Coaggregation between *Prevotella oris* and *Porphyromonas gingivalis*

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**KEYWORDS**

Biofilm formation; Gingipain; Periodontal disease; Periodontopathogens

**Background/Purpose:** The coaggregation of bacteria has been defined as one of the most important processes in the oral infection such as periodontitis. *Prevotella oris* and *Porphyromonas gingivalis*, which are two of the periodontopathogens, are frequently detected in severe forms of periodontal diseases. However, the interaction between *P. oris* and *P. gingivalis* is still unknown. In this study, the coaggregation of *P. oris* with nine oral bacterial species including *P. gingivalis* was examined.

**Methods:** All bacteria used in this study were cultured anaerobically and suspended in coaggregation buffer. Each cell suspension was mixed in a test tube and subjected to shaking at room temperature for 1 hour. Subsequently, the coaggregation values were scored. Furthermore, the effects of various chemical reagents, and heat, proteinase K, and serum treatment were examined.

**Results:** In this study, *P. oris* coaggregated only with *P. gingivalis*. A heat-stable, nonproteinous component of *P. oris* and a heat-labile, proteinous component of *P. gingivalis* play important roles in this coaggregation. In addition, this coaggregation was inhibited by L-arginine, L-lysine, and Nα-p-tosyl-L-lysine. Therefore, it was considered that a cell surface protein on *P. gingivalis*, such as gingipain, may be involved in the coaggregation. Furthermore, the coaggregation was not inhibited by serum treatment.

**Conclusion:** This is the first report to describe the coaggregation of *P. oris* and *P. gingivalis*. Our study proposes the possibility that *P. oris* may promote the colonization of *P. gingivalis* in an early stage of biofilm formation. Furthermore, this coaggregation may contribute to the initiation and progression of periodontitis.

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Coaggregation of P. oris and P. gingivalis

Introduction

Chronic periodontitis is a destructive inflammatory disease of the periodontal tissues, caused by various periodontopathogens. To cause infection, it is necessary for periodontopathogens to coaggregate with early colonizing bacterial species to form an oral biofilm on the tooth surface or on the gingival epithelium. As such, the coaggregation of bacteria has been defined as one of the most important processes in the formation of periodontopathic biofilms.

Prevotella oris is a nonpigmented, anaerobic, Gram-negative, rod-shaped bacterium frequently isolated from infectious oral lesions, such as those found in periodontal disease, endodontic infection, dentoalveolar abscess, and spreading odontogenic infection, and also from systemic infectious lesions, such as those found in empyema, cervical spinal epidural abscesses, and meningitis. In previous studies, it has been reported that P. oris produces immunoglobulin A protease, hyaluronidase, and beta-lactamase as pathogenic factors. However, there are no reports regarding the coaggregation of P. oris and other bacteria, except in the case of Actinomyces israelii and Actinomyces odontlyticus.

Porphyromonas gingivalis is a member of the red complex group of bacteria frequently detected in severe forms of periodontal diseases. Previously, it has been reported that P. gingivalis coaggregates with Actinomyces naeslundii, Actinomyces viscosus, Streptococcus gordonii, Streptococcus oralis, Prevotella intermedia, Treponema denticola, Treponema medium, and Fusobacterium nucleatum. It is considered that these coaggregations may play important roles in the colonization of P. gingivalis with early colonizing bacteria.

In this study, the coaggregation of P. oris with nine oral bacterial species with potentially important roles in the formation of oral biofilms and in the progression of periodontitis was studied. To the best of our knowledge, this is the first report to describe that P. oris coaggregates with P. gingivalis in an in vitro setting. In addition, we sought to reveal the mechanism behind the coaggregation of P. oris and P. gingivalis, along with the effect of heat and proteinase K treatments, and treatment with various other reagents (including serum), on the coaggregation observed in the present study.

