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The N-Terminal Lipopeptide of a 44-kDa Membrane-Bound Lipoprotein of Mycoplasma salivarium Is Responsible for the Expression of Intercellular Adhesion Molecule-1 on the Cell Surface of Normal Human Gingival Fibroblasts

Ken-ichiro Shibata,* Akira Hasebe,* Takeshi Into,* Masanori Yamada,† and Tsuguo Watanabe*

The activities to induce TNF-α production by a monocyte cell line, THP-1, and ICAM-1 expression and IL-6 production by human gingival fibroblasts were detected in plural membrane lipoproteins of Mycoplasma salivarium. Although SDS-PAGE of the lipoproteins digested by proteinase K did not reveal any protein bands with molecular masses higher than approximately 10 kDa, these activities were detected in the front of the gel. A lipoprotein with a molecular mass of 44 kDa (Lp44) was purified. Proteinase K did not affect the ICAM-1 expression-inducing activity of Lp44, but lipoprotein lipase abrogated the activity. These results suggested that the proteinase K-resistant and low molecular mass entity, possibly the N-terminal lipid moiety, played a key role in the expression of the activity. The N-terminal lipid moiety of Lp44 was purified from Lp44 digested with proteinase K by HPLC. Judging from the structure of microbial lipopeptides as well as the amino acid sequence and infrared spectrum of Lp44, the structure of the N-terminal lipid moiety of Lp44 was speculated to be S-(2,3-bisacyloxypropyl)-cysteine-GDPKHPKSFTEW-. Its analogue, S-(2,3-bispalmitoyloxypropyl)-cysteine-GDPKHPKSF, was synthesized. The lipopeptide was similar to the N-terminal lipid moiety of Lp44 in the infrared spectrum and the ICAM-1 expression-inducing activity. Thus, this study suggested that the active entity of Lp44 was its N-terminal lipopeptide moiety, the structure of which was very similar to S-(2,3-bispalmitoyloxypropyl)-cysteine-GDPKHPKSF.

Mycoplasmas, the smallest self-replicating microbes, are characterized by a wall-less envelope. Mycoplasmas are generally commensal parasites in human, but some species are real pathogens capable of causing a wide variety of diseases (1). Although mycoplasmas do not possess bacterial modulins such as LPS, lipoteichoic acid, or murein components, they are potent activators of various types of mammalian cells, such as monocytes/macrophages, lymphocytes, and other nonimmune system cells (2). Evidence is accumulated that mycoplasmal membrane-bound lipoproteins are responsible for activating monocytes/macrophages or fibroblasts (2–4). Mühler et al. (5, 6) identify a 2-kDa lipopeptide, macrophage-activating lipopeptide (MALP-2), which is capable of activating monocytes/macrophages, and determine that the structure of MALP-2 is S-(2,3-bispalmitoyloxypropyl)-cysteine-GNNDESNSFKEK. Recently, it was found that lipoproteins in the cell membranes of Mycoplasma salivarium triggered the transcription of ICAM-1 mRNA in normal human gingival fibroblasts (HGF) and induced its cell surface expression by a mechanism distinct from that of Escherichia coli LPS (4). In addition, the lipid moiety of the lipoproteins was suggested to play a key role in the expression of the activity (4). M. salivarium is a member of oral microbial flora and inhabits preferentially in gingsival sulci and is suspected to play an etiological role in some cases of oral infections including periodontal diseases (7–9). Chronic periodontal diseases are characterized by dense infiltrations of lymphocytes and macrophages in the connective tissue (10). Cell adhesion molecules are involved in the infiltration of activated leukocytes in inflammatory sites. Evidence for the importance of ICAM-1 in periodontal diseases has been accumulated (11–14). Therefore, we are very much interested in the ICAM-1-inducing activity of the organism.

In this study, it was found that plural membrane-bound lipoproteins of M. salivarium were capable of inducing the ICAM-1 expression on the cell surface of HGF, and their active entities were N-terminal lipopeptide moieties. Furthermore, the membrane-bound lipoprotein of M. salivarium with a molecular mass of 44 kDa was characterized, and the putative structure of its N-terminal lipopeptide was partially defined.

