde-3-phosphate dehydrogenase	36031	gi | 31645	18 (638)	9 (348)	Enzyme	5
			1 (57)	7 (280)		7
Transaldolase I	37516	gi | 5803187	10 (373)	9 (409)	Enzyme	5
Haptoglobin precursor	45176	gi | 306882	9 (318)	15 (455)	Blood	5
			3 (116)	6 (211)		9
Aldolase A	39307	gi | 28614	8 (366)	10 (361)	Enzyme	5
Phosphoglycerate kinase I	44586	gi | 4505763	7 (287)	7 (360)	Enzyme	5
α1-Antitrypsin	46677	gi | 177831	ND	5 (271)	Immunity	5
Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, ζ polypeptide	27696	gi | 68085578	15 (566)	ND	Others	6
Tyrosine 3-monooxygenase/ tryptophan 5- monooxygenase activation protein, β polypeptide	28065	gi | 4507949	14 (557)	ND	Others	6
Stratifin	27757	gi | 5454052	14 (511)	ND	Others	6
Phosphoglycerate mutase 1	28786	gi | 4505753	9 (431)	3 (115)	Enzyme	6
Lactate dehydrogenase A isoform 1	36665	gi | 5031857	9 (349)	5 (199)	Enzyme	6
Tyrosine 3-monooxygenase/ tryptophan 5- monooxygenase activation protein, η polypeptide	28201	gi | 4507951	9 (289)	ND	Others	6
Chain A, crystal structure of 14-3-3 γ in complex with A phosphoserine peptide	28154	gi | 82407948	8 (305)	ND	Others	6
Carbonic anhydrase I	28852	gi | 4502517	7 (279)	11 (364)	Enzyme	6
Tyrosine 3-monooxygenase tryptophan 5- monooxygenase activation protein, ϵ polypeptide	29155	gi | 5803225	7 (268)	ND	Others	6
Carbonyl reductase 1	30356	gi | 4502599	6 (321)	ND	Enzyme	6
Chain L, comparison of the three-dimentional structures of a humanized and a chimeric Fab of an anti-γ-interferon antibody	23586	gi | 5542066	ND	12 (244)	Immunity	6
			ND	7 (212)		8
Immunoglobulin light chain	23261	gi | 194173397	5 (297)	ND	Immunity	6
Nucleoside phosphorylase	32097	gi | 157168362	5 (261)	ND	Enzyme	6
L-lactate dehydrogenase B	36615	gi | 4557032	5 (258)	2 (67)	enzyme	6
Haptoglobin Hp 2	41717	gi | 223976	2 (54)	5 (133)	blood	6
			10 (310)	ND		8
Tyrosine 3/tryptophan 5-monooxygenase activation protein, ζ polypeptide	27728	gi | 4507953	ND	11 (505)	Others	6
Chain A, apo-human serum transferrin (nonglycosylated)	74643	gi | 110590597	ND	6 (206)	Blood	6

Table 1. (Continued)