Methods

Bacterial strains and culture conditions

P. oris JCM 8540T, the sole strain available from bacterial culture collections, and WK 1 isolated from periodontal pocket of a patient with chronic periodontitis were used in the present study. P. gingivalis ATCC 33277T, F. nucleatum JCM 8532, P. intermedia ATCC 25611T, Prevotella denticola JCM 8528, Prevotella loescheii JCM 8530T, Prevotella oralis JCM 12251T, A. viscosus ATCC 19246, S. mutans ingbritt, and Streptococcus sanguinis ATCC 10556 were also used in this study. These strains were cultured anaerobically in tryptic soy broth supplemented with 0.5% yeast extract, 5 μg/mL hemin, and 1 μg/mL menadione at 37°C in an atmosphere of 80% N2, 10% H2, and 10% CO2. The cells were collected in the late exponential growth phase by centrifugation at 10,000×g for 15 minutes and washed three times with coaggregation buffer solution (1 mM Tris–HCl, 0.1 mM CaCl2, 0.1 mM MgCl2, 0.15 M NaCl, and 0.02% NaN3, pH 7.5).

Coaggregation assay

The cell suspensions were adjusted to 1.8 (OD 600 nm) for P. oris, P. loescheii, P. intermedia, A. viscosus, S. mutans, and S. sanguinis, and 0.65 for P. gingivalis and F. nucleatum, using a UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). The coaggregation assay was performed according to Cisar et al. Each P. oris cell suspension (200 μL), additional cell suspensions (200 μL), and chemical reagents or coaggregation buffer (100 μL) were mixed in a test tube and subjected to shaking at room temperature on a shaker (150 rpm) for 1 hour. Subsequently, the coaggregation values were scored from negative to 4+ described in Cisar et al. Each experiment was performed at least twice on separate days.

Effect of heat and proteinase K treatments on coaggregation

P. oris and P. gingivalis cells were treated at 100°C for 10 minutes or treated individually with 2 mg/mL proteinase K at 37°C for 1 hour. After washing three times with the coaggregation buffer, the coaggregation values of the treated cells were measured.

Effects of various chemical reagents on coaggregation

The addition of 12.5 to 100 mmol/L amino acids (L-arginine, L-lysine, L-cysteine, L-proline, L-threonine, L-glycine, L-serine, L-alanine, L-glutamine, L-valine, L-isoleucine, L-methionine, L-histidine, L-lysine, and L-phenylalanine) and 100 mmol/L sugars (D-mannitol, xylose, maltose, xylohexoside, D-arabinose, D-fructose, D-galactose, D-glucose, L-rhamnose, D-mannose, D-sorbitol), in addition to 0.6 mmol/L EDTA and 10–20 mmol/L Nα-p-tosyl-L-lysine (TLCK), was examined with respect to their effects on cell coaggregation. Following the mixing of bacterial cells together with these reagents, the coaggregation of P. oris and P. gingivalis was measured.

Effect of serum on coaggregation

After collection of horse blood, an equal volume of Alsever’s solution was added immediately, and the mixture was refrigerated at 4°C until use. To obtain the serum, the blood was centrifuged at 1500×g for 10 minutes. The P. oris and P. gingivalis cells were pretreated with serum for 10 minutes at room temperature and washed with coaggregation buffer three times, after which coaggregation values were obtained.
Results

Coaggregation of P. oris with other bacterial species

In this study, coaggregation of two P. oris strains, including JCM 8540T and WK 1, with nine additional bacterial species was examined (Table 1). In these bacterial species, self-aggregations were not observed except for A. viscosus and F. nucleatum. For all combinations of bacterial cells used as partner species, the two strains of P. oris formed aggregation clumps with A. viscosus, F. nucleatum, or P. gingivalis. However, because self-aggregations were observed for A. viscosus and F. nucleatum in the coaggregation buffer solution, P. oris could coaggregate strongly with P. gingivalis only. Following this result, the coaggregation of P. oris and P. gingivalis was studied further.

