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Original

# The cell extract of *Porphyromonas gingivalis* promotes attachment of *Prevotella nigrescens* cells to hydroxyapatite

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**Abstract: Large quantities of *Prevotella nigrescens* ATCC 25261 (*P. nigrescens*) cells adhere to hydroxyapatite (HA) treated with extract from *Porphyromonas gingivalis* 381 (*Pg*-Ext), but not to HA coated with human serum albumin (HSA) or human serum globulin (HSG). The duration of HA treatment with *Pg*-Ext and several other conditions were tested to determine the factors causing *Pg*-Ext to promote *P. nigrescens* cell adhesion. *Pg*-Ext adsorbed rapidly to HA in less than 5 min. The maximum adherence of *P. nigrescens* cells to HA was observed after treatment of HSA and HSG and then retreatment of HA with *Pg*-Ext. It was found that *Pg*-Ext heated at 80°C for 30 min did not lose its propensity to promote attachment of *P. nigrescens* to HA and that it also remained stable at 4°C for at least 6 days. The trypsin-like enzyme activity of *Pg*-Ext was also measured, with BAPNA as the substrate and commercially purchased trypsin as the standard, and was approximately 0.12 units/mg. These data suggest that the presence of *Pg*-Ext is one of the essential factors responsible for *P. nigrescens* cell attachment to apatitic surfaces, and that with its trypsin-like activity, *Pg*-Ext may be considered an extremely important substance for the establishment of *P. nigrescens* in the periodontal pocket and the development of periodontal disease. (J. Oral Sci. 45, 99-106, 2003)**

Key words: *Prevotella nigrescens*; *Porphyromonas gingivalis*; bacterial adsorption; hydroxyapatite.

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## Introduction

Adult inflammatory periodontal diseases are a result of the interaction of host immune and inflammatory systems with subgingival plaque bacteria. Proteases from diverse origins have been implicated in the process of tissue destruction. In addition, proteases originating from plaque bacteria may contribute directly to proteolytic tissue destruction. Proteolytic activity has been demonstrated in several periodontitis-related pathogens: *Bacteroides* and *Peptostreptococcus*, and Capnocytophaga spp. (1-3), *Treponema denticola* (4), *Fusobacterium nucleatum* (5), *Porphyromonas gingivalis* (6), and *Actinobacillus actinomycetemcomitans* (7-9).

The complexity of the subgingival microorganisms has been recognized since the very first microscopic examination of these flora by Van Leeuwenhoek in 1683. Numerous similar studies have been performed since that time, and it is now estimated that 400 or more species of subgingival microorganisms reside in the periodontal pocket. Culture, immunologic and DNA samples of plaque have demonstrated that certain species frequently occur together in subgingival plaque samples. *P. gingivalis*, for example, is almost always observed in samples that harbor *Bacteroides forsythus* (10). Other combinations that have been observed include *P. gingivalis* and *T. denticola* (11), *F. nucleatum* and *Prevotella intermedia* (12) and *Prevotella nigrescens*, *P. intermedia*, *B. forsythus* and *P. gingivalis* (13). Understanding the relationships between these bacterial microorganisms is useful in developing strategies for their control.

The *P. intermedia/nigrescens* group can be found in the oral cavity as well as extra-oral sites in both healthy and

diseased conditions (14-18). However, some differences may exist within this group. For example, *P. intermedia* has been reported in association with oral infections, such as periodontitis, more frequently than *P. nigrescens* (19-22), whereas *P. nigrescens* seems to be more common in children than *P. intermedia* (20,22).

There is considerable evidence to suggest that *P. nigrescens* may play an important role in the occurrence of periodontitis. However, until recent studies conducted by this laboratory (23,24), it was not known how *P. nigrescens* adheres to non-desquamating hard surfaces like the root surface in the periodontal pocket.

This study examined the characteristics and role of extract from *Porphyromonas gingivalis* 381 (*Pg*-Ext) in promoting the adhesion of *P. nigrescens* cells in the periodontal pocket.