Materials and Methods

Chemicals

S-(2,3-bispalmitoyloxypropyl)-N-palmitoyl-cysteine (Pam3-cysteine) was purchased from Bachem AG (Bubendorf, Switzerland); and mAb to ICAM-1 (HA58) used for Cell-ELISA from PharMingen (San Diego, CA). All of the other chemicals were obtained from commercial sources and were of analytical or reagent grade.

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Preparation of lipoproteins by the Triton X-114 phase separation

*M. salivarium* cells were treated with Triton X-114 to extract lipoproteins (TXLP) according to the method described previously (3). TXLPs in the Triton X-114 phase were precipitated by methanol and used for stimulation after being suspended in sterile PBS by light sonication. Protein concentration was determined by the method of Duly and Grieve (15).

Preparation of lipoproteins by using n-octyl-β-D-glucopyranoside

Cell membranes of *M. salivarium* were prepared according to the method described previously (16). The lipoproteins were extracted from the cell membranes by using *n*-octyl-β-D-glucopyranoside (OG) according to the method of Mühlradt et al. (5) modified slightly. Briefly, a 10-ml volume of cell membrane suspensions (10 mg of protein/ml) was treated twice with 10 ml of chloroform-methanol (2:1, v/v) at room temperature. The delipidated interphase was freed of the organic solvents in vacuo at 37°C and lyophilized to remove water. The lyophilized material was suspended in 50 mM OG in PBS, sonicated, treated for 6 min in boiling water, and centrifuged at 20,000 × g for 30 min. The supernatant was collected, filtrated through a 0.45-μm-pore-size filter, and used as lipoproteins (OGLP).

ICAM-1 expression on HGF and cytokine production by THP-1 cells

HGF preparation and used in the previous study (4) were cultured in DMEM (Life Technologies) containing 10% (v/v) FBS (Life Technologies), penicillin G (100 U/ml), and streptomycin (100 μg/ml) in plastic culture dishes. In this study, HGF between passages 6 and 8 were used.

The activity to induce the expression of ICAM-1 on the cell surface of HGF was measured by the Cell-ELISA according to the method of Hayashi et al. (17). Briefly, 10^5 of HGF were seeded into a 96-well flat-bottom microplate. After HGF reached confluence, the cells were stimulated. The culture supernatant was collected and examined for IL-6 production by using a TiterZyme ELISA kit (PerSeptive Diagnostics, Cambridge, MA). The cells were fixed with 3% (w/v) paraformaldehyde in PBS supplemented with 8% (v/v) saccharose. Nonspecific binding was blocked by the addition of PBS containing 10% (v/v) horse serum. The cells were reacted with anti-ICAM-1 mAb (HA58) and then with peroxidase-conjugated goat anti-mouse IgG Ab. Peroxidase activity was measured by the addition of tetramethylbenzidine peroxide substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and stopped by the addition of equal volume of 1.8 M sulfuric acid. The OD at 450 nm was measured by using a microplate reader.

THP-1 cells, a human myelomonocytic cell line, were obtained from Health Science Research Resources Banks (Osaka, Japan) and cultured in RPMI 1640 culture medium (Life Technologies) containing 10% FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml). After a 0.2-ml volume of cell suspensions of THP-1 (5 × 10^5 cells) in each well of a 96-well tissue culture plate was incubated at 37°C for 15 h with OGLP (40 μg of protein) in the culture medium supplemented with 1% (v/v) human serum, the culture supernatant was collected, filtered at 400 × g for 10 min, TNF-α in the supernatant was determined by using a TiterZyme kit (PerSeptive).

