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ORIGINAL ARTICLE

Relationship of periodontal bacteria and *Porphyromonas gingivalis fimA* variations with phenytoin-induced gingival overgrowth

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OBJECTIVES: We investigated the relationship between phenytoin-induced gingival overgrowth (GO) and the harboring of periodontal bacteria.

MATERIALS AND METHODS: Periodontal conditions and subgingival bacterial profiles were examined in 450 sites of 75 subjects. A polymerase chain reaction method was used to detect six bacterial species; *Porphyromonas gingivalis* (Pg), *Actinobacillus actinomycetemcomitans* (Aa), *Tannerella forsythia*, *Treponema denticola* (Td), *Prevotella intermedia* (Pi), and *Prevotella nigrescens* (Pn). Genetic variations of the Pg *fimA* gene were also examined. Bacterial occurrence was compared with the severity of GO, and alterations in the bacterial occurrence rate and quantities were monitored following periodontal treatment.

RESULTS: The occurrences of Aa, Td, Pi, Pn, and Pg with type II *fimA* (type II Pg) were significantly associated with the severity of GO. Td occurrence was reduced in association with gingival improvement following ultrasonic scaling, however, no such relationship was observed with Aa, Pi, Pn, and Pg. In addition, Pg and Pi markedly persisted after treatment. Clinical improvement of the sites, following an Er:YAG laser treatment, significantly associated with quantitative reduction of Pg in improved sites, however, not that of Pi.

CONCLUSION: Type II Pg and Td were each found to have a significant relationship with the development and deterioration of GO.

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Introduction

Gingival overgrowth (GO) or enlargement is one of the most frequent and troublesome adverse effects associated with administrations of the anticonvulsant drug phenytoin, calcium channel blockers such as nifedipine, and the immunosuppressant cyclosporine (Seymour *et al*, 2000). Of these, phenytoin is known to stimulate responsive subpopulations of gingival fibroblasts to accumulate extracellular matrix components such as collagen and glycosaminoglycans, resulting in GO (Vernillo and Schwartz, 1987). However, the pathogenic mechanisms of phenytoin-induced GO seem to be affected by other factors, such as dental plaque, as several studies have found a relationship between the quantity of accumulated dental plaque and phenytoin-induced GO (Stinnett *et al*, 1987; Hassell and Hefti, 1991; Thomason *et al*, 1993; Botha, 1997; Hassell, 1990). Further, it was recently indicated that dental plaque accumulation is the most important determinant of phenytoin-induced GO (Majola *et al*, 2000). It is now considered that an enhanced matrix synthesis by fibroblasts responsive to phenytoin can be triggered or enhanced by chronic inflammation due to dental plaque.

Periodontal diseases including adult periodontitis are basically infectious disorders caused by a small subset of periodontal pathogens (Darveau *et al*, 1997), among which *Porphyromonas gingivalis* (Pg) is considered to be a bona fide periodontal pathogen (Ezzo and Cutler, 2003). Pg has fimbriae, hair-like appendages on the bacterial surface, that are thought to be critical virulence factors to mediate bacterial interactions with and invasion of host tissues (Amano, 2003), and are classified into six genotypes (I–V and Ib) based on the diversity of *fimA* genes encoding FimA (a subunit protein of fimbriae) (Amano *et al*, 1999, 2004). The bacterial clones with type II *fimA* revealed a significantly greater virulence *in vitro* and *in vivo* (Nakagawa *et al*, 2002; Amano *et al*, 2004; Nakano *et al*, 2004). Thus, Pg

with type II *fimA* is strongly suggested to be a causative factor of adult periodontitis.

Pg with a specific *fimA* type and some periodontal bacteria may be related to other types of periodontal diseases, such as phenytoin-induced GO. However, sufficient qualitative analyses of dental plaque have not been performed to determine the exact microbial agents related to phenytoin-induced GO. In the present study, the occurrences of six periodontal bacteria, including Pg *fimA* variations, were surveyed in epileptic patients who were receiving phenytoin. We also monitored alterations in the occurrence of the tested bacteria as well as their quantities following periodontal treatment in the same subjects.

Materials and methods

Study population

All subjects were enrolled following self and/or parental informed consent, according to a protocol approved by the Ethics Committee of Osaka University Graduate School of Dentistry. Seventy-five epileptic subjects aged 10–35 years old (mean age; 24.3 ± 7.0 years, 44 males and 31 females) who were taking phenytoin were enrolled, after being referred to the Division of Special Care Dentistry at Osaka University Dental Hospital for dental/periodontal treatment. All of the subjects manifested epilepsy and mental disabilities, and had not received professional teeth cleaning or systemic medication, excluding antiepileptic drugs, during the 6 months prior to the study. Each had at least 20 teeth. All demonstrated poor oral hygiene [plaque control record >80% (O'Leary *et al*, 1972)] due to mental disability, when our initial clinical examination and plaque sample collections were performed.

Periodontal examinations

Clinical parameters were measured by a single skilled examiner (S.A.), and included probing pocket depth (PD) and bleeding on probing (BOP). The dentition was divided into six segments, which were the anterior canine to canine, left premolars and molars, and right-posterior sextants in each arch. PD was used as a marker of the development and progression of GO (none: PD ≤ 3 mm, moderate: PD 3–5 mm, severe: PD > 5 mm). The tooth with the most serious gingival condition in each sextant was selected as representative.

