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Cleaved inflammatory lactoferrin peptides in parotid saliva of periodontitis patients

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Abstract

Lactoferrin (Lf) is a member of the transferrin family of iron-binding anti-bacterial proteins, present in most exocrine secretions, such as saliva, and plays an important role in mucosal defense. In this study, we identified small Lf peptides with Con A low-affinity in the parotid saliva of chronic periodontitis patients by Con A two-dimensional immunoelectrophoresis, Con A affinity chromatography and Western blotting using anti-human Lf polyclonal Ab. N-terminal amino acid sequencing of the four Con A low-affinity Lf peptides confirmed them to be fragments of intact Lf. The detection ratio of the proteinase 3 (PR3)-like activity was elevated in the parotid saliva of periodontitis patients and was associated with the severity of clinical symptoms. PR3 protein was also detected in the parotid saliva of periodontitis patients, and PR3, but not human leukocyte elastase and cathepsin G, degraded intact Lf. Con A low-affinity saliva Lf peptides showed no anti-bacterial activity against *Escherichia coli*, and had a reduced iron-chelating capacity. Con A low-affinity saliva Lf peptides, PR3-treated Lf preparation and two of four synthetic polypeptides induced the production of interleukin IL-6, monocyte chemoattractant protein-1 and IL-8, and the activation of NF-κB in human oral epithelial HSC-2 cells. Furthermore, concentrations of the Lf peptides in the parotid saliva of periodontitis patients were increased with a correlation to the severity of clinical symptoms. These results suggest that Lf in the parotid saliva of periodontitis patients was degraded into small peptides by the PR3-like activity with the capability to induce inflammatory mediators.

Keywords: Lactoferrin; Saliva; Inflammation; Periodontitis

1. Introduction

Saliva, a complex mix of fluids from major (parotid, submandibular and sublingual) and minor salivary glands, is a most valuable oral fluid that is critical to the preservation and maintenance of oral health, such as oral mucous membrane and teeth (Mandel, 1987). Saliva contains a number of antimicrobial agents, including lactoferrin (Lf), secretory IgA, proteins (glycoproteins, statherins, agglutinins, histidine-rich proteins and proline-rich proteins), mucins, antimicrobial peptides (Humphrey and Williamson, 2001; Tenovuo, 1998), and a recognition molecule of pathogen-associated molecular patterns, CD14 (Uehara et al., 2003; Takayama et al., 2003). Saliva also contains many enzymes, such as lysozyme, peroxidase, elastase, transferases and glycosidases, which originate from gingival crevicular fluids (GCF), leukocytes and epithelial cells in addition to salivary glands (Nakamura and Slots, 1983; Ueno et al., 1996). The concerted action of these agents is thought to provide a multifunctional protective network against microorganisms.

Lf is a member of the transferrin family of iron-binding proteins and present in most exocrine secretions, such as milk, saliva, nasal exudate, bronchial mucus, gastrointestinal fluid, pancreatic fluid and tears (Lönnerdal and Iyer, 1995; Weinberg, 2001). Lf is also stored in the secondary specific granules of neutrophils (Lönnerdal and Iyer, 1995; Weinberg, 2001). As

Abbreviations: Lf, lactoferrin; GCF, gingival crevicular fluid; HLE, human leukocyte elastase; MCP-1, monocyte chemoattractant protein-1; PR3, proteinase 3; SLPI, secretory leukocyte protease inhibitor

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almost all bacteria require iron for growth, Lf can principally inhibit the spread of bacteria by chelating iron under certain conditions (Lönnerdal and Iyer, 1995; Weinberg, 2001). In addition to the iron-binding capability, Lf and especially the pepsin-digested N-terminal region, termed lactoferricin, distinct from the iron-binding sites, have broad-spectrum activity against various microorganisms, including Gram-positive and -negative bacteria (Bellamy et al., 1992; Ellison and Giehl, 1991; Yamauchi et al., 1993), viruses (van der Strate et al., 2001) and fungi (Lupetti et al., 2003), and have antitumor activity (Iigo et al., 1999). Moreover, Lf interacts with LPS and its active moiety lipid A (Appelmelk et al., 1994; Elass-Rochard et al., 1995), and consequently neutralizes the function of endotoxin (Zhang et al., 1999). Thus, Lf exhibits important functions for host defense mechanisms.

Periodontitis is one of the major diseases afflicting mankind and caused by a bacterial infection leading to gingival inflammation, the destruction of periodontal tissues, loss of alveolar bone, and culminating in tooth loss (Holt and Bramanti, 1991). Many investigators have tried to find indicators that will be able to evaluate the status of periodontitis to date, and most studies have assessed enzymes, inflammatory mediators or the number of leukocyte in GCF. Some reports demonstrated that in periodontitis, Lf is increased in concentration in the GCF (Friedman et al., 1983; Adonogianaki et al., 1996), that Lf is an effective marker of crevicular neutrophil numbers (Adonogianaki et al., 1993), and that human leukocyte elastase (HLE) increases the concentration in GCF, correlating with Lf in periodontitis patients (Murray et al., 1995). It was also reported that the concentration of Lf in the GCF correlated with clinical parameters, and it is a more sensitive indicator of periodontal pathology than traditional clinical indices (Tsai et al., 1998).

As the analysis of GCF is restricted to specialists, saliva has been considered to be a potential valuable oral sample for evaluating periodontal health. Elevated levels of enzymes, such as HLE (Uitto et al., 1996) and collagenase (Uitto et al., 1990), in the oral fluid have been reported in periodontitis. However, the stringent analysis of saliva itself for evaluating the periodontal status has not been reported. To address this issue, we analyzed the properties of Lf in saliva by various electrophoresis methods and N-terminal amino acid sequencing. In addition to whole saliva, we analyzed parotid saliva to exclude possible contamination of the GCF and other materials from leukocytes, epithelial cells or oral bacteria. We also examined the anti-bacterial activity against *Escherichia coli*, the iron-binding capacity and the inflammation-inducing activity by measuring the production of IL-6, monocyte chemoattractant protein-1 (MCP-1) and IL-8, and the activation of NF-κB in oral epithelial cells in culture.

