Human Trial of Liposomal Lactoferrin Supplementation for Periodontal Disease

Atsushi Ishikado, Syoko Uesaki, Hirohisa Suido, Yoshio Nomura, Kazuhide Sumikawa, Mariko Maeda, Mutsumi Miyauchi, Takashi Takata, and Taketoshi Makino

* R&D Department, Sunstar Inc.; 3-1 Asahi-machi, Takatsuki, Osaka 569-1195, Japan; b Sunstar Clinic, STARLECS Inc.; 5-30-1 Kamihamuro, Takatsuki, Osaka 569-1044, Japan; and c Department of Oral and Maxillofacial Pathobiology, Graduate School of Biomedical Sciences, Hiroshima University; 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.

Received July 9, 2010; accepted July 27, 2010; published online July 30, 2010

New approaches to periodontal health have been in strong demand in addition to conventional local plaque control. In this study, liposomal bovine lactoferrin (L-bLF) was orally administered to subjects with periodontal disease to investigate whether it could be a useful treatment. L-bLF composed of soy phosphatidylcholine was given as a supplement for four weeks in tablet form (180 mg bLF/d) to twelve subjects with multiple sites of more than 3 mm probing depth (PD). PD, bleeding on probing (BOP), gingival crevicular fluid (GCF) volume and the levels of tumor necrosis factor (TNF-α), interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein-1 (MCP-1) in GCF were evaluated for 51 sites with more than 4 mm PD in five subjects. Blood samples of all subjects were collected 0, 2 and 4 weeks after supplementation. Isolated peripheral blood mononuclear cells (PBMCs) were incubated for 24 h with or without lipopolysaccharide (LPS) (100 ng/ml) from Porphyromonas gingivalis, and TNF-α, IL-1β, IL-6 and MCP-1 in the culture media were measured. Toll-like receptor 2 (TLR2) and TLR4 mRNA expressions of isolated PBMCs were also quantitatively analyzed using real-time reverse transcription-polymerase chain reaction (RT-PCR). The PD was significantly reduced by L-bLF supplementation, but the BOP and GCF volume were not significantly changed. The MCP-1 level in GCF was significantly reduced, while levels of other cytokines were not changed. Four-week L-bLF supplementation also showed significant decreases of LPS-induced cytokine production from PBMCs. Relative gene expressions of TLR2 and TLR4 did not change. These results suggest that L-bLF supplementation can be effective in the treatment of periodontal disease, although prospective controlled large-scale studies are required.

Key words lactoferrin; liposome; periodontal disease; peripheral blood mononuclear cell; inflammatory cytokine

Periodontal disease is one of the most highly prevalent diseases in the world population, and it is considered to be an inflammatory disease caused by a variety of periodontopathic bacteria when the balance between bacterial infection and host protective response is disrupted. In particular, lipopolysaccharides (LPSs) from gram-negative bacteria such as Porphyromonas gingivalis (P. gingivalis) and Aggregatibacter actinomycetemcomitans building dental plaque on the subgingival tissue induce the inflammatory cytokites such as tumor necrosis factor (TNF-α), interleukin (IL)-1β, IL-6 and monocyte chemoattractant protein-1 (MCP-1) via the Toll-like receptors (TLRs)–nuclear factor-kappa B (NF-κB) signaling pathway in gingival cells and infiltrated immune cells. Further consecutive inflammatory reaction in the alveolar bone resorption through osteoclast formation by inflammatory cytokines. Peripheral blood mononuclear cells (PBMCs) are the major source of these cytokines, and some studies suggest that hypersensitivity of PBMCs reflects the severity of periodontal disease.

Plaque control or supportive care using antibacterial agent has basically been emphasized for the treatment of periodontal disease, however, there is little decline in its prevalence. Recent studies have demonstrated that the systemic condition from aging as well as from metabolic problems such as obesity and diabetes affects the clinical condition of periodontal disease. It has also been reported that the condition of periodontal disease influences the glycemic control of diabetes, and periodontal disease increases the risk of atherosclerosis. These reports suggest that periodontal disease is deeply associated with systemic condition, and therefore it can be considered that introduction of new methods on the basis of systemic condition becomes more important to maintain periodontal health in addition to a conventional plaque control-based local approach.

