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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; Uitto 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).