2. Materials and methods

2.1. Reagents

Purified human Lf and affinity-purified rabbit anti-human Lf polyclonal Ab were purchased from ICN Pharmaceuticals (Aurora, OH). Purified human proteinase 3 (PR3) and goat anti-human PR3 polyclonal Ab were obtained from Elastin products (Owensville, MO). HLE and cathepsin G were obtained form Calbiochem (San Diego, CA). Lf peptides were synthesized by Takara Bioproduct (Kyoto, Japan). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise indicated.

2.2. Saliva samples

The diagnosis of chronic periodontitis was established on the basis of the clinical criteria at the Department of Periodontics, Tohoku University Hospital (Sendai, Japan). Japanese patients who had no general diseases were divided into four groups based on the severity of chronic periodontitis characterized on the basis of the amount of clinical attachment loss (Armitage, 1999) as follows: healthy (<1 mm, aged 21–50), slight periodontitis (1 or 2 mm, aged 27–77), moderate periodontitis (3 or 4 mm, aged 21–85), and severe periodontitis (≥5 mm, aged 23–70). Whole saliva was collected into sterile plastic tubes. Parotid saliva was collected with the aid of Schaefer cups placed over the parotid duct beforeundergoing periodontal therapy (Schaeffer et al., 1977). The saliva samples were immediately clarified by centrifugation at 14,000 × *g* for 5 min at 4 °C and passed through sterile membrane filters (0.45 μm pore size). Clarified saliva samples were immediately used or aliquotted and frozen at −80 °C until use. The Ethical Review Board of Tohoku University Graduate School of Dentistry approved the experimental procedures (Sendai, Japan).

2.3. Con A two-dimensional immunoelectrophoresis

The carbohydrate structure of intact Lf and Lf preparations used in this study was analyzed by Con A two-dimensional immunoelectrophoresis, as described previously (Komine et al., 2005).

2.4. Purification of Lf molecules in parotid saliva

Parotid saliva was precipitated with 50% saturated ammonium sulfate. The precipitate was suspended in PBS and dialyzed against PBS. Lf in the parotid saliva was purified by affinity chromatography, which coupled rabbit anti-human Lf polyclonal Ab to CNBr-activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ). Elution was carried out with 4.5 M $MgCl_2$. The fraction of human Lf was dialyzed in 20 mM Tris–HCl, 0.5 M NaCl, pH 7.2 and concentrated with a freeze dry method. For further purification, the fraction eluted with the Lf affinity column was applied to a column of Con A Sepharose (Amersham Biosciences) equilibrated with 20 mM Tris–HCl, 0.5 M NaCl, pH 7.2. Elution was carried out stepwise with 0.001, 0.01 and 0.1 M α-D-methylmannoside. Each eluted fraction was dialyzed against deionized water.

The protein concentration of each eluted fraction was measured by DC protein assay (Bio-Rad Laboratories, Hercules, CA). We confirmed that the endotoxin concentration was less than 10^{-4} EU/ml with an endotoxin detection kit (Endospecy test; Seikagaku, Tokyo, Japan) (Obayashi et al., 1985).

2.5. SDS-PAGE and western blotting

The molecular weights of Lf in samples were measured by 15% SDS-PAGE using Laemmli's method (Laemmli, 1970). The purified Lf was confirmed to have antigenicity to anti-human Lf polyclonal Ab by Western blotting. After SDS-PAGE, proteins were blotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The blotted membrane was blocked with 3% OVA, 0.05% Tween 20, 20 mM Tris–HCl and 137 mM NaCl, pH 7.2 for 2 h at room temperature. After blocking, the membrane was incubated with HRP-conjugated rabbit anti-human Lf polyclonal Ab at 4 °C overnight. The resulting Ag–Ab complexes were detected with ECL Western blotting detection reagents (Amersham Biosciences) in Chemi Imager 4000 (Alpha Innotech, San Leandro, CA).

To detect the PR3 protein in the saliva, saliva samples were concentrated by vacuum centrifuge, and 30-times concentrated saliva samples were subjected to Western blotting using goat anti-human PR3 polyclonal Ab, as described previously (Sugawara et al., 2001).

2.6. N-terminal amino acid sequence determination

After the SDS-PAGE and blotting, the blotted membrane was stained Coomassie Brilliant Blue. After drying, detected bands on the stained membrane were excised and analyzed as to their N-terminal amino acid sequence. The N-terminal amino acid sequences of each Lf molecules were determined using a protein sequencer (G1005A Protein Sequencing System; Hewlett Packard, Palo Alto, CA).

2.7. Growth inhibition of bacterium by Lf preparations

Growth inhibitory effect of each Lf preparation on *E. coli* NIHJ was measured using the paper disk method (Komine et al., 2005). Paper disks were soaked with each Lf preparation at 0.5 and 1 mg in PBS.

2.8. Capacity of iron-binding to Lf preparations

The iron-binding capacity was assessed using a chelating sepharose chromatography method (Hitrap Chelating HP column; Amersham Biosciences). The gel was conjugated with iron using an Fe solution (pH 3.2) containing 0.1 M $FeCl_3$, 0.05 M glycine, 0.01 M ammonium bicarbonate and 8 mM HCl. Five milligrams of the Lf preparations were applied to the gel. Then, the gel was washed with sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl, and the non-iron-binding fraction was collected. After the washing, the iron-binding fraction was collected with an elution buffer composed of phosphate buffer containing 0.05 M EDTA, pH 7.2. Absorbance was measured at 280 nm with a spectrophotometer (DU 640; Beckman Coulter, Fullerton, CA). The iron-binding capacity was calculated from the ratio of the Lf concentration of the iron-binding fraction to the total Lf concentration.