Protein name	Relative molecular mass	Accession number	Number of detected peptides (Mascot score)		Classification	Fraction number†
			H	P		
Chain A, crystal structure of lipid-free human apolipoprotein A-1	28061	gi | 90108664	34 (1240)	69 (1084)	Lipid	7
Serum albumin	69321	gi | 28592	15 (594)	27 (759)	Blood	7
Glutathione *S*-transferase	23367	gi | 2204207	14 (602)	8 (514)	Enzyme	7
Chain A, germline V-genes sculpt the binding site of a family of antibodies neutralizing human cytomegalovirus	23687	gi | 258588258	21 (556)	ND	Others	7
Rho GDP dissociation inhibitor (GDI) β	22974	gi | 56676393	10 (309) 10 (136)	3 (176) 12 (168)	Others	7 8
Immunoglobulin λ light chain VLJ region	28236	gi | 21669581	8 (378)	ND	Immunity	7
Triosephosphate isomerase 1 isoform 1	26653	gi | 4507645	8 (344)	ND	Enzyme	7
Neutrophil lipocalin	20535	gi | 4261868	6 (248)	4 (159)	Immunity	7
Small proline-rich protein 3	18128	gi | 63021422	5 (197)	ND	Cytoskeleton	7
Chain A, Crystal structure of the Fab fragment of a human monoclonal Igm cold agglutinin	23266	gi | 10835792	ND	35 (662)	immunity	7
Protein NIG64 λ, Bence-Jones	22661	gi | 351205	ND	10 (363)	Others	7
Triosephosphate isomerase 1	26625	gi | 17389815	ND	7 (368)	Enzyme	7
Peptidylprolyl isomerase A (cyclophilin A)	18000	gi | 13937981	19 (531)	15 (360)	Enzyme	8
Cofilin 1 (nonmuscle)	18491	gi | 5031635	13 (447)	13 (514)	Cytoskeleton	8
Transthyretin precursor	15877	gi | 4507725	13 (587)	ND	Blood	8
Thioredoxin peroxidase PMP20	21976	gi | 6166493	10 (334)	ND	Enzyme	8
Nonmetastatic cells, protein (NM23A) expressed isoform a	19641	gi | 38045913	10 (270)	4 (161)	Others	8
Glia maturation factor, γ	16790	gi | 4758440	7 (172)	4 (165)	Cell Function	8
Calmodulin	17152	gi | 825635	2 (50)	5 (141)	Cell Function	8
Haptoglobin isoform 1 preproprotein	45177	gi | 4826762	ND	24 (478)	Blood	8
hCG2016877, isoform CRA_c	43255	gi | 119594653	ND	9 (296)	Others	8
Transthyretin	20186	gi | 114318993	ND	9 (503)	Blood	8
α-Actin	42081	gi | 178027	ND	7 (329)	Cytoskeleton	8
Albumin, isoform CRA_h	68568	gi | 119626071	ND	6 (172)	Blood	8
β-Globin	18919	gi | 183817	50 (662)	ND	Blood	9
S100 calcium-binding protein A9 (S100A9)	13234	gi | 4506773	22 (361)	14 (403)	Immunity	9
Profilin 1	15045	gi | 4826898	18 (360)	7 (300)	Cytoskeleton	9
α2 Globin	15248	gi | 183801	17 (478)	ND	Blood	9
SH3 domain binding glutamic acid-rich protein like	12766	gi | 4506925	5 (285)	1 (87)	Others	9
Coactosin-like 1	15935	gi | 1624607	6 (134)	ND	Cytoskeleton	9
Fatty acid binding protein 5 (psoriasis-associated)	15155	gi | 4557581	6 (119)	ND	Lipid	9
Chain D, neutron structure analysis of deoxy human hemoglobin	15869	gi | 161760892	ND	126 (814)	Blood	9
Chain A, hemoglobin (α V1m) mutant	15149	gi | 3891367	ND	75 (496)	Blood	9

ND, not detected.

* Proteins with more than five detected peptides in H or P gingival crevicular fluid sample are shown.

† Fraction number is the number of gel fraction shown in Fig. 1A. H, health sample; P, periodontitis sample.

Table 2. Liquid chormatography coupled with tandem mass spectrometry analysis of proteins with low molecular weight* in gingival crevicular fluid from periodontitis

Fraction number†	Protein name	Relative molecular mass	Accession number	Number of detected peptides (Mascot score)	Classification
F 1	Matrix metalloproteinase-9 (MMP-9) precursor	78377	gi | 116863	4 (262)	Enzyme
F 2	Nm23 protein	20398	gi | 35068	1 (56)	Enzyme
	Nudix-type motif 3	19459	gi | 5729804	1 (53)	Enzyme
	Interleukin 1 family, member 9	18709	gi | 9665234	1 (40)	Inflammation
F 3	Hemoglobin β chain	15954	gi | 4378804	15 (262)	Blood
	Hemoglobin α2	15280	gi | 22671717	7 (168)	Blood
	Haptoglobin-related protein	43049	gi | 1495458	3 (89)	Blood
	Psoriasin (S100A7)	11450	gi | 190668	1 (42)	Immunity
	Histone H2A	6050	gi | 510990	1 (41)	Cell
F 4	S100 calcium binding protein A11 (S100A11)	11733	gi | 5032057	4 (195)	Cell Function
		10828	gi| 21614544	7 (187)	Immunity
	S100 calcium binding protein A8 (S100A8)	7136	gi | 112180539	2 (113)	Cell Function
		21613	gi | 10835256	1 (73)	Others
	TMSB4X protein	11411	gi | 9966777	1 (72)	Inflammation
	PYD and CARD domain containing isoform a	12887	gi | 21956645	1 (63)	Others
	Resistin	11450	gi | 190668	1 (58)	Immunity
	Myotrophin				
	Psoriasin (S100A7)				

* These proteins were detected in the fraction of <27 kDa in Fig. 1B and not identified in two gingival crevicular fluid samples of Fig. 1A.
† Fraction number is the number of the gel fraction shown in Fig. 1B.

