Inhibitory Effects of Lactoferrin on Growth and Biofilm Formation of Porphyromonas gingivalis and Prevotella intermedia

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Lactoferrin (LF) is an iron-binding antimicrobial protein present in saliva and gingival crevicular fluids, and it is possibly associated with host defense against oral pathogens, including periodontopathic bacteria. In the present study, we evaluated the in vitro effects of LF-related agents on the growth and biofilm formation of two periodontopathic bacteria, Porphyromonas gingivalis and Prevotella intermedia, which reside as biofilms in the subgingival plaque. The planktonic growth of P. gingivalis and P. intermedia was suppressed for up to 5 h by incubation with ≥130 μg/ml of human LF (hLF), iron-free and iron-saturated bovine LF (apo-bLF and holo-bLF, respectively), and ≥6 μg/ml of LF-derived antimicrobial peptide lactoferricin B (LFcin B); but those effects were weak after 8 h. The biofilm formation of P. gingivalis and P. intermedia over 24 h was effectively inhibited by lower concentrations (≥8 μg/ml) of various iron-bound forms (the apo, native, and holo forms) of bLF and hLF but not LFcin B. A preformed biofilm of P. gingivalis and P. intermedia was also reduced by incubation with various iron-bound bLFSs, hLF, and LFcin B for 5 h. In an examination of the effectiveness of native bLF when it was used in combination with four antibiotics, it was found that treatment with ciprofloxacin, clarithromycin, and minocycline in combination with native bLF for 24 h reduced the amount of a preformed biofilm of P. gingivalis compared with the level of reduction achieved with each agent alone. These results demonstrate the antibiofilm activity of LF with lower iron dependency against P. gingivalis and P. intermedia and the potential usefulness of LF for the prevention and treatment of periodontal diseases and as adjunct therapy for periodontal diseases.

MATERIALS AND METHODS

LF-related agents. hLF was purified from the milk of healthy volunteers by a previously reported method (34). bLF was produced by Morinaga Milk Industry (Tokyo, Japan) and was used as native bLF. Iron-free apo-bLF was prepared by the depletion of iron through the incubation of native bLF in a 0.1 M citric acid solution and dialysis against distilled water. Iron-saturated holo-bLF was prepared from native bLF by the method reported previously (3). The binding of iron to the metal-chelating site of bLF was confirmed by measuring the maximum absorbance at 465 nm (3). The metal contents of these preparations were mea-
FIG. 1. Inhibition of planktonic growth of \( \textit{P. gingivalis} \) by LF-related agents. About \( 10^4 \) cells/ml of \( \textit{P. gingivalis} \) JCM 8525 were incubated with the indicated concentrations of hLF (A), apo-bLF (B), holo-bLF (C), or LFcin B (D) in TSB for 8 h. Bacterial growth was determined by ATP-based luminescence quantification and is expressed as RLU. Values are means ± SDs (\( n = 3 \)) of one representative result from two similar results. *, \( P < 0.05 \) compared with the result for the control (no test agent) at 5 or 8 h; **, \( P < 0.001 \) compared with the result for the control (no test agent) at 5 or 8 h.

Susceptibility tests with planktonic bacteria. To determine the growth-inhibitory activity, about \( 10^6 \) bacteria/ml were incubated in 2 ml of TSB in the presence of fourfold serial dilutions of the test agents for 8 h. Bacterial growth was determined at defined intervals by ATP-based luminescence quantification. One hundred microliters of the bacterial culture was incubated with 100 µl of BacTiter-Glo microbial cell viability assay reagent (Promega, Madison, WI) in each well of a 96-well luminescence white plate (Greiner Bio-One, Tokyo, Japan). The luminescence was recorded by a Veritas microplate luminometer (Promega). Bacterial growth was calculated by subtracting the number of relative luminescence units (RLUs) at 0 h from that at the defined time. To determine bactericidal activity, about \( 10^6 \) bacteria/ml were incubated in physiological saline (0.85% NaCl) with or without the test agents for 4 h. At defined intervals, each culture was collected, serially diluted, and incubated on a TSA plate for 3 to 5 days; and the numbers of CFU were counted.