## Materials and Methods

### Bacterial strain and culture conditions

*P. nigrescens* cells were obtained from the culture collection of our laboratory. Stock cultures were stored in 1% skim milk at -80°C until used. The *P. nigrescens* strain was preincubated in GAM broth (Nissui, Japan) inside an anaerobic jar for 24 h at 37°C, in an atmosphere of 95% $N_2$ and 5% $CO_2$. The fresh bacterial cells were then inoculated into GAM broth supplemented with 740 kBq of [$^3$H]-thymidine (ICN Biochemicals, CA, USA) per ml, and grown to the early stationary phase at 37°C in the an anaerobic condition (BBL GasPak Anaerobic System, Becton Dickinson Microbiology Systems, MD, USA). The bacterial cells were harvested by centrifugation and washed twice with buffered KCl (0.05 M KCl containing 1 mM $K_2HPO_4$, 1 mM $KH_2PO_4$, 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ at pH 6.2). The washed cells were then suspended in buffered KCl supplemented with 2.5 mg per ml of human serum albumin (HSA: Sigma Chemical, MO, USA) (HSA-KCl). The suspensions were adjusted to a level of $6x10^8$ bacteria per ml based on a standard curve relating $OD_{550}$ to the number of bacterial cells, as determined by microscopic counting.

### Bacterial adhesion assays

Bacterial adhesion to HA (Nihon Chemical Industries, Lt. Japan) treated with *Pg*-Ext (preparation procedure shown in Table 1) was studied.. Before the assay, 5 mg of the HA beads were equilibrated overnight in buffered KCl at room temperature (RT). The HA was treated with HSA & HSG-KCl, each supplemented with 1.25 mg per ml of HSA and HSG (Sigma Chemical Co., MO, USA), for 30 min at RT to mimic the internal condition of the periodontal pocket. The HA beads were then washed twice with buffered KCl and incubated with an adequate concentration of *Pg*-Ext solution at RT. The HA beads were again washed and the liquid was removed. The *Pg*-Ext-coated HA was incubated with an adequate number of [$^3$H]-labeled *P. nigrescens* cells in 125 µl of HSA-KCl. After one hour of continuous rotation at RT, the HA beads were washed twice with buffered KCl and transferred to scintillation vials. The number of *P. nigrescens* cells that had become attached to the HA beads was determined by direct scintillation

Table 1 Preparation on *Pg*-Ext from *Porphyromonas gingivalis* 381

Preincubation of 5 ml of GAM broth
↓
Incubation of 1,000 ml of GAM broth
↓
Harvest with centrifugation
↓
Wash two times
↓
Treatment of cells with 0.1% Sodium Deoxycholate (37°C for 30 min)
↓
Removal of cells with cenrifugation 5,000×g for 30 min
↓
Filtration (0.45 µm → 0.22 µm)
↓
Dialysis against distilled water containing 0.004% $NaN_3$
↓ (*Pg*-Ext: 12 mg prot./ml)
Lyophilization (Stored at -80°C)

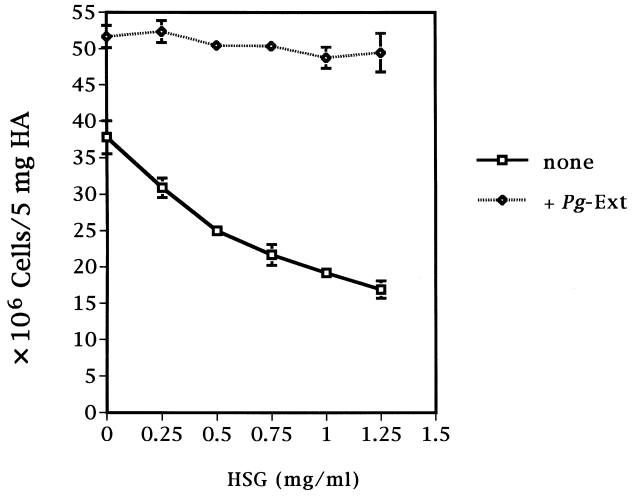


Fig. 1 Effect of HA-treatment with HSA (1.25 mg/ml) and HSG(0-1.25 mg/ml) on *P. nigrescens* attachment. Bars represent SD of 4 assays.

counting (LSC-5200: Aloka, Japan). The influence of *Pg*-Ext on *P. nigrescens* attachment to HSA and HSG-HA was also studied by mixing with *P. nigrescens* cells.