2.9. Measurement of PR3-like activity in saliva

Saliva samples were concentrated 10 times using the freeze dry method and pretreated with 2 μM secretory leukocyte protease inhibitor (SLPI) (R&D System, Minneapolis, MN) for 30 min before use. Amidolytic activity in the saliva samples was assayed at 25 °C for 10–90 min with 0.625 mM Boc-Ala-*p*-nitrophenyl ester (Boc-Ala-ONp; Bachem, Bubendorf, Germany) in 0.1 M HEPES buffer containing 0.1 M NaCl, 10 mM $CaCl_2$, 0.005% Triton X-100, and 5% DMSO, pH 7.5 (Sugawara et al., 2001). The liberation of *p*-nitrophenol was monitored at 405 nm. More than OD_{405} 0.5 at 90 min was estimated as positive and the detection ratio was calculated as follows: (the number of saliva samples more than OD_{405} 0.5 at 90 min)/(the total number of saliva samples examined) × 100 (%).

2.10. Treatment of human Lf with serine proteases

Human Lf (1 mg/ml) was incubated with PR3, HLE or cathepsin G at the indicated concentrations at 37 °C for 1 h. After the incubation, the samples were mixed with Laemmli's sample buffer and boiled for 3 min. Then, the molecular weights in these samples were examined by SDS-PAGE and Western blotting using rabbit anti-human Lf polyclonal Ab. In addition, 100 μl of human Lf solution (2 mg/ml) was incubated with 0.5 unit of PR3 for 1 h, and the reaction was stopped by 100 μl of α_1-antitrypsin (500 μg/ml). Then, it was used for the stimulation of oral epithelial cells and electrophoresis.

2.11. Cytokine and chemokine production from oral epithelial cells

A human oral epithelial cell line, HSC-2 (Momose et al., 1989), established from squamous cell carcinoma, was obtained from the Cancer Cell Repository, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and grown in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) with 10% FCS (Gibco; Invirogen, Carlsbad, CA).

HSC-2 cells were stimulated with human intact Lf, PR3-treated Lf, purified Con A low-affinity saliva Lf peptides and synthetic Lf peptides (10 μg/ml each) for the time indicated at 37 °C in a CO_2 incubator. After the incubation, the cultures were centrifuged at 2500 × *g* for 20 min, and the supernatants were collected. The levels of IL-6, IL-8 and MCP-1 in the supernatants were determined with a human IL-6 ELISA kit (GT, Minneapolis, MN) and human IL-8 and MCP-1 ELISA kits (American Research Products, Belmont, MA).

2.12. Assay for NF-κB activity

Activated NF-κB was measured with an NF-κB assay kit specific for the p65 subunit according to the manufacturer's instructions (Active Motif, Carlsbad, CA), as described previously (Uehara et al., 2004).

2.13. Measurement of Lf and Lf peptides in saliva

Antiserum against the Lf peptide was raised by immunizing the synthetic peptide pep4 (GQKDLLFKDSAl) into rabbits using the method described previously (Kai et al., 2002). The antiserum was purified to IgG by ammonium sulfate precipitation and DE52 cellulose (Whatman, Maidstone, England) columns (Kai et al., 2002). The purified anti-Lf peptide polyclonal Ab was labeled with HRP by the method described previously (Nygren et al., 1979).

Concentrations of whole Lf and Lf peptides were measured with sandwich ELISA using rabbit anti-human Lf polyclonal Ab and rabbit anti-Lf peptide polyclonal Ab, respectively, according to the method described previously (Kuroishi et al., 2003). The detection range was 300 ng/ml to 100 μg/ml and the percentage of the coefficiency of variation was less than 5%.

2.14. Statistical analysis

All of the experiments were conducted at least three times to confirm the reproducibility of the results. Experimental values are given as the mean ± S.E.M. of triplicate assays. The statistical significance of the difference between the two means was evaluated using the two-tailed Student's *t*-test, and $P < 0.05$ was considered significant.

3. Results

3.1. Detection of Lf peptides with Con A low-affinity in parotid saliva of periodontitis patients

We first examined the property of Lf in parotid saliva of chronic periodontitis patients by Con A two-dimensional electrophoresis. Peak 1 and peak 2 showed Con A high- and low-affinity, respectively (Fig. 1). Lf in the parotid saliva of each clinical case revealed two peaks, and peak 2 was abundant in the saliva of moderate and severe cases. A low level of peak 2 was also detected in a mild case. In contrast, peak 1 was mainly detected in the healthy controls, as in the case of human intact Lf.

We next examined the molecular weight and antigenicity of saliva Lf with SDS-PAGE and Western blotting using anti-human Lf polyclonal Ab. Parotid saliva samples were pooled and two-step purified using anti-Lf-coupled and Con A Sepharose affinity columns. Con A two-dimensional immunoelectrophoresis confirmed that purified Con A high- and low-affinity saliva Lf reparations revealed the single peak with high- and low-affinity to Con A, respectively (Fig. 2A). Human intact Lf was able to detect the single band of approximately 86 kDa, and the Con A low- and high-affinity saliva Lf could detect multiple bands (Fig. 2B). The Con A low-affinity saliva Lf showed the major bands of approximately 48, 32, 23, 22 and 19 kDa. In contrast, the Con A high-affinity saliva Lf exhibited high molecular weight bands (86, 72, and 56 kDa). These results indicate that Lf in the parotid saliva of periodontitis patients was degraded into small peptides with Con A low-affinity.