Susceptibility tests with biofilms. The amount of bacterial biofilm was measured by a microtiter plate biofilm assay by use of a previously reported protocol (28). In brief, about \( 10^7 \) bacteria/ml were incubated in 100 µl of TSB supplemented with hemin (5 µg/ml), vitamin K1 (0.5 µg/ml), vitamin K3 (5 µg/ml), and yeast extract (1 mg/ml) in the presence of fourfold serial dilutions of test agents in the wells of round-bottom polystyrene 96-well microtiter plates (BD) for 24 h. After removal of the planktonic bacteria by washing the plates with water, 125 µl of 0.1% crystal violet solution was placed in each well and the plates were incubated for 10 min. The optical density at 550 nm (OD550) of 125 µl of the solution was measured with a microplate reader (MTP-32; Corona Electric, Ibaraki, Japan) and was used to define the amount of biofilm. For microscopic observation, the biofilm was developed in the wells of flat-bottom polystyrene 96-well microtiter plates (BD) and stained with crystal violet, and phase-contrast micrographs were taken. To determine the effects of the test agents on the preformed biofilm, the bacteria were incubated anaerobically in polystyrene 96-well microtiter plates for 24 h, as described above for incubation without test agents. After the planktonic bacteria were removed, the biofilm was incubated anaerobically with 100 µl of physiological saline containing the test agents for 5 or 24 h. The amount of biofilm was then measured by crystal violet staining. The viability of the bacteria in the biofilm was measured by ATP-based luminescence quantification, as indicated above, except that each well was incubated with 100 µl of the BacTiter-Glo reagent for 15 min, after the removal of the planktonic bacteria, and 80 µl of the resultant solution was transferred to the luminescence white plate for measurement.

Statistical analysis. Data are expressed as means ± standard deviations (SDs). Statistical analysis was done between two groups by two-tailed Student’s \( t \) test. \( P \) values of <0.05 were considered to indicate a significant difference.
RESULTS

Effects of LF on planktonic P. gingivalis and P. intermedia. Approximately 10^4 cells/ml of P. gingivalis JCM 8525 (Fig. 1) and P. intermedia ATCC 25611 (Fig. 2) were incubated with different concentrations of LF-related agents in TSB not supplemented with an iron source for 8 h, and the growth was monitored by a viability assay by the use of ATP-based luminescence quantification and is expressed as RLU. All agents tested, including hLF, apo-bLF, and holo-bLF at 0.13 to 8 mg/ml and LFcin B at 0.006 to 0.4 mg/ml, suppressed the growth of P. gingivalis for up to 5 h (Fig. 1). The RLU for the control bacteria (which were incubated with no test agent) declined at 8 h, which may reflect growth arrest in a medium free of an iron source. Inhibition of the RLU (viability) by the test agents was weak or was not seen after 8 h, especially by lower concentrations of the test agents (0.13 to 2 mg/ml of LF and 0.006 mg/ml of LFcin B). The growth of P. intermedia was suppressed by hLF through 8 h of incubation (Fig. 2A). apo-bLF and holo-bLF suppressed the growth of P. intermedia until 5 h, but the effect was weak or tended to be opposite of the earlier effect at 8 h (Fig. 2B and C). The inhibitory effect of LFcin B against P. intermedia was seen more clearly at 8 h than at 5 h (Fig. 2D). Because LFcin B exhibits broad bactericidal activity (10) and because even hLF shows killing activity against some bacteria, including S. mutans, in saline (7), we tested the effects of bLF and LFcin B on the numbers of CFU of P. gingivalis and P. intermedia in saline.

Approximately 10^7 cells/ml of P. gingivalis JCM 8525 and P. intermedia ATCC 25611 were incubated with native bLF (8 mg/ml) or LFcin B (0.4 mg/ml) in physiological saline for 4 h (Table 1). The numbers of CFU of P. gingivalis and P. intermedia spontaneously decreased in saline. However, LFcin B further reduced the numbers of CFU of P. gingivalis and P. intermedia after 2 or 4 h of incubation compared with numbers of CFU in the control cultures, although bLF did not show such an inhibitory effect.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Treatment</th>
<th>CFU/ml at the following times (h):</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. gingivalis</td>
<td>Control</td>
<td>3.1 × 10^7</td>
</tr>
<tr>
<td>Native bLF</td>
<td>NA</td>
<td>2.0 × 10^7</td>
</tr>
<tr>
<td>LFcin B</td>
<td>NA</td>
<td>8.0 × 10^6</td>
</tr>
<tr>
<td>P. intermedia</td>
<td>Control</td>
<td>2.1 × 10^7</td>
</tr>
<tr>
<td>Native bLF</td>
<td>NA</td>
<td>4.8 × 10^6</td>
</tr>
<tr>
<td>LFcin B</td>
<td>NA</td>
<td>4.3 × 10^5</td>
</tr>
</tbody>
</table>

*P. gingivalis JCM 8525 or P. intermedia ATCC 25611 was incubated with 8 mg/ml of native b LF or 0.4 mg/ml of LFcin B in physiological saline for 4 h. The numbers of CFU of the bacteria were determined by counting the colonies in diluted samples grown on a TSA plate. The detection limit was 5 × 10^2 CFU/ml. The values indicate one representative result from two similar results. NA, not assayed.
FIG. 3. Inhibition of biofilm formation of *P. gingivalis* and *P. intermedia* by LF-related agents. About 10^7 cells/ml were incubated in TSB with hemin, vitamin K₁, and yeast extract in polyvinylchloride plates for 24 h. Biofilm formation was determined by crystal violet staining and is expressed as the OD₅₅₀.