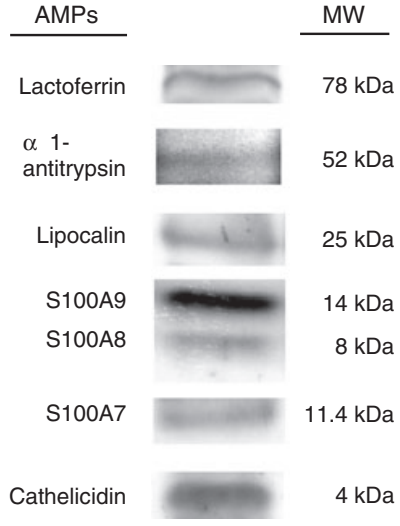

Fig. 2. Western blotting of antimicrobial peptides (AMPs) in gingival crevicular fluid. Gingival crevicular fluid samples were collected from periodontal pockets of six periodontitis patients and subjected to SDS-PAGE (25–30 μg of protein per lane) with 10, 12.5 or 15% gel. The proteins on the gels were transferred to polyvinylidene difluoride membranes and specific AMP proteins were immunoreacted with each anti-AMP antibody and then horseradish peroxidase-labeled secondary antibody. The molecular weight (MW) of each AMP protein is shown on the right.

related proteins, lipid-related proteins and others.

The components in gingival crevicular fluid are similar to serum components (24). In the present study, circulating blood proteins, including macroglobulin, albumin, globins and several glycoproteins, were detected with high frequency in gingival crevicular fluid samples from both healthy and periodontitis sites. Heme-binding proteins, such as transferrin and hemopexin, calcium-binding proteins, such as S100A7, S100A8, S100A9, S100A11, calmodulin and ceruloplasmin, and copper-carrying proteins were identified in gingival crevicular fluid. Haptoglobin and orosomucoid (α1 acid glycoprotein) detected in gingival crevicular fluid appear to bind to hemoglobin released from erythrocytes and the charged lipophilic compounds, respectively. Furthermore, transthyretin, a carrier of thyroxine, and vitamin D-binding proteins were also contained in gingival crevicular fluid. These blood-related proteins are supposed to infiltrate from blood vessels and carry metals and chemical elements to periodontal tissues.

Gingival crevicular fluid contains some species of enzymes that are expressed in periodontal tissues, cells and blood. In the present study, glycolytic enzymes, including glycogen phosphorylase, pyruvate kinase, enolase, glucose phosphate isomerase, aldolase A, phosphoglycerate kinase, glyceraldehydes-3-phosphate dehydrogenase, phosphorglycerate mutase and triose-phosphate isomerase, were detected in gingival crevicular fluid of healthy and periodontitis sites. These glycolytic enzymes appear to be derived from actively metabolized oral epithelial cells, gingival fibroblasts and alveolar bone-related cells that are degraded by periodontitis, because these enzymes have not been reported in normal blood. Lactate dehydrogenase A and B detected in the healthy and periodontitis samples are glycolytic enzymes that catalyse the conversion between pyruvate and lactate, and lactate dehydrogenase in gingival crevicular fluid is known as a tissue-degraded marker for periodontal diseases because the enzyme is induced by cell death (25). Matrix metalloproteinase-8 and MMP-9 (92 kDa type IV collage-

nase), which are proteolytic enzymes, were observed in gingival crevicular fluid samples from periodontitis sites (Table 2 and Table S1). Matrix metalloproteinase-8 is mainly produced by neutrophils and released into gingival crevicular fluid in periodontal diseases (26). Matrix metalloproteinase-9 is expressed by some cells, including neutrophils, macrophages, keratinocytes, fibroblasts and osteoclasts, and a high level of MMP-9 was reported in gingival crevicular fluid from periodontitis sites (27). The research presented here and analysis by Ngo *et al*. (12) showed peptidylprolyl isomerase A (cyclophilin A) in gingival crevicular fluid at a high level. This enzyme catalyses the *cis–trans* isomerization of peptide bonds at the N-terminal to proline residues and binds to ciclosporin A, and was shown to induce inflammatory infiltration and alveolar bone resorption in experimental periodontitis (28). Glutathione *S*-transferase, an oxidative stress marker, was present in saliva and gingival tissues with periodontal diseases (29,30) and was detected at high levels in gingival crevicular fluid samples from healthy sites and periodontitis sites (Table 1). Thioredoxin peroxidase PMP20 (peroxiredoxin) was observed in gingival crevicular fluid as well as saliva and may play a role in the defense of periodontal tissues because this enzyme protects cells from reactive oxygen species (31).