Identification of lipoproteins responsible for the ICAM-1-inducing activity by the monocyte Western blotting

Lipoproteins in TXLP or OGLP responsible for the ICAM-1-inducing activity were determined by the monocyte Western blotting described previously (18). SDS-PAGE of TXLP or OGLP was performed in 10% gel according to the method of Hager and Burgess (20). Briefly, the gels were rinsed with distilled water (DW) and stained with ice-cold 0.25 M KCl and 1 mM DTT. Gel pieces containing Lp44 were cut out and soaked for 15 min in DW and 1 mM DTT. The gel pieces were crushed in 50 mM Tris-HCl modified method of Hager and Burgess (20). Briefly, the gels were rinsed with distilled water (DW) and stained with ice-cold 0.25 M KCl and 1 mM DTT. Gel pieces containing Lp44 were cut out and soaked for 15 min in DW and 1 mM DTT. The gel pieces were crushed in 50 mM Tris-HCl

### Table 1. TXLP-induced cytokine production by HGF and THP-1 cells

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Cytokine Concentration (pg/ml)*</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF</td>
<td>None</td>
<td>0</td>
<td>16 ± 4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TXLP</td>
<td>0</td>
<td>1120 ± 149</td>
<td>0</td>
</tr>
<tr>
<td>THP-1 cells</td>
<td>None</td>
<td>0</td>
<td>NT</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td>TXLP</td>
<td>45 ± 10</td>
<td>NT</td>
<td>12,469 ± 490</td>
</tr>
</tbody>
</table>

*Confluent monolayers of HGF grown in a flat-bottom 96-well microplate or 5 × 10^5 cells/well of THP-1 cells in a round-bottom microplate were incubated at 37°C for 16 h in the presence or the absence of TXLP at a final protein concentration of 20 μg/ml. Data, expressed as means ± SDs from triplicate wells, were obtained by a representative of three separate experiments. NT, Not tested.
buffer (pH 7.9) containing 0.1 mM EDTA, 5 mM DTT, BSA (0.1 mg/ml), and 0.15 M NaCl, incubated at 25°C for 2 h with agitation, and centrifuged at 2000g to pellet the crumbled gel. Lp44 was precipitated by adding 4 volumes of cold acetone prechilled at −20°C to the supernatant. The precipitate was dissolved in 6 M guanidium hydrochloride and dialyzed against DW. The resulting white aggregate was suspended in 20 mM OG in PBS by light sonication and incubated at 50°C for 30 min.

Amino acid sequence analysis

SDS-PAGE of the Lp44 extracted was performed in 10% gels. The proteins were blotted onto Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) and stained with Coomassie brilliant blue. The Lp44 was excised and analyzed in an automated gas-phase procise sequencer model 492 (PE Biosystems, Foster City, CA).

HPLC and infrared (IR) spectrometry

HPLC was performed on preparative Nucleosil 120-7C18 column (10 × 300 mm) (Chemco Scientific, Osaka, Japan). The fractionation was done with the program: time zero, 5% N,N′-dimethylformamide (DMF)/95% water; at 15 min, 5% DMF/95% water; at 60 min, 5% DMF/95% 2-propanol; and at 70 min, 5% DMF/95% 2-propanol. The flow rate was 1.5 ml/min. Each fraction was dried in vacuo at 60°C, dissolved in 20 mM OG in PBS, and used for stimulation of HGF. IR absorption spectrum of the dried fractions in KBr pellet was measured with Foulier transform IR spectrometer (RT-210; Horiba, Kyoto, Japan). Pam3-cysteine was used as a standard.

Synthesis of S-(2,3-bispalmitoyloxypropyl)-cysteine-GDPKHPKSF

The side chain-protected GDPKHPKSF was built up with an automated peptide synthesizer, model 433 (Applied Biosystems, Foster City, CA). Fmoc-S-(2,3-bispalmitoyloxypropyl)-cysteine (Novabiochem, Laufelfingen, Switzerland) was manually coupled to the peptide-resin by using a solvent system of 1-hydroxy-7-azabenzotriazole-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/CH₂Cl₂-DMF. The Fmoc and resin were removed from the lipopeptide by trifluoroacetic acid. The lipopeptide

---

**FIGURE 2.** The IL-6 or TNF-α production-inducing activity in fractions of TXLP separated by SDS-PAGE. SDS-PAGE of TXLP (A) or TXLP digested by proteinase K (B) was performed in 10% gel and transferred to the nitrocellulose membrane. The membrane was cut into 4-mm strips and dissolved in DMSO. Lipoprotein-coated particles were formed by the addition of 50 mM sodium carbonate buffer (pH 9.6) and washed three times with sterile PBS. HGF (A) or THP-1 cells (B) were stimulated with the particles. The amount of TNF-α or IL-6 was determined by ELISA.