*P. gingivalis* JCM 8525, ATCC 33277, and ATCC 53978 and *P. intermedia* ATCC 25611 and ATCC 49046 were treated with the indicated concentrations of native bLF (A). *P. gingivalis* ATCC 33277 (B) and *P. intermedia* ATCC 25611 (C) were treated with the indicated concentrations of hLF, apo-bLF, native bLF, holo-bLF, or LFcin B. Values are means ± SDs (n = 3) of one representative result from two similar results. *, P < 0.05 compared with the result for the control (no test agent); **, P < 0.001 compared with the result for the control (no test agent).
**Inhibition of biofilm formation by LF.** We investigated whether LF-related agents affected the biofilm formation of *P. gingivalis* and *P. intermedia*. The level of biofilm formation of *P. gingivalis* ATCC 33277, ATCC 53978 and *P. intermedia* ATCC 25611 and ATCC 49046 in the presence of several concentrations of native bLF in polyvinylchloride round wells was estimated by measuring the OD_{550} after staining of the bacteria with crystal violet (Fig. 3A). The amount of biofilm that was formed varied among the bacterial strains. However, native bLF inhibited biofilm formation by all strains tested. Since *P. gingivalis* ATCC 33277 and *P. intermedia* ATCC 25611 formed large amounts of biofilm, these strains were used for the subsequent experiments. hLF, apo-bLF, native bLF, and holo-bLF inhibited the biofilm formation of *P. gingivalis* ATCC 33277 at a lower concentration (0.008 mg/ml, a concentration lower than that required to inhibit the planktonic growth) in a dose-dependent manner (Fig. 3B). LFcin B at lower concentrations (0.006 and 0.025 mg/ml) promoted biofilm formation, but at 0.4 mg/ml, the peptide markedly inhibited biofilm formation. For *P. intermedia* ATCC 25611, apo-bLF, native bLF, and holo-bLF at more than 0.031 mg/ml or hLF at more than 0.13 mg/ml inhibited biofilm formation in a dose-dependent manner (Fig. 3C). The inhibition of biofilm formation by LFcin B was apparent at 0.4 mg/ml. For microscopic observation, the biofilm of *P. gingivalis* ATCC 33277 was formed on polystyrene flat-bottom wells and stained with crystal violet. Culture of *P. gingivalis* for 24 h generated a substantial biofilm on the surface of each well (Fig. 4A). Addition of native bLF at 0.031 mg/ml (Fig. 4B) or 0.5 mg/ml (Fig. 4C) to the culture of *P. gingivalis* drastically inhibited biofilm formation, with only single cells or clumps attached to the plates being observed. Microscopically, it seems likely that the LF inhibition of biofilm formation by *P. gingivalis* is more drastic when the organism is cultured on a polystyrene plate than on a polyvinylchloride plate (data not shown).

**Reduction of preformed biofilms by LF.** Next, we examined whether LF-related agents could reduce preformed biofilms of *P. gingivalis* and *P. intermedia*. Biofilms of *P. gingivalis* ATCC 33277 and *P. intermedia* ATCC 25611 formed by 24 h of culture were treated with saline containing hLF, apo-bLF, native bLF, holo-bLF, and LFcin B for 5 h. The remaining biofilm was then stained with crystal violet. hLF and apo-bLF reduced the amount of the *P. gingivalis* biofilm in a dose-dependent manner starting at 0.008 mg/ml (Fig. 5A). The effects of native bLF and holo-bLF were weaker than the effect of apo-bLF. LFcin B was more effective at a lower concentration (at 0.0063 mg/ml but not at 0.0016 mg/ml) than at higher concentrations. For *P. intermedia*, treatment with all types of LFs resulted in dose-dependent reductions in the level of biofilm formation (Fig. 5B). LFcin B at concentrations ranging from 0.0063 to 0.4 mg/ml reduced the level of *P. intermedia* biofilm formation by similar amounts.