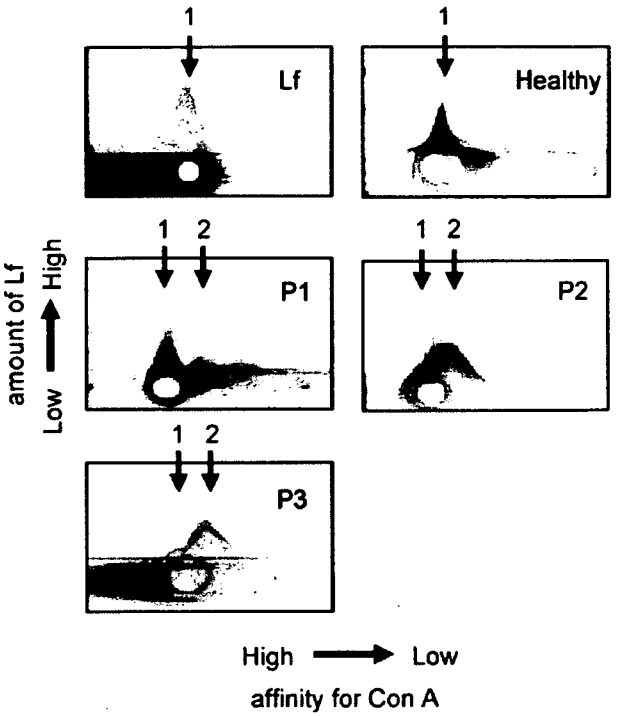

Fig. 1. Detection of Lf with Con A low-affinity in the parotid saliva of periodontitis patients. Human intact Lf (Lf) and the parotid saliva of healthy control and slight (P1), moderate (P2) and severe (P3) cases of periodontitis were separated by Con A two-dimensional immunoelectrophoresis. The abscissa and ordinate indicate the affinity for Con A and the amount of Lf, respectively. Peak 1 and peak 2 are Con A high- and low-affinity peaks, respectively.

3.2. N-terminal amino acid sequences of Con A low-affinity saliva Lf peptides

N-terminal amino acid sequences of Con A low-affinity saliva Lf peptides were analyzed by a protein sequencer. The N-terminal sequences of the peptides contained in the Lf were RRSVQWCAVSQPEAT for the 32-kDa peptide, KPVDKFKD-CHLARV for the 23-kDa peptide, KFQLFGSPSGQKDLLF for the 22-kDa peptide, and AARRARVVWCAVGEQ for the 19-kDa peptide. The position of each Lf peptide in human Lf is shown in Fig. 3. It was indicated that the 32-kDa Lf peptide has an anti-bacterial domain, termed lactoferricin (Bellamy et al., 1992; Yamauchi et al., 1993). The position of the 23- and 22-kDa Lf peptides was in the N-lobe fragment. On the other hand, the 19-kDa Lf was a C-lobe fragment containing the end of the N-lobe amino acid arginine (R). The 48- and 32-kDa fragments reacted with anti-lactoferricin Ab (data not shown), indicating that the sequences are highly overlapped. Therefore, we excluded the 48-kDa peptide for the sequencing.

3.3. No anti-bacterial activity and reduced iron-binding capacity of Con A low-affinity saliva Lf peptides

Lf has a chelating effect on ferrous ions in bodily fluid and inhibits the growth of bacteria, such as *E. coli* (Ellison and Giehl, 1991). Therefore, the anti-bacterial activity of purified Con A high- and low-affinity saliva Lf peptides was examined

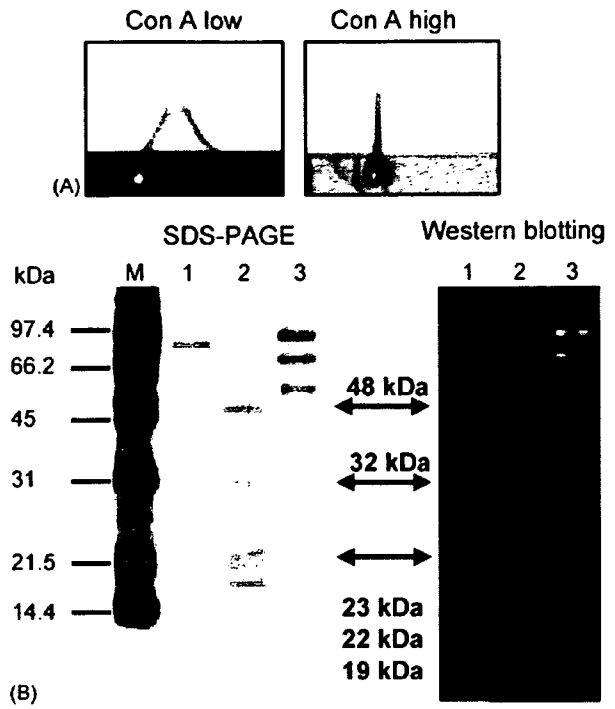

Fig. 2. The analysis of purified parotid saliva Lf with Con A high- and low-affinity. (A) Parotid saliva samples were pooled and two-step purified using anti-Lf-coupled and Con A Sepharose affinity columns, and analyzed by Con A two-dimensional immunoelectrophoresis. (B) Samples in A were subjected to 10% SDS-PAGE and western blotting using anti-human Lf polyclonal Ab. M, molecular weight marker; lane 1, human intact Lf; lane 2, Con A low-affinity saliva Lf preparation; lane 3, Con A high-affinity saliva Lf preparation.

against *E. coli* as a target bacteria with the paper disk method in vitro. Human intact Lf was used as a positive control. The intact Lf and the Con A high-affinity saliva Lf peptides at both 0.5 and 1.0 mg/disk definitely inhibited the growth of *E. coli*, whereas the Con A low-affinity saliva Lf peptides showed no anti-bacterial effect at both concentrations (Table 1). The iron-binding capacities of the intact Lf and Con A high-affinity saliva Lf peptides were 0.94 and 0.88 for the iron-binding Lf/total Lf ratio, respectively. However, the iron-binding Lf/total Lf ratio (0.21) of the Con A low-affinity saliva Lf peptides was approximately 4.5-fold lower than that of intact Lf. These results clearly indicate that the Con A low-affinity Lf saliva peptides had no anti-bacterial activity and a reduced iron-chelating capacity.