**FIGURE 3.** ICAM-1-inducing activity of OGLP. SDS-PAGE of a protein marker (A) and OGLP (B) was performed in 10% gel. Lipoproteins in OGLP separated by SDS-PAGE were transferred to the nitrocellulose membrane. The membrane was cut into 4-mm strips and dissolved in DMSO. Ag-coated particles were formed by the addition of 50 mM sodium carbonate buffer (pH 9.6) and washed three times with sterile PBS. HGF were stimulated with the particles. ICAM-1 expression was determined by Cell-ELISA.
was extracted into 90% acetic acid and lyophilized. The lipopeptide was purified by preparative HPLC with reverse-phase C18 column (30 × 250 mm). The purity (97.9%) was confirmed by analytical HPLC with reverse-phase C18 column (4.6 × 150 mm).

Results

Plural lipoproteins are responsible for inducing ICAM-1 expression and cytokine production by HGF

Lipoproteins in TXLP responsible for inducing ICAM-1 expression on the cell surface of HGF were analyzed by the monocyte Western blotting. The activity was detected in plural lipoproteins with molecular mass ranges of 40–50 kDa and 60–80 kDa (Fig. 1). Previously, we found that lipoprotein lipase abrogated the ICAM-1 expression-inducing activity of TXLP, whereas proteinase K does not affect the activity of TXLP (4). Therefore, the activity of TXLP digested by proteinase K was also examined by the monocyte Western blotting. SDS-PAGE of TXLP digested by proteinase K did not reveal any protein bands with molecular masses higher than approximately 10 kDa, but the activity was detected in the front of the gel (Fig. 1). This result suggests that proteinase K-resistant entities with molecular masses lower than approximately 10 kDa common in plural lipoproteins play a key role in the expression of the activity.

Previously, it was found that water-soluble and insoluble materials prepared from M. salivarium cells induced the production of IL-6 and IL-8 by HGF (3) or TNF-α by THP-1 cells (unpublished data). Therefore, it was examined whether or not TXLP possessed the cytokine production-inducing activity. TXLP was found to induce TNF-α production by THP-1 cells or IL-6 production by HGF (Table I). Lipoproteins involved in the cytokine production-inducing activity were also identified by the monocyte Western

Table II. Effect of enzymes on the ICAM-1-inducing activity of Lp44 extracted from SDS-PAGE gels

<table>
<thead>
<tr>
<th>Treated with</th>
<th>ICAM-1-Inducing Activity$^a$ ($A_{450}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.09 ± 0.07 (100)</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>1.21 ± 0.09 (111)</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>0.04 ± 0.01 (4)</td>
</tr>
</tbody>
</table>

$^a$ Two hundred microliters of Lp44 extracted from SDS-PAGE gels (5 µg/ml of 20 mM OG in PBS) was treated with at 37°C for 2 h with 0.2 µg of each enzyme, and then 20 µl of the reaction mixture was added to the monolayer of HGF grown in 200 µl of DMEM in each well of a 96-well flat-bottom microplate. After a 16-h incubation, ICAM-1 expression on HGF was measured by Cell-ELISA. Enzyme or buffer control was run at the same time. Absorbance at 450 nm ($A_{450}$) of enzyme or buffer control was the same as that for the control medium. Therefore, $A_{450}$ was expressed as $A_{450}$ of each well – the mean $A_{450}$ of three medium control wells. Data, expressed as means ± SDs from triplicate wells, are representative of three separate experiments. Values in the parentheses are 100 × (mean $A_{450}$ of a specimen treated with enzyme/mean $A_{450}$ of a specimen left untreated).
blotting. The lipoprotein profile (Fig. 2A) with IL-6 production-inducing activity was similar to that (Fig. 2B) with TNF-α production-inducing activity. Also, each of them was similar to that (Fig. 1) with the ICAM-1 expression-inducing activity. The cytokine production-inducing activity of TXLP digested by proteinase K migrated to the front of the gel (Fig. 2, A and B) as the ICAM-1 expression-inducing activity did (Fig. 1).