**Effects of combinations of antibiotics and LF on preformed biofilms.** The effects of combinations of LF with antibiotics were examined by a biofilm reduction test. A biofilm of *P. gingivalis* ATCC 33277 formed through 24 h of culture was treated for 24 h with saline containing 0.5 mg/ml of native bLF and antibiotics at several concentrations, including ABPC, CPFX, CAM, and MINO (Fig. 6A). The amount of remaining biofilm was then determined by crystal violet staining. CPFX, CAM, or MINO in combination with native bLF reduced the amount of the pre-formed biofilm, but ABPC in combination with native bLF did not (Fig. 6A). The combination of native bLF and 1 or 10 μg/ml of CPFX significantly reduced the amount of the preformed biofilm compared with the level of reduction achieved with each agent alone. Furthermore, the combination of native bLF and 1 μg/ml of CAM or 10 μg/ml of MINO significantly reduced the amount of preformed biofilm compared with the level of reduction achieved with each agent alone. The effects of combinations of antibiotics and apo-bLF on the viability of the bacteria in the preformed biofilm were examined (Fig. 6B). Although, overall, the results obtained with the combinations seemed to be relatively modest, the combination of 0.5 mg/ml of apo-bLF and 10 μg/ml of CPFX significantly reduced the viability of the bacteria in the biofilm compared with that achieved with each agent alone.

**DISCUSSION**

The iron-withholding ability of LF is the central mechanism of its antimicrobial activity (13, 31). Our results indicated that
bLf has the ability to suppress the growth of planktonic *P. gingivalis* and *P. intermedia* independently of the iron-bound form of bLf used. Furthermore, we found that various iron-bound forms of bLf inhibit the biofilm formation of these bacteria even at lower concentrations and that LF alone or in combination with antibiotics reduces the amounts of preformed biofilms of these bacteria.

Previous studies showed that hLf has growth-inhibitory activity against *P. gingivalis* but not *P. intermedia* or *Prevotella nigrescens* in a conventional turbidity assay (2, 35). The activity of LF against *P. gingivalis* was not seen in a medium supplemented with hemin (35), and by a turbidity assay, the growth of *P. gingivalis* was not detected in a medium not supplemented with hemin (2). For the more sensitive detection of the susceptibility of planktonic *P. gingivalis* and *P. intermedia* cells to LF in a medium not supplemented with hemin, we measured the ATP-based luminescence of the bacteria during a short-term incubation starting with a small number of cells (10⁴/ml). The results indicated that *P. intermedia*, as well as *P. gingivalis*, is susceptible to the growth-inhibitory activity of LF during a 5-h incubation. However, the effect of LF appears to be weak or absent after a longer incubation (8 h). It is reported that LF is degraded by the proteases produced by *P. gingivalis* and *P. intermedia* (5, 15). The decline in the antibacterial activity of LF after longer incubations with *P. gingivalis* and *P. intermedia* may be due to the degradation of this protein. Our results revealed that both iron-free apo-bLf and iron-saturated holo-bLf suppress the short-term growth of *P. gingivalis* and *P. intermedia*, suggesting that the growth-inhibitory activity against these bacteria is independent of an iron-withholding
mechanism. It was suggested that hLF exhibits a bacteriostatic action on \( P. \) gingivalis by removal of a hemoglobin receptor from the bacterial cell surface in a medium containing hemoglobin as the sole iron source but not in a medium containing hemin as the sole iron source (35). Although we used a medium not supplemented with heme-related compounds in our assay, the bacteria may have used a trace amount of hemin from the inoculum culture for their growth. It is conceivable that LF may have an additional antibacterial mechanism by which the growth of \( P. \) gingivalis and \( P. \) intermedia is suppressed, apart from the iron-withholding mechanism and the removal of the hemoglobin receptor.

LFcin B is a potent antimicrobial peptide that shows activity against various microorganisms at concentrations of 0.3 to 45 \( \mu \)g/ml (10, 43), and it is recognized as the bactericidal domain of bLF (42). However, the growth-inhibitory activity of LFcin B against \( P. \) gingivalis and \( P. \) intermedia did not appear to be very strong. Although the number of \( P. \) gingivalis and \( P. \) inter-
media cells in physiological saline spontaneously decreased. LFcin B at a relatively high concentration (0.4 mg/ml) further reduced the numbers of these bacteria. These results obtained with LFcin B seem to be in accord with those obtained in previous studies with other antimicrobial peptides, in which their activities against P. gingivalis and P. intermedia were weak or were not seen at all (8, 29, 32).