3.4. Degradation of human Lf by PR3 and the generation of Con A low-affinity Lf peptides

Neutrophils are critically involved in inflammation, and activated neutrophils produce serine proteases, such as HLE, cathepsin G and PR3. In addition, human epithelial cells are able to express and produce PR3 after inflammatory stimuli (Uehara et al., 2004; Sugawara, 2005). As neutrophil counts were negligible in the parotid saliva of periodontitis patients (data not shown), we examined the possible involvement of PR3 in Lf degradation. PR3-like activity in the 10-times concentrated saliva samples was measured using Boc-Ala-ONp as a substrate and expressed as the detection ratio (see Section 2), since the activity was low in the original saliva samples. The detection ratio of the PR3-like activity in the parotid saliva was increased in all cases of periodontitis and correlated with the severity of periodontitis

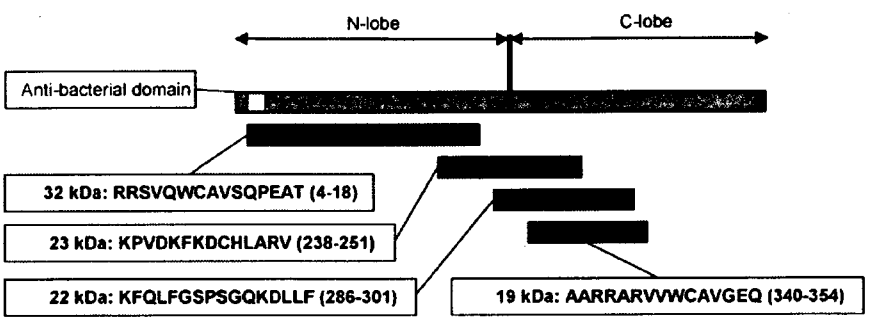

Fig. 3. Amino acid sequences of Con A low-affinity saliva Lf peptides. The cleaved 32-, 23-, 22- and 19-kDa Con A low-affinity saliva Lf peptides blotted to a polyvinylidene difluoride membrane were excised and analyzed as to their N-terminal amino acid sequence using a protein sequencer. The position of each Lf peptide and known anti-bacterial lactoferricin domain (Yamauchi et al., 1993) are shown.

Table 1
The anti-bacterial effect and iron-binding capacity of Con A high- and low-affinity saliva Lf peptides

	E. coli growth inhibition circle (diameter, mm)		Iron-binding capacity (iron-binding Lf/total Lf)
	Lf[a]: 0.5 mg	Lf[a]: 1 mg	
PBS	ND[b,c]	ND[b,c]	ND[b]
Human Lf	19 ± 0.3	26 ± 0.1	0.94
Con A high-affinity saliva Lf	17 ± 0.1	24 ± 0.2	0.88
Con A low-affinity saliva Lf	ND[b,c]	ND[b,c]	0.21

[a] The amount of each Lf preparation per disk.
[b] ND, not detectable.
[c] $P < 0.05$ compared with human Lf.

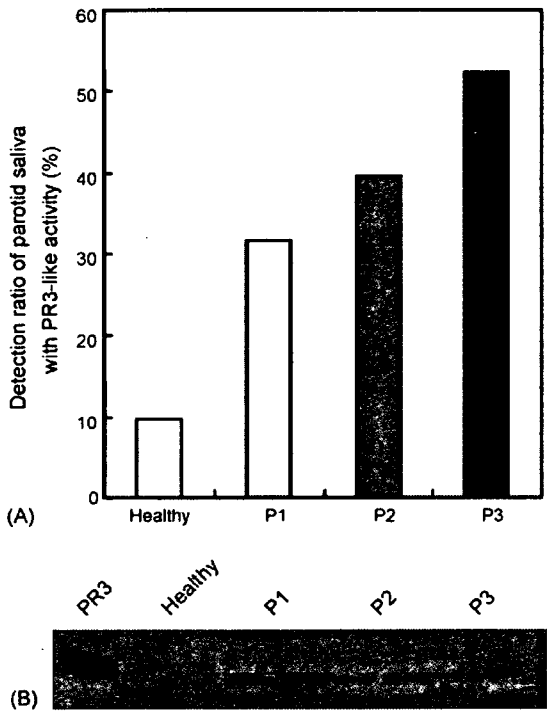

Fig. 4. Detection of PR3-like activity and PR3 protein in the parotid saliva of periodontitis patients. (A) Parotid saliva of healthy subjects without periodontitis (healthy, $n=37$) or patients with slight (P1, $n=39$), moderate (P2, $n=42$) and severe (P3, $n=34$) periodontitis were 10-times concentrated and pretreated with 2 μM SLPI for 30 min. Then, the enzymatic activities in the samples were quantified using Boc-Ala-ONp, and more than OD_{405} 0.5 at 90 min was estimated as positive, and the results are expressed as the detection ratio. (B) Parotid saliva (30 × concentrated) of healthy subjects without periodontitis (normal) or patients with slight (P1), moderate (P2), and severe (P3) periodontitis was subjected to Western blotting using anti-human PR3 polyclonal Ab. Purified PR3 (5 μg/lane) was loaded as a control.

(Fig. 4A). The activity was blocked by phenylmethylsulfonyl fluoride, an inhibitor of serine proteases, including PR3 (data not shown). PR3 protein in the saliva samples was also detected in periodontitis cases but not healthy controls by Western blotting (Fig. 4B).

We next examined the effect of HLE, cathepsin G and PR3 on human Lf in vitro by SDS-PAGE and Western blotting using anti-human Lf polyclonal Ab. PR3 efficiently degraded Lf into small peptides among three serine protease (Fig. 5A). The 29-kDa band that appeared in Lf treated with cathepsin G in SDS-PAGE was cathepsin G, because the band was not detected in Western blotting. Con A two-dimensional immunoelectrophoresis confirmed that PR3-treated Lf revealed a single peak with low-affinity to Con A (peak 2) (Fig. 5B). Intact Lf and the parotid saliva of severe periodontitis patients were used as controls. These results indicate that PR3-like activity is responsible for the degradation of Lf and the generation of Lf peptides in parotid saliva.