Actin- and myosin-related proteins, which are important cytoskeleton-related proteins, were contained in gingival crevicular fluid at high levels (Table 1). Actin, a component of actin filament present in all eukaryotic cells, was identified in gingival crevicular fluid and saliva by several proteome studies (12,31). Actin-binding proteins, including actinin, cofilin, coronin, gelsolin and profilin, were detected in gingival crevicular fluid in the present study, and also by Ngo *et al*. (12) and Grant *et al*. (20). These proteins appear to be derived from periodontal tissue cells, blood cells and immune cells, and regulate the turnover of periodontal tissues by binding between cells and extracellular matrix or cells (32,33). Myosin is involved in cell movement and cell division in connection with actin filaments, and the high frequency of myosin detection in mass spectrometric analysis may be due to degradation of periodontal tissues and bleeding by inflammation. Fibronectin, a major component of extracellular matrix, in gingival crevicular fluid samples appears to be derived from periodontal tissues and blood because fibronectin binds to collagen, fibrin and integrins (34).

Gingival crevicular fluid contained immune-system-related factors that inhibit the invasion of microbes through the interface of periodontal tissues composing the gingival crevice or periodontal pocket. Multiple immunoglobulins (IgG, IgM, IgA and IgE) are reported in gingival crevicular fluid (5), and some Ig components (α- and β-heavy chains, and κ- and λ-light chains and their fragments) were also detected in gingival crevicular fluid by mass spectrometry in the present analysis. Immunoglobulins in gingival crevicular fluid appear to contribute to defense against microbial infection in dental plaque and calculus as part of acquired immunity. In addition, gingival crevicular fluid contained some AMPs, including α1-antitrypsin, cathelicidin, lactoferrin (lactotransferrin), lipocalin, S100A7, S100A8 and S100A9, and complement components that play important roles in the innate immunity of periodontal tissues. α1-Antitrypsin, a serum trypsin inhibitor, is produced by the liver and secreted into bodily fluids, including blood, saliva, tears and gingival crevicular fluid, and may inhibit the degradation of periodontal tissues by blocking neutrophil elastase in gingival crevicular fluid (35). Cathelicidin (LL-37), expressed in neutrophils and epithelial cells, shows antimicrobial activity, migration and chemotaxis (36), and its level in gingival crevicular fluid was determined using SELDI-TOF-MS (10) and was high in gingival crevicular fluid from periodontitis sites (37). Lactoferrin (lactotransferrin) is a glycoprotein contained in milk, tears, saliva and gingival crevicular fluid, and shows broad antimicrobial activity, and the lactoferrin level in gingival crevicular fluid was found to correlate with the gingival index, probing depth and volume of gingival crevicular fluid (15). Neutrophil lipocalin is expressed in epithelial cells as well as neutrophils and shows antimicrobial activity by binding to iron siderophores; it increases in gingival connective tissues with inflammation (38,39). S100A7 is expressed in healthy skin and mucosal epithelium, and its expression is increased by inflammatory cytokines and keratinocyte differentiation modulator in oral epithelial cells (40–42). S100A8 and S100A9 are expressed in neutrophils, macrophages and epithelial cells, and show antimicrobial activity by zinc-chelating action (43). We previously identified calprotectin, S100A8/S100A9 complex, in gingival crevicular fluid by western blotting and ELISA, and showed that the calprotectin level was high in gingival crevicular fluid samples from periodontitis sites (13,14). Furthermore, S100A8 and S100A9 were also detected in gingival crevicular fluid using electrophoresis and several mass spectrometric systems (12,19). Antimicrobial peptides in gingival crevicular fluid may function in defense against infection in the surrounding periodontal tissues in co-operation with other AMPs. Gingival crevicular fluid contains complement components at 70% of the level of serum complements; complement component 1 (C1) was detected in gingival crevicular fluid, and its activity was reduced by dental plaque (44,45). In the present study, complement component 3 (C3) and complement component C4A (C4) were detected in gingival crevicular fluid (Table 1 and Table S1). C3 and C4 each form complexes with other components and appear to play roles in innate immunity to periodontal diseases by activating other complement components and inducing opsonization of bacterial pathogens in the periodontal pocket.