All assays were conducted in quadruplet and most experiments were performed at least twice.

## Adsorption of *Pg*-Ext to HSA & HSG-HA

Five milligrams of HA beads pretreated with HSA and HSG and an adequate amount of *Pg*-Ext were incubated to determine the adsorption time of *Pg*-Ext to the HA beads. *P. nigrescens* cells ($6 \times 10^8$) were then incubated for one hour at RT. After incubation, the HA beads were washed twice with buffered KCl and transferred to scintillation vials for counting. Similarly, the quantity of *Pg*-Ext which promotes maximum attachment of *P. nigrescens* cells to the HA was determined.

## Trypsin-like enzyme assay of *Pg*-Ext

The enzyme substrate used for the assay was *N*-benzoyl-L-arginine-ρ-nitroanilide (BAPNA) with a final concentration of 2.5 mM in an assay buffer containing 50% DMSO. The assay buffer consisted of 0.05 M Tris-HCl containing 0.2 M NaCl and 5 mM dithiothreitol, pH 7.5.

Ten µl of the sample was preincubated for 5 min with 40 µl of the assay buffer in the wells of a 96-well microtiter plate, and 50 µl of the substrate was then added. The plate was incubated at 37°C, and the release of ρNA was followed spectrophotmetrically by $OD_{405}$ readings, using a microtiter plate reader (BIO RAD, Model 550, CA, USA).

# Results

## Examination of the concentrations of HSA and HSG for HA treatment

The cell count was the same for bacteria that bound to HA treated with HSA (1.25 mg/ml) and HSG (0-1.25 mg/ml). Even when the concentration of HSG was higher than the minimum number of *P. nigrescens* cells attached to the HA (the negative control; non-treatment with *Pg*-Ext). It therefore was concluded that the HSA and HSG concentrations (1.25 mg/ml each) used for treating the HA were sufficient (Fig. 1).

## Examination of concentrations of HSA and HSG for *P. nigrescens* cell treatment

The cell count was found to be dose-dependent as the HSA concentration went up. No change was observed in the positive control (cells bound to HA treated with *Pg*-Ext), so an HSA concentration of 2.5 mg/ml was considered to be adequate for the treatment of *P. nigrescens* cells (Fig. 2).

## Influence of *Pg*-Ext on the adhesion of *P. nigrescens* cells to HA

The attachment of *P. nigrescens* to HA treated with increasing amounts of *Pg*-Ext, ranging from 0 to 3.0 mg prot./ml, was tested. In the absence of *Pg*-Ext, *P. nigrescens* cells did not become attached to HA. In the presence of *Pg*-Ext, *P. nigrescens* cells became attached to HA in a dose-dependent manner up to a *Pg*-Ext concentration of 0.1 mg prot./ml. At higher concentrations of *Pg*-Ext, the number

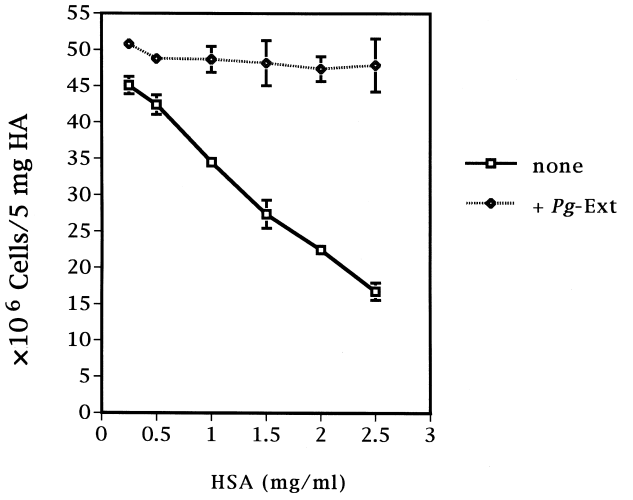


Fig. 2 Effect of *P. nigrescens* cell-treatment with HSA on its attachment to HSA and HSG-treated HA. Bars represent SD of 4 assays.