3.5. Cytokine and chemokine production from oral epithelial cells and NF-κB activation in the cells by Con A low-affinity saliva Lf peptides, PR3-treated Lf and synthetic peptides

We synthesized the polypeptides (pep1 to pep4) of Con A low-affinity saliva Lf peptides without an anti-bacterial domain or the C-lobe fragment of Lf (Table 2) and examined whether the cleaved and the synthetic Lf peptides stimulate the production of proinflammatory cytokines and chemokines from oral epithelial HSC-2 cells. The production of IL-6, MCP-1 and IL-8 in response to the Lf preparations peaked at 48-, 15- and 24-h stimulation, respectively, and the production showed a higher value on the stimulation of Con A low-affinity saliva Lf peptides, PR3-treated Lf, pep1 and pep4 than intact Lf (Fig. 6A–C). In

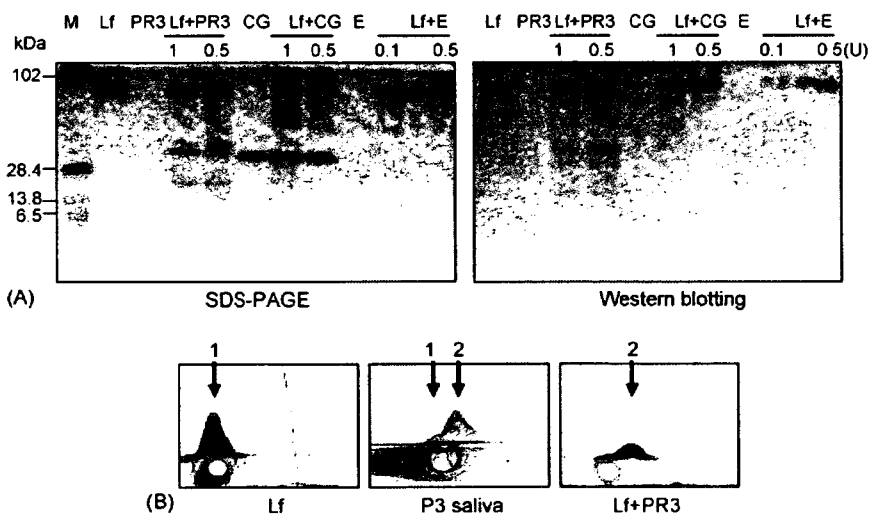

Fig. 5. The degradation of Human Lf with PR3. (A) Human Lf was treated with buffer alone or the indicated concentrations of PR3, cathepsin G (CG) and HLE (E) at 37 °C for 1 h, then subjected to 15% SDS-PAGE, and western blotting using anti-human Lf polyclonal Ab. M, molecular weight marker. (B) Human intact Lf was treated with PR3 (1 U) for 1 h and separated by Con A two-dimensional immunoelectrophoresis (Lf + PR3). Intact Lf (Lf) and parotid saliva of a severe periodontitis patient (P3 saliva) were used as a control. The abscissa and ordinate indicate the affinity for Con A and the amount of Lf, respectively. Peak 1 and peak 2 are Con A high- and low-affinity peaks, respectively.

Table 2
Amino acid sequences of synthesized human Lf peptides

	Amino acids	Position in human Lf
pep1	FKDCHLA	243–249
pep2	VPSHAVVAR	251–259
pep3	FQLFGSP	287–293
pep4	GQKDLLFKDSAI	295–306

contrast, intact Lf, pep2 and pep3 showed only marginal activity in the induction of IL-6, MCP-1 and IL-8. Consistent with this, Con A low-affinity saliva Lf peptides, PR3-treated Lf, pep1 and pep4 induced the marked activation of NF-κB in HSC-2 cells (Fig. 6D). Con A high-affinity saliva Lf showed the same activity as intact Lf (data not shown). These results indicate that cleaved Lf peptides in parotid saliva acquired the capability to induce inflammatory cytokines and chemokines through the activation of NF-κB.

3.6. Increase in Lf peptides in parotid saliva of periodontitis patients

As pep4 is one of the inflammatory Lf peptides, pep4 was used to immunize rabbits to obtain anti-Lf peptide polyclonal Ab, and concentrations of whole Lf, including intact Lf and Lf peptides, and Lf peptides in the whole and parotid saliva of periodontitis patients were examined using ELISA. The concentrations of whole Lf were significantly increased in moderate and severe cases in whole saliva, whereas they were slightly increased in moderate cases in parotid saliva, compared with healthy controls (Fig. 7A). In contrast, concentrations of Lf peptides were markedly increased in all cases of periodontitis in parotid saliva, but only slightly increased in severe case of periodontitis in whole saliva, compared with healthy controls (Fig. 7B). These results indicate that the increased concentrations of Lf peptides in parotid saliva were associated with periodontitis.

4. Discussion

The present study showed that the levels of cleaved Con A low-affinity Lf peptides were elevated in the parotid saliva of chronic periodontitis patients in association with the severity of clinical symptoms, and that the cleaved saliva Lf peptides induced the production of IL-6, MCP-1 and IL-8 from oral epithelial cells and the activation of NF-κB in the cells. As the production of IL-6, MCP-1 and IL-8, and the activation of NF-κB are associated with inflammation, we designate the cleaved Con A low-affinity Lf peptides as 'inflammatory Lf peptides'. In addition to whole saliva, parotid saliva was analyzed to exclude the possible contamination of GCF and other materials from leukocytes, epithelial cells or oral bacteria. As parotid saliva was directly collected from the orifice of parotid duct, it is unlikely that the parotid saliva was contaminated with other materials.

Human Lf is resistant to proteolysis (Davidson and Lönnerdal, 1987), and the present study provided the first evidence that cleaved Lf peptides were secreted from a human exocrine gland. A recent study reported that human milk Lf is a serine protease that cleaves *Haemophilus* surface proteins (Hendrixson et al., 2003). However, the analyses with SDS-PAGE, Western blotting using anti-human Lf Ab and Con A