P. gingivalis and P. intermedia colonize and grow as biofilms within subgingival plaque (14, 27). Recently, our preliminary clinical trial indicated that the bLF orally administered to patients with chronic periodontitis was transferred to the subgingival pocket and led to decreases in the numbers of total bacteria, P. gingivalis, and P. intermedia in the subgingival plaque (23). Therefore, we explored the effects of LF-related agents on the biofilms of P. gingivalis and P. intermedia in vitro. hLF, apo-bLF, native bLF, and holo-bLF inhibited the biofilm formation of P. gingivalis in a dose-dependent manner; and these agents were effective even at a low concentration of 0.008 mg/ml. Likewise, hLF at 0.13 mg/ml and apo-bLF, native bLF, and holo-bLF at 0.031 mg/ml inhibited P. intermedia biofilm formation. There are reports indicating that LF prevents the biofilm formation of Pseudomonas aeruginosa, Burkholderia cepacia, enteraggregative Escherichia coli, and an oral pathogen, S. mutans (12, 18, 30, 33, 36). It has been documented that hLF inhibited the biofilm formation of P. aeruginosa by an iron-chelating mechanism (36). In contrast, apo-bLF increased the biofilm formation of S. mutans, while holo-bLF decreased the biofilm formation of S. mutans (18). bLF both with and without iron inhibited the biofilm formation of enteraggregative E. coli (30). The inhibition of P. gingivalis and P. intermedia biofilm formation by LF was independent of the iron status of the protein, and the mechanism of action of LF against P. aeruginosa and S. mutans biofilm formation seems to be different from that against P. gingivalis and P. intermedia biofilm formation but similar to that against enteraggregative E. coli biofilm formation. LFcin B inhibited the biofilm formation of P. gingivalis and P. intermedia only at a concentration of 0.4 mg/ml, suggesting that the LFcin region is not solely responsible for the biofilm-inhibitory activity of LF. It was reported that the cleavage of hLF by cathepsin results in the loss of its inhibitory effect on biofilm formation (33). It is assumed that an intact LF structure may be important for its inhibitory effect on biofilm formation.

We obtained results showing that various iron-bound forms of bLF, hLF, and LFcin B also reduce the amounts of preformed biofilms of P. gingivalis and P. intermedia. In this experiment, LFcin B was effective even at a low concentration (0.0063 mg/ml). It was reported that hLF and bLF bind to P. gingivalis and P. intermedia cells (20). bLF also interferes with the binding of P. intermedia to subepithelial matrix proteins, including fibronectin, collagen types I and IV, and laminin, as well as fibroblasts and epithelial cells (4, 6). Therefore, LF may have interacted with the cell surface of P. gingivalis and P. intermedia and interfered with or disconnected the binding of biofilm-forming cells to the plastic wall. It is thought that the biofilm-inhibiting and -reducing effects of LF at least partly contributed to the reductions in the amounts of P. gingivalis and P. intermedia in the subgingival plaque of patients with chronic periodontitis, which was observed in a small-scale clinical study (23). The physiological concentration of hLF in stimulated whole saliva of patients with chronic periodontitis was 9.8 µg/ml in our previous clinical study (data not shown) and 9.0 µg/ml in another study (19). The hLF concentration in the GCF of patients with chronic periodontitis was calculated to be 51 µg/ml in our previous clinical study (data not shown) and 80 µg/ml in another study (19), on the basis of the assumption that 2 µl of GCF was collected. These concentrations of hLF in saliva and GCF may have a role in the inhibition of biofilm formation by P. gingivalis and P. intermedia. However, the oral administration of LF would boost the antibiofilm environment in the oral cavity because a higher LF concentration is more effective at inhibiting biofilm formation.

Furthermore, specific concentrations of CPFX, CAM, or MINO combined with bLF significantly reduced the amounts of preformed P. gingivalis biofilms in vitro. Although a combination of CPFX and bLF also suppressed the viability of the bacteria in the biofilm, the overall effectiveness of combinations of antibiotics and bLF on the viability of the bacteria in the biofilm was somewhat modest. These results imply that the use of combinations of antibiotics and LF may be effective at reducing the biofilm materials rather than suppressing bacterial activity. In relation to these results, hLF has been reported to enhance the B. cepacia biofilm formation-inhibitory activities of antibiotics such as CPFX (12). Although tetracycline antibiotics, including MINO, have been employed as local drug delivery systems for the treatment of periodontal diseases (22), the low levels of activity of antibiotics, including tetracyclines, against P. gingivalis and P. intermedia biofilm formation provide a reason for the difficulty of developing antibiotic therapy for periodontal diseases (17, 24, 37). LF may aid with the chemotherapy of periodontitis with antibiotics such as MINO, CPFX, and CAM. Thus, larger-scale clinical tests are warranted for assessment of the preventive and therapeutics effects of LF alone or LF with antibiotics on periodontal diseases.

REFERENCES