Effects of the heat and proteinase K treatments on coaggregation

After the individual treatment of P. oris JCM 8540T, WK 1, and P. gingivalis bacterial cells with heat or proteinase K, the degree of coaggregation between the treated cells was measured (Table 2). The results show that both heated strains of P. oris coaggregated with the nonheated P. gingivalis, although the coaggregation value fell from 3+ to 2+. In contrast, when heated, P. gingivalis did not coaggregate with either the heated or nonheated P. oris strains.

Comparatively, when coaggregations of the cells treated with proteinase K were examined, the treated P. oris coaggregated strongly with the nontreated P. gingivalis. However, the treatment of P. gingivalis inhibited the coaggregation with the nontreated P. oris, demonstrated by an aggregation score reduction from 3+ to 1+.

Inhibitory effects of various chemical reagents and serum on coaggregation

The coaggregation between P. oris and P. gingivalis was effectively inhibited with L-arginine, L-lysine, and TLCK, and was strongly concentration dependent. However, there were no effects observed with any of the other amino acids.

Table 2 Effects of the heat and proteinase K treatments on the coaggregation of P. oris and P. gingivalis

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Coaggregation</th>
<th>Coaggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JCM 8540T</td>
<td>P. gingivalis</td>
</tr>
<tr>
<td></td>
<td>WK 1</td>
<td>ATCC 33277</td>
</tr>
<tr>
<td>P. oris JCM 8540T</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>P. oris JCM 8540</td>
<td>2+</td>
<td>(H) ATCC 33277</td>
</tr>
<tr>
<td>P. oris WK 1</td>
<td>0</td>
<td>ATCC 33277 (H)</td>
</tr>
<tr>
<td>P. oris WK 1 (H)</td>
<td>2+</td>
<td>ATCC 33277</td>
</tr>
<tr>
<td>P. oris WK 1</td>
<td>0</td>
<td>ATCC 33277</td>
</tr>
<tr>
<td>P. oris JCM 8540</td>
<td>4+</td>
<td>(P) ATCC 33277</td>
</tr>
<tr>
<td>P. oris WK 1 (P)</td>
<td>4+</td>
<td>ATCC 33277</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3 Effects of various reagents and serum treatment on coaggregation of P. oris and P. gingivalis

<table>
<thead>
<tr>
<th>Tested reagent (mmol/L)</th>
<th>Coaggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. oris JCM 8540T</td>
</tr>
<tr>
<td>Buffer</td>
<td>3+</td>
</tr>
<tr>
<td>Sugars 100 mmol/L</td>
<td>3+</td>
</tr>
<tr>
<td>EDTA 0.6 mmol/L</td>
<td>3+</td>
</tr>
<tr>
<td>L-Arginine 100 mmol/L</td>
<td>2+</td>
</tr>
<tr>
<td>L-Lysine 100 mmol/L</td>
<td>2+</td>
</tr>
<tr>
<td>Other amino acids 100 mmol/L</td>
<td>3+</td>
</tr>
<tr>
<td>TLCK 10 mmol/L</td>
<td>1+</td>
</tr>
<tr>
<td>Blood serum</td>
<td>3+</td>
</tr>
</tbody>
</table>

Discussion

There have been no reports describing the coaggregation of P. oris and periodontopathogens, such as P. gingivalis and...
Prevotella species. In the present study, we observed that P. oris coaggregates with P. gingivalis only (Table 1) when the coaggregation of two strains of P. oris with nine bacterial species, including five Prevotella species, was examined. Therefore, to the best of our knowledge, this is the first report to describe the coaggregation of P. oris and P. gingivalis. It is known that P. gingivalis is isolated from the infected gingival crevice and that it coaggregates with many bacterial species, including A. viscosus, S. gordonii, S. mutans, F. nucleatum, and T. denticola. This coaggregation is an important step in the formation of oral biofilms.