Regarding inflammation-related proteins, HSP-70, IL-1 and resistin were detected in gingival crevicular fluid samples in the present analysis. Heat shock protein, a stress protein, is expressed upon bacterial infection, inflammation and in the presence of reactive oxygen species (46), and gingival crevicular fluid HSP 70 is released

from periodontal tissue cells and may protect such cells from stress conditions caused by infection and inflammation. Inflammatory cytokines, including IL-1α, IL-6 and tumor necrosis factor-α, were identified in gingival crevicular fluid by ELISA (2,5), but these cytokines were detected at a low level or hardly observed when gingival crevicular fluid samples were analysed by mass spectrometry and electrophoresis in the present study and in other studies (12,19). Although we did not elucidate the reason for this, the detection sensitivity of mass spectrometric analysis appears not to be high compared with that of ELISA, and the biological stability of cytokines is not sufficiently high for detection by mass spectrometry.

Several apolipoproteins, including A, B, C, D and H, were observed in gingival crevicular fluid in the present study. Apolipoprotein A1, a high-density lipoprotein, is contained in plasma and saliva (31) and was also detected in gingival crevicular fluid (Table S1). Apolipoprotein B, a major low-density lipoprotein and a marker for cardiovascular diseases, was identified in gingival crevicular fluid by western blotting (47) and LC-MS/MS (20), and its concentration was determined by ELISA (47). Although apolipoprotein B in gingival crevicular fluid has not been well characterized, its concentration has a broad range (0.8–267.8 μg/mL; 47).

Some gingival crevicular fluid components detected in the present study have not previously been reported. These proteins were carbonic anhydrase 1, ceruloplasmin, glutathione *S*-transferase, glycogen phosphorylase, phosphoglycerate mutase 1, psoriasin (S100A7), S100A11 and resistin, among others. S100A11, a member of the calcium-binding S100 protein family, is expressed in diverse tissues, including skin, placenta, heart, kidney, liver and lung, and also in human skin and gingival keratinocytes (37,38,48). S100A11 shows pleiotropic functions (stimulation and arrest of cell proliferation, regulation of enzyme activity and induction of apoptosis; 49), but the role of S100A11 in gingival crevicular fluid and periodontal tissues is unknown. Resistin is an adipocytokine that is detected in adipose tissue, blood, skeletal muscle and pancreas, and its level is associated with obesity and diabetes in mice; however, in humans, resistin is expressed in monocytes/macrophages, neutrophils and lymphocytes and increases in inflammatory diseases, such as rheumatic diseases, atherosclerosis and inflammatory bowel diseases (50,51). We determined resistin in gingival crevicular fluid by ELISA and showed that the resistin level of gingival crevicular fluid from periodontitis sites was higher than that of a healthy site (data not shown). Resistin induces the secretion and production of proinflammatory cytokines and chemokines in neutrophils, macrophages, endothelial cells, adipocytes and osteoclasts (50,51), and may be a modulator in periodontal diseases.

The level of each component in gingival crevicular fluid was considered to be reflected by the number of detected peptides when the number of detected peptides of the same protein in the identical fraction of gingival crevicular fluid samples from healthy and periodontitis sites was compared in mass spectrometric analysis (52). Hemoglobin, haptoglobin, anti-agglutinin antibody, MMP8, MMP9 and anti-γ-interferon antibody were detected more in gingival crevicular fluid samples from periodontitis sites than from healthy sites, suggesting that gingival crevicular fluid samples from periodontitis sites contain blood components and proteases that are associated with inflammation and tissue degradation. Some proteins in gingival crevicular fluid are thought as markers for periodontal diseases, and LC-MS/MS system makes it possible to detect multiple proteins in gingival crevicular fluid at the same time. However, only three gingival crevicular fluid samples were analysed in the present study. To obtain information about gingival crevicular fluid from various periodontal disease states, the analysis of more gingival crevicular fluid samples will be necessary in future. A mass spectrometry system can analyse a very small volume of one gingival crevicular fluid sample. Therefore, mass spectrometric analysis appears to be useful for accurate diagnosis of periodontal diseases. The analysis of gingival crevicular fluid using an LC-MS/MS system may become a diagnostic method for systemic diseases as well as periodontitis because gingival crevicular fluid contains many components derived from blood and is collected by a noninvasive procedure.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Proteins with the number of detected peptides of less than 4 in two GCF samples on Table 1.

Table S2 Proteins* detected in the fraction of more than 27 kDa in Figure 1B.

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