Lactoferrin (LF) is an 80 kDa iron-binding protein contained mainly in mother’s milk and also in tears, saliva, nasal secretion, semen and neutrophilic leukocytes of mammals. It has been considered to be a bioactive milk protein that plays versatile roles in the immune system responses and helps to protect the body from various infections. Bovine lactoferrin (bLF) has already been manufactured as an available raw material for functional foods and cosmetics. Many studies have already demonstrated that oral administration of bLF shows anti-inflammatory and immuno-modulatory effects, which suggests that intake of bLF could be a new way to maintain periodontal health and prevent periodontal disease. However, it can be considered that bLF is susceptible to being digested and also has low permeability for the intestinal membrane, therefore, we have already developed bLF encapsulated into liposome, which is spherical artificial vesicle composed of bilayers of phosphatidylcholine. Our previous studies have shown that the oral administration of liposomal bLF (L-bLF) exhibited more suppressive effects than non-liposomal bLF on LPS-induced TNF-α production from mouse PBMCs, as well as on osteoclast increase or TNF-α expression in marginal periodontal tissue of a rat LPS-induced periodontitis model. In the present study, L-bLF was orally administered to subjects with periodontal disease to investigate whether it can be useful for treating periodontal disease.

* To whom correspondence should be addressed. e-mail: atsushi.ishikado@jp.sunstar.com © 2010 Pharmaceutical Society of Japan
MATERIALS AND METHODS

Subjects Fourteen in-house volunteers (11 males, 3 females) 37—59 years were enrolled. Approval for the study was obtained from the institutional ethical committee according to the Helsinki Declaration. The patients received an explanation of the details of this study, and written informed consents were obtained. The criteria for inclusion were as follows: (1) no allergies to cow’s milk or soy; (2) no medications affecting systemic or periodontal status; (3) no pregnancy or lactation; (4) presenting at least five sites with periodontal pocket of 3 mm or more deep; (5) no receipt of prophylaxis procedures, including instruction about tooth brushing, within the preceding six months and all test periods.

Study Design and Supplement The study was conducted as an open intervention trial with 4-week L-bLF supplementation. All subjects underwent full-mouth periodontal probing a week before the test periods. As a result of screening, only five subjects who had at least one site with more than 4 mm PD were selected for periodontal examinations. Four tablets of supplements were administrated to each subject for a day (180 mg of bLF per day). The tested supplement composed of multi-lamellar vehicles was prepared by hydrating the food grade soy phosphatidylycerol (Tsuji Oil Mill Co., Ltd., Japan) with the aqueous solution containing bLF (Morinaga Milk Industry Co., Ltd., Japan). Compliance with the supplement intake was assessed by pill counting and intake record at weeks 0, 2 and 4.

Assessments Blood samples were collected by venipuncture, and general, biological and immunological blood examinations as described hereinafter were conducted at weeks 0, 2 and 4 of the study. At the same time, periodontal measurements were registered at the mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual tooth surfaces of all teeth except the third molars.

Periodontal Examinations Periodontal examination consisted of assessments of probing depth (PD) and bleeding on probing (BOP) and collection of gingival crevicular fluid (GCF). All parameters were assessed by a single periodontist. Conventional manual periodontal probes (PCP UNC15, Hu-Friedy, Chicago, IL, U.S.A.) were used to clinically examine the periodontal status. The test sites were selected based on the highest scored PD. For evaluation, the above parameters were used in the following manner: (1) PD was measured from the gingival margin to the base of clinical pockets with the probe tip parallel to the long axis of the tooth at a unit of 1 mm; (2) BOP was evaluated based on the presence or absence of gingival bleeding on probing; (3) After gently drying the area with a blast of air syringe, supragingival plaque was removed without touching the marginal gingiva. The area was isolated by using cotton rolls to prevent saliva contamination, and GCF was collected with paper strips (Periopaper, Proflow Inc., Amityville, NY, U.S.A.) at the bottom of the gingival sulcus. GCF volume was measured by PERIOTRON 8000 (Oraflow Inc., Smithtown, NY, U.S.A.) and represented as PERIOTRON score. Paper strips were eluted into 50 μl phosphate-buffered saline (PBS) for 30 min at room temperature. The supernatant, after centrifugation at 10000 rpm for 5 min, was applied to the Bio-Plex Assay System (Bio-Rad, Hercules, CA, U.S.A.) to analyze concentrations of TNF-α, IL-1β, IL-6 and MCP-1.