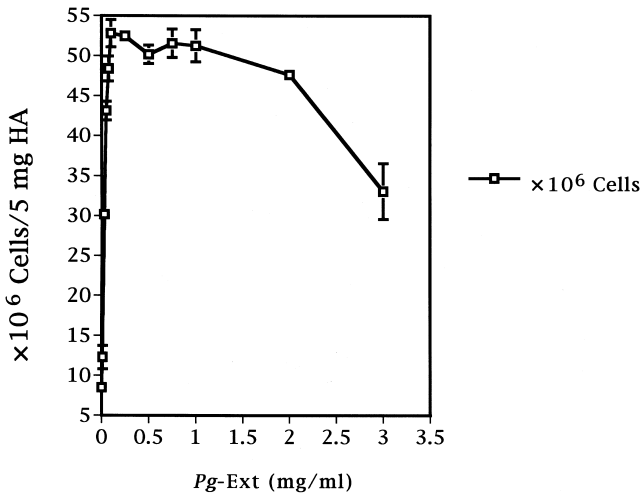


Fig. 3 Effect of *Pg*-Ext on *P. nigrescens* attachment. Bars represent SD of 4 assays.

of cell attachments gradually decreased (Fig. 3). In a similar experiment (HA treated with a mixture of *Pg*-Ext and *P. nigrescens* cells) the data showed almost identical results, but a higher concentration of *Pg*-Ext was needed to reach maximum attachment (Fig. 4).

## Effect of the duration of *Pg*-Ext treatments of HA on the attachment of *P. nigrescens*

Treating HA with *Pg*.-Ext for various durations (6, 10, 20, 30, 40 min) did not significantly alter the number of bacteria binding to HA. These data showed that *Pg*-Ext adsorbed rapidly to HA that had been pretreated with HSA and HSG. It therefore was concluded that treating HA with *Pg*-Ext for a period of 30 min was sufficient to produce the maximum attachment (Fig. 5).

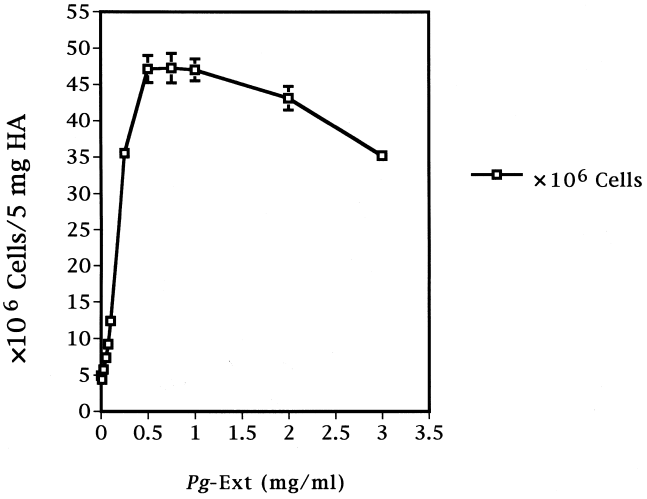


Fig. 4 Effect of *Pg*-Ext on *P. nigrescens* attachment (Mixture of *Pg*-Ext and *P. nigrescens* cells).
Bars represent SD of 4 assays.

## Effect of heating Pg-Ext (to 80°C) on *P. nigrescens* attachment

*P. nigrescens* cells attached to HA after being treated with heated *Pg*-Ext. However, when *Pg*-Ext was heated for 30 min, the number of attached cells decreased somewhat. It was concluded that *Pg*-Ext is a very heat stable substance as a receptor for *P. nigrescens* cell attachment to HA (Fig. 6).