It has been assumed that P. oris is one of the early colonizers in periodontal pockets, as P. oris has been isolated from the periodontal pocket before and after periodontal therapy. Thus, our findings also suggest that P. oris may be actively involved in the formation of periodontopathic biofilms by acting as a target organism for P. gingivalis together with other early colonizer bacteria.

The coaggregation was inhibited by the heat or proteinase K treatment of P. oris (Table 2). These results suggest that a heat-stable, nonproteinous component of P. oris together with a heat-labile proteinous component of P. gingivalis, is involved in the coaggregation. It is interesting that a strong coaggregation was observed when using P. oris cells treated with proteinase K (Table 2). This result may indicate that a proteinous component of P. oris, such as fimbria, was decomposed by proteinase K, and as a result, the coaggregation factor of P. oris was exposed on the cell surface. Thus, it was assumed that the proteinous components of P. oris partially inhibit coaggregation with P. gingivalis, although the significance of this phenomenon remains unknown.

The coaggregation of P. oris and P. gingivalis was not inhibited by either sugars or EDTA (Table 3). These results suggest that lectin-like binding, such as that observed with the coaggregation of F. nucleatum and P. gingivalis, and bicationic metal ions are not involved in the coaggregation. This coaggregation was inhibited by TLCK (Table 3), indicating the involvement of gingipain, a major proteinase in or secreted from P. gingivalis, because TLCK is an inhibitor of gingipain. The coaggregation of P. intermedia, one of the black-pigmented Prevotella bacterial species, and P. gingivalis was also inhibited by TLCK. It is already clear that the gingipain—adhesin complex is involved in the coaggregation between P. intermedia and P. gingivalis. Although the Arg-gingipain genes are encoded from rgpA and rgpB and Lys-gingipain gene is encoded from kgp, Kamaguchi et al. demonstrated that the gingipain—adhesin complex on P. gingivalis interacts with P. intermedia when using the rgpA rgpB, rgpA kgp, rgpA kgp bgp, and rgpA kgp hagA mutants of P. gingivalis. Furthermore, Kamaguchi et al. revealed that the P. gingivalis adhesion molecules involved in the coaggregation with P. intermedia were encoded by the gingipain genes. The coaggregation of P. oris and P. gingivalis may be similar to that observed between P. intermedia and P. gingivalis.

In this study, the coaggregation of P. oris and P. gingivalis was inhibited by l-lysine and especially l-arginine. The coaggregation of P. intermedia and P. gingivalis is also inhibited by arginine. Similarly, the inhibition of coaggregation by l-arginine was also described for the coaggregation of P. gingivalis and S. oralis, A. viscosus, T. denticola, and T. medium. Some studies have reported that proteinous components derived from saliva or serum inhibit the coaggregation of periodontal bacteria. In fact, periodontopathogens in the gingival sulcus or periodontal pockets are bathed with saliva and gingival cervical fluid containing many proteinous components. Therefore, it is important to understand whether or not bacterial species coaggregate with each other in the saliva or gingival cervical fluid. Thus, we examined the effect of serum treatment on this coaggregation. As shown in Table 3, after the serum treatment, the coaggregation of P. oris and P. gingivalis was determined to have the same coaggregation values as with the nonserum treatment. This result suggests that the coaggregation of P. oris and P. gingivalis may actually occur in vivo.

In conclusion, the coaggregation of P. oris and P. gingivalis was observed in an in vitro setting for the first time, and that a heat-stable, nonproteinous component on P. oris and a heat-labile, proteinous component of P. gingivalis were involved in the coaggregation. Our study proposes the possibility that P. oris may promote the colonizing of P. gingivalis in an early stage of biofilm formation. Furthermore, this coaggregation may contribute to the initiation and progression of periodontitis. Further studies are needed to clarify the identity of the coaggregation factors and the mechanism underlying the coaggregation of P. oris and P. gingivalis.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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References