P. gingivalis LPS-Induced Inflammatory Cytokines Production in PBMCs Approximately 10 ml of heparinized blood was obtained by venipuncture, and PBMCs were isolated by means of Ficoll–Conray gradient centrifugation. 2.0×10^6 isolated PBMCs were seeded on 48-well plate, and cultured at 37 °C and 5% CO₂ in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in the presence or absence of LPS (100 ng/ml) from P gingivalis, and concentrations of TNF-α, IL-1β, IL-6 and MCP-1 in the supernatant of culture media after incubation for 24 h were analyzed by the Bio-Plex Assay System. Each cytokine level from cells treated without LPS was subtracted from that with LPS, and the value was regarded as each cytokine level of each subject.

RNA Extraction and Real-Time Polymerase Chain Reaction (PCR) Analysis Total RNA was extracted from redundant PBMCs for inflammatory cytokine analyses using a Total RNA Mini Kit (Bio-Rad, Hercules, CA, U.S.A.). Single-strand cDNA was synthesized from 0.3 μg of total RNA using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). Quantitative analyses of TLR2 and TLR4 mRNA were performed by real-time PCR using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Japan). Premix Ex Taq (Takara Bio) and Assay-on-Demand, Gene Expression Products (Hs01872448_s1 for TLR2, Hs00152939_m1 for TLR4, Hs99999905_m1 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Applied Biosystems, Foster City, CA, U.S.A.) were used for the quantitative real-time PCR analysis. All the quantitative data were normalized by the expression level of GAPDH.

Blood Examinations Safety assessments were followed by blood chemistry tests; total protein, aspartate aminotransferase (AST), alanine transaminase (ALT), total cholesterol, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, triglyceride, alkaline phosphatase, albumin, A/G ratio, γ-glutamyltransferase (γ-GT), amylase, urea, nitrogen, ureic acid and creatinine, general blood tests; glucose level, HbA1c, erythrocyte count, hemoglobin, hematocrit value, differential count of leukocytes and platelet count erythrocyte-related values (mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC)). All parameters were measured by SRL, Inc. (Tokyo, Japan).

Statistical Analysis All analyses were carried out using SPSS version 17.0J for Windows (SPSS Japan Inc.). The Friedman test was used to determine whether there was any evidence of a difference among 0, 2 and 4 weeks. Individual differences between weeks were investigated by the Wilcoxon’s signed rank test. All analyses were considered statistically significant at p<0.05.

RESULTS

Subjects Two subjects were dropped from the study due to the lack of compliance and extenuating circumstances. The remaining twelve subjects were included in the final analyses. Characteristics of the subjects are summarized in Table 1.

Periodontal Examinations As a result of screening, five subjects had at least one site with more than 4 mm PD. Fifty-one gingival crevicular fluid samples were collected from all
the sites with more than 4 mm PD. As shown in Table 2, PD at 4 weeks was significantly reduced compared to 0 weeks, although BOP and PERIOTRON scores showed no significant difference. TNF-α and IL-1β in GCF were not significantly changed (Fig. 1). On the other hand, MCP-1 in GCF at 2 and 4 weeks were significantly decreased compared with 0 weeks. IL-6 in GCF was almost below the detectable limit (data not shown).