## Stability of the attachment promoting propensity of *Pg*-Ext (samples stored at 4°C at concentration of 0.08 mg prot./ml)

The attachment of *P. nigrescens* cells to HA was studied using diluted samples of *Pg*-Ext (conc.: 0.08 mg prot./ml) stored at 4°C. The data showed that *Pg*-Ext retained its function as a receptor for *P. nigrescens* cell attachment to HA for at least 6 days (Fig. 7).

## Estimation of trypsin-like enzyme activity of *Pg*-Ext

With respect to the enzyme assay, the intensity of *Pg*-Ext absorbance was linearly dependent on the concentration of the commercially purchased trypsin used as standard (Fig.8). *Pg*-Ext exhibited 0.12 units/mg prot. of typsin-like activity compared with the standard enzyme.

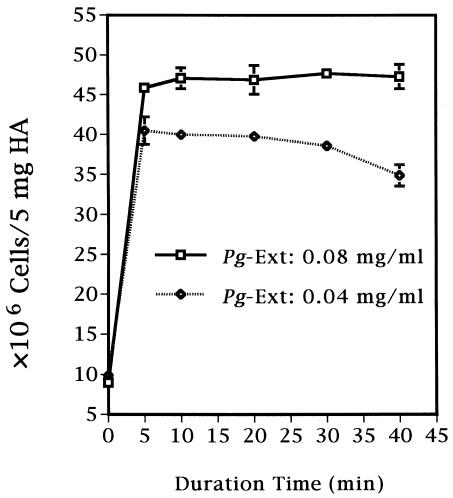


Fig. 5 Adsorption time of *Pg*-Ext to HSA and HSG-coated HA.
Bars represent SD of 4 assays.

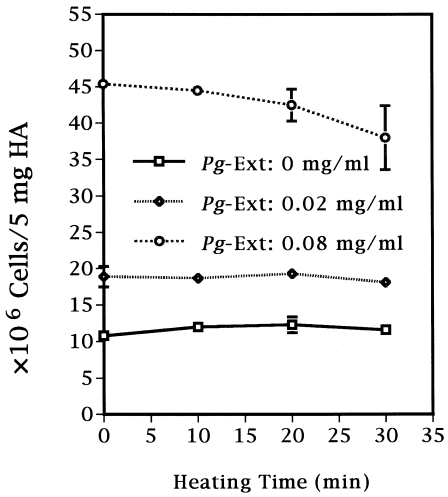


Fig. 6 Effect of heat (at 80°C)-treatment of *Pg*-Ext on *P. nigrescens* attachment.
Bars represent SD of 4 assays.

## Discussion

*P. gingivalis* was much more widespread than *P. intermedia* group organisms in adult periodontitis, and the prevalence of *P. gingivalis* was found to be greater than in studies using culture methods by Slots el al. (45%) (25), Dzink et al. (22%) (26), Moore et al. (50%) (27) and Rosenburg et al. (8%) (28).

The attachment of periodontal pathogens to oral hard surfaces is undoubtedly important in the etiology and progression of periodontal disease. *P. intermedia* and *P. nigrescens*, as well as *P. gingivalis*, are black-pigmented anaerobic gram-negative bacteria. These bacterial species are frequently found in the periodontal sites of patients with gingivitis and periodontitis (29-32). It is therefore suspected that these species are involved in the causation of periodontal diseases. Although pathogenic activities involving these species have been reported, such as cytotoxic end-product formation (33,34) and extracellular enzyme (35,36), only limited information such as amino acid metabolism (37) and glucose metabolism (38) is available on their metabolic properties, which provide the source of energy and cell materials essential for their physiological activity.

The properties of gingival crevicular fluid (GCF) are assumed to be derived from serum, host epithelial and connective tissues. Most proteins that have been identified in GCF are of serum origin, such as albumin (39,40), immunoglobulins (41), complement components (41,42), transferrin (43), and fibrinogen (44). The proteins in serum consist mostly of albumin (50-60%) and globulin (40%). The protein concentration has been reported as 60 to 80 mg/ml. To modulate the environment of the internal periodontal pocket, HSA and HSG were used for HA coating, both at concentrations of 1.25 mg/ml. *P. nigrescens* cells were treated with only HSA to prevent activity of the protease inhibitors contained in the globulin. The concentration was 2.5 mg/ml.