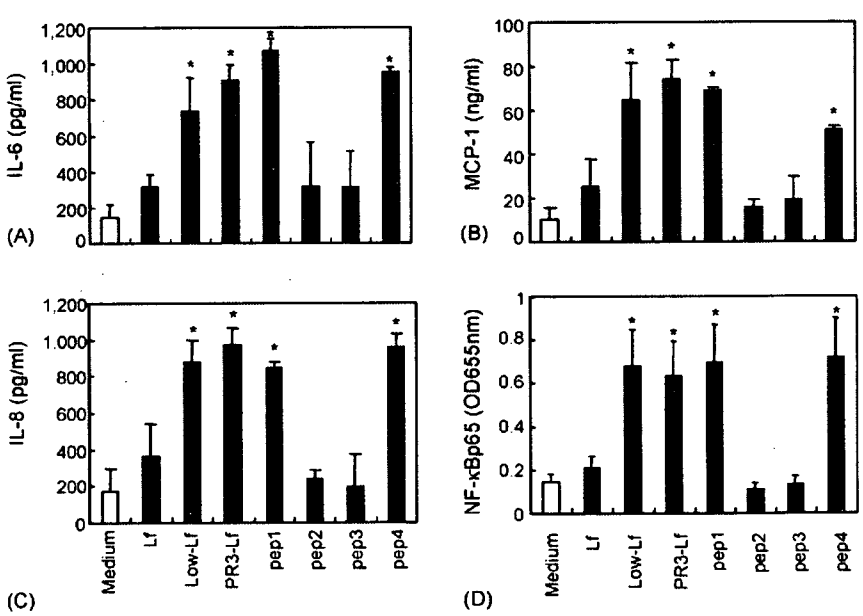

Fig. 6. IL-6, MCP-1 and IL-8 production from oral epithelial cells and NF-κB activation in the cells by Lf preparations. (A–C) HSC-2 cells were incubated with medium alone (medium), human intact Lf (Lf), Con A low-affinity saliva Lf peptides (low-Lf), PR3-treated Lf (PR3-Lf) and synthetic Lf peptides (pep1 to pep4) (10 μg/ml each) for up to 48 h. The levels of IL-6 (A), MCP-1 (B) and IL-8 (C) in the supernatants were determined using ELISA. The peak values of IL-6 at 48 h, MCP-1 at 15 h, and IL-8 at 24 h are depicted. (D) HSC-2 cells were incubated for 4 h in the presence or absence of Lf preparations and synthetic Lf peptides (10 μg/ml each) used in A–C at 37 °C, and active NF-κB was measured. The data are expressed as means ± S.E.M. of triplicate assays. $^{*}P<0.01$ compared with the value of intact Lf.

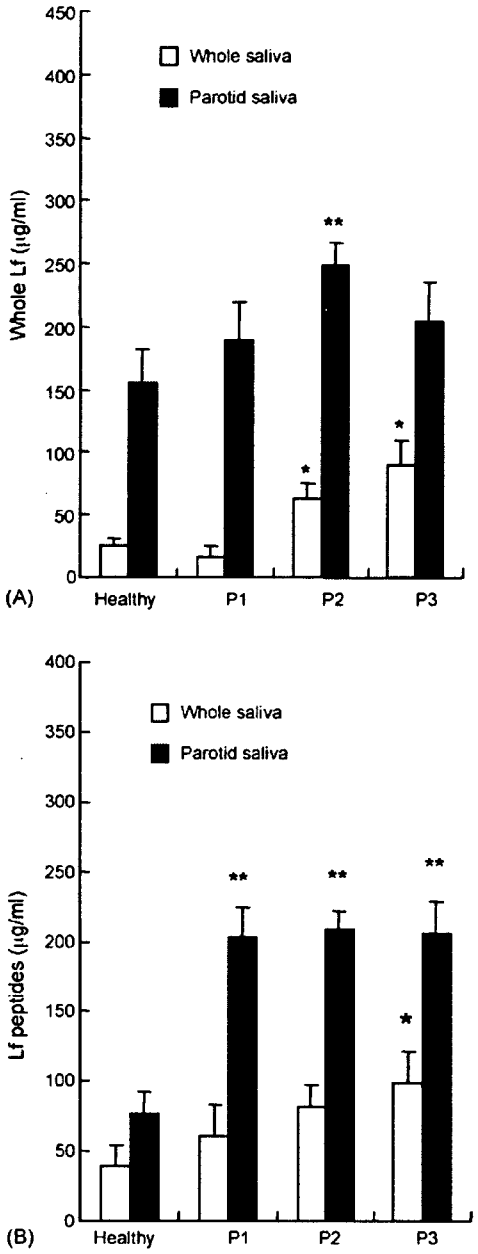

Fig. 7. Concentrations of whole Lf and Lf peptides in the saliva of periodontitis patients. (A and B) Whole and parotid saliva was collected from healthy subjects without periodontitis (healthy, $n=37$) or patients with slight (P1, $n=39$), moderate (P2, $n=42$) and severe (P3, $n=34$) periodontitis, and the concentrations of Lf (A) and Lf peptides (B) in the samples were measured using ELISA. The data are expressed as means ± S.E.M. $^{*}P<0.1$ and $^{**}P<0.01$ compared to the healthy subjects.

two-dimensional immunoelectrophoresis showed that the incubation of human intact Lf alone did not generate cleaved Lf peptides. These results exclude the possibility that the cleavage of saliva Lf resulted from autolysis, instead suggesting that cleaved Lf peptides were generated by proteolysis.

Con A has specificity for α-D-mannose and α-D-glucose, and has been used in the affinity purification of a variety of glycoproteins (Samyn Petit et al., 2003). The present study showed that intact Lf and cleaved saliva Lf peptides exhibited Con A high- and low-affinity, respectively. The results indicate that the cleavage of intact Lf resulted in the generation of Lf fragments with the reduced number of glycosylation sites compared to intact Lf. It is also possible that some Lf fragments without glycosylation are generated and missed by Con A affinity chromatography. The properties of the missed fragments are unknown and remain to be determined.

Neutrophils are critically involved in inflammation, and activated neutrophils produce serine proteases, such as HLE, cathepsin G and PR3. In fact, HLE levels were elevated in the whole saliva of periodontitis patients, and a major source of HLE was considered to be human neutrophils from GCF (Uitto et al., 1996), but we confirmed that neutrophil counts were negligible in parotid saliva of periodontitis patients (data not shown). Therefore, it is unlikely that the parotid gland has inflammation during the course of periodontitis. The average daily flow of whole saliva in healthy individuals amounts to 1–1.5 l, and about 15–20% and 45–50% of whole saliva come from parotid saliva in resting and stimulated conditions, respectively (Sreebny, 2000). Therefore, it is also possible that a steady supply of saliva removes neutrophils, even if neutrophils enter saliva by the proinflammatory effects of the Lf fragments.