Thus, it was found that M. salivarium possessed plural membrane-bound lipoproteins responsible for the ICAM-1 expression- and cytokine production-inducing activities, the active entities of which were proteinase K-resistant low molecular mass molecules, possibly their lipid moieties.

Identification of a lipoprotein with a molecular mass of 44 kDa is one of the lipoproteins responsible for the ICAM-1 expression-inducing activity

OGLP extracted from the cell membrane of M. salivarium by using OG were examined for the ICAM-1 expression-inducing activity by the monocyte Western blotting. Lp44 was found to possess the activity significantly higher than the others (Fig. 3). Therefore, to characterize Lp44, the N-terminal amino acid sequence of Lp44 was examined. However, the N-terminal amino acid of Lp44 was not determined, because any amino acid peak significantly higher than the others was not observed in the amino acid profile of the first cycle of Edmann degradation (Fig. 4). Amino acids after second were easily identified, as shown in Fig. 4. This result suggests that the amino group of the N-terminal amino acid is free. Judging from characteristic of Edmann degradation, the N-terminal amino acid is speculated to be cysteine. This is supported by the previous finding that the N-terminal amino acid of lipoproteins from prokaryotes is cysteine, the Src homology group of which is bound to lipid (21). Therefore, the N-terminal amino acid sequence of Lp44 was speculated to be CGDPKHPKSFTEWVA-. It was found by homology search with GenBank databases that the amino acid sequence of Lp44 had not been reported previously.

Purification of Lp44 and determination of its N-terminal structure

Lp44 extracted from gels of SDS-PAGE was analyzed by SDS-PAGE. It revealed one dense band with a molecular mass of 44 kDa and a faint band with a higher molecular mass, possibly an aggregate of Lp44 (Fig. 5).

The effect of proteinase K or lipoprotein lipase on the ICAM-1 expression-inducing activity of Lp44 was also investigated. The activity was also found to be resistant to proteinase K and sensitive to lipoprotein lipase (Table II), suggesting that the lipid moiety, but not the proteinous moiety, is involved in the expression of the activity. To purify the lipid moiety of Lp44, Lp44 was digested with proteinase K and then fractionated by a reverse-phased HPLC with a linear gradient of isopropanol. The lipid moiety of Lp44 was eluted by about 90% isopropanol, because the ICAM-1 expression-inducing activity was detected in these fractions (Fig. 6). These fractions were dried in vacuo and applied to IR spectrometer to get some information on the structure of the lipid moiety of Lp44. In the spectrometry, Pam3-cysteine was used as a standard. The IR spectra of the lipid moiety of Lp44 and Pam3-cysteine exhibited signals about 2900 cm⁻¹ and 1700 cm⁻¹, which show the presence of fatty acid alkyl chains and typical ester bonds, respectively (Fig. 7, A and B). Lipid is known to be bound to Src homology group of the N-terminal cysteine in lipoproteins from many microbes (22). The structure of N-terminal lipid of murein lipoproteins of E. coli is determined to be S-(2,...
3-bis[3-palmitoyloxypropyl]-N-palmitoyl-cysteine-SSNKIDELSSD-(21). Furthermore, Mühlradt et al. (5, 6) identified a lipopeptide called MALP-2, S-(2,3-bisacyloyloxypropyl)-cysteine-GNNDESNISSK, from *Mycoplasma fermentans* which is capable of activating macrophages/monocytes.

On the basis of these findings, the structure of the lipid moiety of Lp44 is speculated to be S-(2,3-bisacyloyloxypropyl)-cysteine-GDPKHPKSFTEWVA-. To confirm whether a lipopeptide with the speculated structure possesses the ICAM-1 expression-inducing activity, S-(2,3-bisacyloyloxypropyl)-cysteine-GDPKHPKSF (FSL-1) was synthesized (Fig. 8). IR spectrum of FSL-1 also exhibited signals about 2900 and 1700 cm\(^{-1}\) due to fatty acid alkyl chains and the ester bond as those of the HPLC-purified lipid moiety of Lp44 and Pam3-cysteine (Fig. 7C). In addition, the activity of FSL-1 possessed a strong activity to induce the ICAM-1 expression on the cell surface of HGF (Fig. 9).