Connective tissue destruction is a major feature of chronic periodontitis and proteolytic enzymes are believed to play a role in its pathogenesis. The appearance of proteases in GCF may correlate with the disease activity. GCF contains various proteolytic enzymes of inflammatory cellular and bacterial origin (45). It is now well known that the oral hygiene or periodontal status of a patient is correlated with the levels of various proteolytic enzymes present in samples obtained from subgingival sites (46).

The present study has demonstrated that *Pg*-Ext extracted from *P. gingivalis* 381 that has typsin-like enzyme activity acts as a receptor for *P. nigrescens* attachment to HA. Our finding makes it possible to understand one of the important items of evidence found in the periodontal pocket, and clarifies how various oral bacterial species accumulate in this area. *Pg*-Ext adsorbs rapidly to HSA and HSG-coated HA, revealing the attachment-promoting propensity of the *P. nigrescens* cell. *Pg*-Ext strongly promoted *P. nigrescens* attachment at relatively low concentrations (up to 1.0 mg prot./ml), but with higher concentrations that attachment was inhibited. This reduction was judged to be a typical case of competitive inhibition. *Pg*-Ext exhibited trypsin-like enzyme activity, but its propensity for *P.*

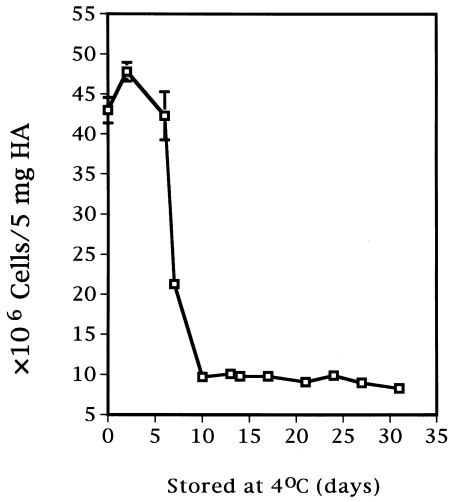


Fig. 7 Stability of attachment promoting ability of *Pg*-Ext. Bars represent SD of 4 assays.

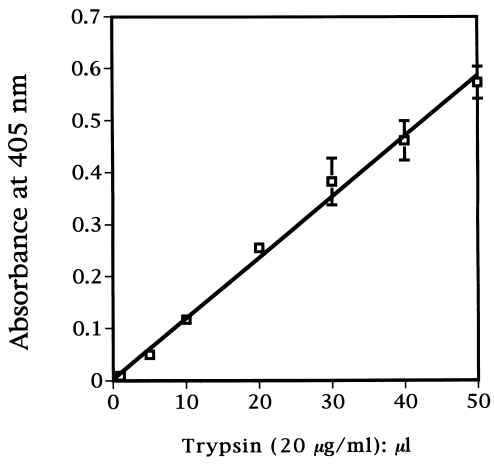


Fig. 8 The relationship of the absorbance intensity at 405 nm to the amount of trypsin. Bars represent SD of 4 assays.

*nigrescens* cell attachment to HA was not lost by heat treatment at 80°C. It was also relatively stable in storage at 4°C.

Considering that *P. intermedia* and *P. nigrescens* can be found in both healthy and diseased oral cavities, these organisms could not be regarded as secondary settlers. In these strains, *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC 33563, *Pg*-Ext did not promote attachment to the experimental root surface. It follows that as the severity of the periodontitis worsens, *P. gingivalis* could promote some of the *P. nigrescens* attachment to hard surfaces and eventually settle in plaque attached to the periodontal pocket.

This study suggests that attachment of *P. nigrescens* cells to human tooth surfaces exposed to the experimental GCF fluid may advance in relation to the increase of the *P. gingivlais* already present.

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