Recent studies showed that the expression of PR3 at mRNA and protein levels is induced in endothelial and epithelial cells by inflammatory cytokines, such as IL-1, TNF-α and IFN (Uehara et al., 2004; Sugawara, 2005), although it is controversial whether endothelial cells express PR3 (King et al., 1995; Pendergraft et al., 2000). In addition, SLPI, which was originally isolated form parotid saliva (Thompson and Ohlsson, 1986), functions as an inhibitor of HLE and cathepsin G, but does not inhibit PR3 (Rao et al., 1991). Therefore, we examined the PR3-like activity by the pretreatment of saliva samples with SLPI. The present study showed that the detection ratio of PR3-like activity in parotid saliva of periodontitis patients was increased and correlated with the severity of periodontitis (Fig. 4A), and PR3 protein was detected in the parotid saliva of the patients (Fig. 4B). Furthermore, PR3, but not HLE and cathepsin G, efficiently cleaved Lf in vitro (Fig. 5). It was reported that HLE can degrade lactoferrin by incubation for 48 h (Britigan et al., 1993), whereas our data in Fig. 6 are the result of 1-h incubation. We found that HLE at high concentration can cleave human Lf, but the proteolytic activity of human PR3 was stronger than that of HLE, and human cathepsin G hardly cleaved human Lf. These results suggest that saliva Lf is preferentially cleaved by PR3. It is conceivable that the source of PR3 in the parotid saliva was the parotid salivary gland epithelial cells and that inflammatory cytokines induced at the site of periodontitis may influence the expression of PR3 in salivary gland epithelial cells by way of circulation, resulting in the cleavage of saliva Lf and the elevation of Lf peptides in the parotid saliva of periodontitis patients. However, it is still unclear whether cleaved Lf peptides are generated before or after secretion in saliva. Further studies are required to clarify these points.

It was reported that the N-terminal region of Lf (residues 1–11), termed lactoferricin, exhibited anti-bacterial activity (Bellamy et al., 1992; Ellison and Giehl, 1991; Yamauchi et al.,

1993), and that the first three residues, GRR, play an essential role in the activity (Nibbering et al., 2001). Fig. 3 shows that the 32-kDa cleaved Lf peptide started from the fourth residue of Lf and was partially overlapped with lactoferricin, but the cleaved Con A low-affinity Lf peptides showed no anti-bacterial activity against *E. coli* (Table 1), suggesting that the GRR domain of Lf in the parotid saliva of periodontitis patients was cleaved off by proteolysis, and consequently the cleaved Lf peptides lost their anti-bacterial activity.

Lf has various domains in N-lobe, which shows anti-bacterial effects (Bellamy et al., 1992; Ellison and Giehl, 1991; Yamauchi et al., 1993), anti-inflammatory effects (Zhang et al., 1999), immunoreactive effects (Tanida et al., 2001) and immunosuppressive effects (Siemion et al., 1995). Therefore, we selected the sequences in N-lobe without an anti-bacterial domain and with a highly homology to cow, sheep, goat and dog, and pep1–4 were synthesized. The cleaved Lf peptides and the synthetic pep1 and pep4, but not pep2 or pep3, induced the production of inflammatory cytokines and the activation of NF-κB in oral epithelial HSC-2 cells (Fig. 6). As pep1 and pep4 share FKD residues in the sequences, it is possible that the FKD motif is important for the cell activation. Recently, the human intestinal Lf receptor has been cloned (Suzuki et al., 2001). The Lf receptor gene was expressed at high levels in fetal small intestine, adult heart and salivary glands. The Lf receptor is a glycosylphosphatidylinositol-anchored membrane protein that lacks transmembrane and cytoplasmic domains and functions in the cellular internalization of Lf, indicating that the receptor itself does not elicit intracellular signaling events. Therefore, it is possible that the cleavage of Lf results in the exposure of novel ligands and that the cleaved inflammatory Lf peptides and the synthetic peptides (pep1 and pep4) may activate human mucosal cells via signaling receptors other than the intestinal Lf receptor. The receptor remains to be clarified.

We previously reported that PR3 at 1–10 μg/ml is able to activate oral epithelial cells resulting in the production of cytokines and chemokines via the activation of G protein-coupled protease-activated receptor 2 (PAR2) (Uehara et al., 2002), indicating that a high concentration of PR3 in the vicinity of PR3-producing neutrophils is needed for cell activation through PAR2. This study showed that PR3-like activity in saliva was not high, probably due to dilution of the activity by saliva or consumption of the activity by cleaving saliva proteins, including saliva Lf. Therefore, it is possible that PR3-like activity in saliva may not contribute to the activation of PAR2-expressing oral epithelial cells.

IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation and oncogenesis (Naka et al., 2002). MCP-1 plays a critical role in the activation and migration of monocytes, T cells and NK cells, and is an important factor in the development of Th1 and Th2 responses (Gu et al., 2000; Huang et al., 2001). MCP-1 is also produced by cytokine-primed and -differentiated neutrophils, and has been implicated in the regulation of the transition from innate to adaptive immunity (Yamashiro et al., 2001). IL-8 is a major chemokine responsible for the activation of neutrophils and the migration of neutrophils and T cells to inflammatory sites (Baggiolini et al., 1997). Therefore, the production of IL-6, MCP-1 and IL-8 from oral epithelial cells induced by inflammatory Lf peptides shown in Fig. 7 may play an important role in immune regulation in the course of inflammatory processes at the site of periodontitis.

In conclusion, the present study suggests that (1) saliva Lf is degraded by the PR3-like activity, (2) cleaved inflammatory Lf peptides with no anti-bacterial activity and reduced iron-binding capacity possess inflammatory properties in the induction of inflammatory mediators through the activation of NF-κB, (3) levels of inflammatory Lf peptides are increased in the parotid saliva of periodontitis patients in association with the severity of clinical symptoms. A longitudinal study is required to verify whether the elevated levels of the inflammatory Lf peptides will decrease to the levels of healthy control subjects or whether persons who have high levels of the inflammatory Lf peptides in their parotid saliva may possess a predisposition to periodontal disease. If it is the case, measurement of inflammatory Lf peptides might be useful to diagnose the status of periodontitis.

Acknowledgments

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