The fact that FSL-1 analogous to the N-terminal lipopeptide moiety of Lp44 exhibited the ICAM-1 expression-inducing activity strongly suggests that the active entity of Lp44 is the N-terminal lipopeptide moiety, the structure of which is very similar to FSL-1.

**Discussion**

Cells of the innate immune system discriminate between self and nonself by receptors that recognize molecules synthesized exclusively by microbes. These molecules include LPS, peptidoglycans, lipoteichoic acid, and lipoproteins. Wall-less mycoplasmas lack them except for lipoproteins. Lipoproteins of some mycoplasma species are known to activate monocytes/macrophages, lymphocytes, or fibroblasts (2, 3). This study also demonstrated that membrane-bound lipoproteins of *M. salivarium* possessed the activity to activate HGF or THP-1 cells. Lipoproteins exist in cell membranes of all mycoplasma species. Therefore, it may be generalized that mycoplasmas are a potent activator of mammalian cells.

Mycoplasmal active entities responsible for the activation of monocytes/macrophages have not been identified until Mühlradt et al. (5, 6) purified and characterized a 2-kDa lipopeptide, MALP-2, from *M. fermentans*. This study indicated that Lp44, one of plural membrane-bound lipoproteins, was an active entity capable of activating fibroblasts, and its active site was the N-terminal lipopeptide moiety, the structure of which was similar to MALP-2. Therefore, lipopeptides with the N-terminal cysteine residue bound to a diacylated glyceride residue through a thioester linkage may be a potent activator of mammalian cells.

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The relationship of the structure of N-terminal lipopeptides of prokaryote lipoproteins and their biological activities has been partially defined (27–29). The N-terminal structure of *E. coli* murein lipoprotein, which is by far the most frequently present lipoproteins in bacteria (22), is S-(2,3-bisacyloyloxypropyl)-N-palmitoyl-cysteine-SSNKIDELSSD-(Pam3-cysteine-SSNKIDELSSD-) (21). The lipopeptides synthesized based on Pam3-cysteine-SSNKIDELSSD- are known to activate macrophages to induce the production of IL-1, IL-6, and TNF-α (22). A well-defined series of analogues such as Pam3-cysteine-SSNK, Pam3-cysteine-SSN,
Pam3-cysteine-SS, Pam3-cysteine-S, and Pam3-cysteine fail to activate monocytes/macrophages (30).

These findings suggest that both peptide and fatty acid portions of microbial lipoproteins are indispensable for the expression of their biological activities. This speculation is also supported by the present finding that Pam3-cysteine and tripalmitin did not possess the activity to induce the ICAM-1 expression on the cell surface of HGF. Furthermore, mycoplasmal lipoproteins are speculated to be a more potent activator of monocytes/macrophages or fibroblasts than bacterial lipoproteins, because the amino group of the N-terminal cysteine of mycoplasmal lipoproteins or lipopeptides like MALP-2 and the N-terminal lipid moiety of Lp44 is free, whereas that of lipoproteins of many bacteria is bounded to some fatty acid.

Lipoproteins or lipopeptides of E. coli, Treponema pallidum, and Borrelia burgdorferi activate monocytes/macrophages, and the activity resides in their N-terminal lipopeptide moieties (31, 32). As described above, lipoproteins with the structure analogous to E. coli murein lipoproteins exist in many bacterial species (22). Taken together, it is very likely that microbial lipoproteins play important etiological roles in diseases caused by these microbes. Especially, lipoproteins of wall-less microbes such as mycoplasmal may play more important roles, because they interact directly with host cells.

M. salivarium, a member of oral microbial flora, is suspected to play some etiological role in some cases of oral infections, including periodontal diseases (7–9), but its etiological roles remain unknown. The present finding that the organism possesses membrane-bound lipoproteins capable of activating monocytes/macrophages and HGF may give a clue to clarify etiological roles in oral infections, especially periodontal disease.

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References