Effects of L-bLF on the Production of Inflammatory Cytokines in P. gingivalis-Derived LPS-Stimulated PBMCs One subject was excluded from the analysis because of a lack of a sufficient number of PBMCs for incubation. The effect of L-bLF in inhibiting the production of inflammatory cytokines including TNF-α, IL-1β, IL-6 and MCP-1 was tested in P. gingivalis LPS-stimulated PBMCs (Fig. 2). All cytokine levels were significantly decreased at 4 weeks compared with 0 weeks.

**DISCUSSION**

In the present study, PD as one of the principle clinical indices of periodontal disease, but not BOP or GCF volume,
was significantly improved in 51 sites from five subjects with more than 4 mm PD 4 weeks after L-bLF supplementation. Furthermore, inflammatory cytokine levels in GCF were also measured as clinical markers of periodontal disease, and MCP-1, but not TNF-α and IL-1β showed significant decrease after L-bLF supplementation. MCP-1 is a well-known chemotactic cytokine that regulates mononuclear inflammatory cell recruitment. Previous reports have shown that expression of MCP-1 was observed in gingival tissue of chronic periodontal disease patients, and MCP-1 levels in GCF of patients with periodontal disease were significantly higher compared to those of healthy subjects.²¹,²² Furthermore, more recent studies have shown that MCP-1 levels in GCF increased with progression of periodontal disease and decreased after its treatment, and also positively correlated with the clinical parameters of periodontal disease, which proposed MCP-1 as an inflammatory biomarker in periodontal disease.²³,²⁴ Therefore, it is suggested that L-bLF supplementation might improve the status of periodontal disease.

PBMCs are well-known as one of the major cells to produce inflammatory cytokines responsible for periodontitis. It has already been reported that Aggregatibacter actinomycetemcomitans or Escherichia coli LPS-stimulated PBMCs from patients with periodontal disease showed increased inflammatory cytokines.³,⁴ P. gingivalis has been reported to be one of the major periodontopathic bacteria in gingival tissue of Japanese periodontitis patients.²⁵ Thus, in the present study, inflammatory cytokines such as TNF-α, IL-1β, IL-6 and MCP-1 produced from P. gingivalis LPS-stimulated PBMCs were analyzed in all subjects with periodontal disease. L-bLF supplementation for four weeks showed significant decreases of these inflammatory cytokines from PBMCs stimulated by P. gingivalis LPS, which also supports the hypothesis that orally administered L-bLF might be effective for treatment of periodontal disease.

LPS from P. gingivalis has been reported to activate NF-κB via TLR2 or TLR4 pathway, and result in the induction of inflammatory cytokines.²⁶,²⁷ In this study, mRNA expres-
sion of TLR2 or TLR4 was also analyzed using real-time RT-PCR to investigate the biological mechanisms of L-bLF supplementation on the decreased inflammatory cytokines from P. gingivalis LPS-stimulated PBMCs. However, mRNA expression levels of these receptors were not changed throughout the test period, which suggests that suppressive effect of L-bLF on LPS-induced inflammatory cytokines production from PBMCs was not due to the regulation of these receptors.

It has already been reported that bLF and its digestive fragment suppress LPS-induced inflammatory response in the cultured monocyctic cells, although it is not completely understood whether orally ingested bLF is absorbed from the intestine in an intact state. We have already demonstrated that many positive cells to a bLF antibody were immunohistochemically detected in periodontal tissue of L-bLF-fed rats, which suggests that bLF or its fragment absorbed from the intestine might show anti-inflammatory effects in this study, although further study is needed to elucidate the biological mechanism of L-bLF. On the other hand, we have already reported that soy phosphatidylcholine itself inhibited TLR4-mediated MCP-1 expression in vascular cells. Therefore, it can not be denied that soy phosphatidylcholine constructing liposomes contributed synergistically to the anti-inflammatory effects of L-bLF in this study.

Finally, no changes in any parameters of blood examination throughout the test period show higher safety of L-bLF intake.

In conclusion, the present study suggests that L-bLF supplementation can be effective as a novel treatment of periodontal disease, although prospective randomized controlled studies with supplementation of liposomes lacking bLF are clearly required.

REFERENCES