Periodontal treatment

Professional subgingival plaque removal was thoroughly performed at all sites with GO using ultrasonic scaling for 15 selected subjects (total 90 sites with BOP(+) and PD ≥ 5 mm). Bacterial elimination was performed using an Er:YAG laser apparatus (Erwin; J. Morita Mfg. Corp., Kyoto, Japan) without local anesthesia for another group of five subjects (total 30 sites with BOP(+) and PD ≥ 5 mm), during which we irradiated the root surface and subgingival tissue at approximately 30 mJ per pulse, at 10 pulses s^{-1}, under water irrigation. The condition of each site in these 20 patients after treatment was then judged as 'improved' [reduction of PD by more than 1 mm and BOP(−)] or 'unchanged' [PD reduced by 1 mm or less, and/or BOP(+)].

Sample collection

Subgingival plaque samples were collected from the mesial and buccal subgingival sites in each of the six representative sections in all subjects using sterile Gracy curettes, after gently removing supragingival plaque, as previously described (Amano *et al*, 2001). The samples were placed into sterile Eppendorf tubes containing 1 ml of phosphate-buffered saline (pH 7.4) on ice and were immediately stored at −80°C.

Conventional polymerase chain reaction assay

Six bacterial species: Pg, *Actinobacillus actinomycetemcomitans* (Aa), *Tannerella forsythia* (Tf), *Treponema denticola* (Td), *Prevotella intermedia* (Pi), and *Prevotella nigrescens* (Pn), were surveyed in the subgingival plaque samples using a polymerase chain reaction (PCR) method, with bacterial species-specific and universal primer sets as described previously (Amano *et al*, 2000a). Genomic variations of *P. gingivalis fimA* were also assessed as described previously (Amano *et al*, 2000b; Nakagawa *et al*, 2000). Briefly, bacterial genomic DNA was isolated using a DNA isolation kit (Puregene; Gentra Systems, Minneapolis, MN, USA) and PCR was performed with a total volume of 25 μl consisting of PCR beads (Ready-To-Go; Amersham Bioscience, Buckinghamshire, UK), 0.5 μM of each primer, 2–5 μl of the template DNA mixture from the six representative teeth (20–60 μg ml^{-1}), and sterile distilled water. The products were analyzed as described previously (Amano *et al*, 2000b). Positive and negative controls were included in each PCR set and in each sample processing.

Quantitative analysis by real-time PCR

Real-time PCR was carried out using a LightCycler™ system (Roche Diagnostics, Mannheim, Germany) with a TaqMan probe, as described previously (Kuboniwa *et al*, 2004). Briefly, each PCR reaction was performed in a total volume of 20 μl containing 2 μl of ×10 LightCycler-DNA Master Hybridization Probes (Roche Diagnostics), 0.2 μl each of forward and reverse primers (final concentration, 500 nM each), an appropriate dose of the TaqMan probe (final concentration, 200 nM), an appropriate dose of $MgCl_2$ (final concentration, 3–6 mM), 2 μl of template DNA solution, and an appropriate dose of sterilized DNase–RNase free water. Each amplification reaction was performed in the LightCycler with the bacterial species-specific cycling parameters set. Data was analyzed using LightCycler™ analysis software (Roche Diagnostics) and quantification of the target bacteria in clinical samples was performed using external standards as described previously (Kuboniwa *et al*, 2004). The quantity of each species was expressed as the ratio per total amount of microorganisms obtained with a universal primer set. All

assays were routinely performed in triplicate on three separate occasions.

Analytical methods

Multiple comparisons by Ryan's method were used for statistical analysis of the comparative frequencies of bacterial occurrence among the groups of GO severity. Differences with respect to frequencies of bacterial occurrence in each group were verified utilizing McNemar's test. Student's *t*-test was used for a comparative analysis of relative bacterial amount. A *P*-value of <0.05 was considered significant.

Results

Gingival condition and bacterial occurrence in epileptic subjects

Among the 450 sites of the 75 subjects, GO was moderate in 240 (53.3%), severe in 79 (17.6%), and not observed in 131 (29.1%) (Table 1). Bacterial components in subgingival plaque were compared among these three categories. There was no significant relationship between the occurrences of Tf and Pg (all *fimA* types), however, some Pg *fimA* variations were found to be correlated with disease status, as Pg with *fimA* type II was significantly more prevalent with disease deterioration, whereas type IV organisms predominantly occurred in non-diseased sites. No significant association was found between GO and the other *fimA* types. It was also found that the occurrences of Aa, Td, Pi, and Pn were significantly greater in sites with moderate or severe GO. These findings indicated that Aa, Td, Pi, Pn, and Pg with type II *fimA* are possibly related to phenytoin-induced GO.

Table 1 Bacterial occurrence in the sites of epileptic subiects with or without gingival overgrowth (GO)

	Severity of GO and bacterial occurrence (%)[a]		
Bacterial species	*None (n = 131)*	*Moderate (n = 240)*	*Severe (n = 79)*
P. gingivalis (total)	96.9	92.1	96.2
flmA type			
I	25.2	18.8	29.1
II	13.7*	26.7*	40.5
III	3.8	4.6	2.5
IV	38.2*	24.2	16.5
V	1.5	4.2	0.0
Ib	2.3	1.3	0.0
I and IV	0.0	4.6	0.0
Ib and IV	0.0	2.5	0.0
II and IV	0.0	0.4	6.3
Unknown	12.2	5.0	0.0
A. actinomycetemcomitans	37.4*	62.9	69.6
T. forsythia	78.6	83.8	86.1
T. denticola	35.9*	50.8*	75.9
P. intermedia	31.3*	42.9*	67.1
P. nigrescens	58.0*	70.4*	83.5

[a]None: PD ≤ 3 mm, moderate: 3 < PD ≤ 5 mm, severe: 5 mm < PD.
*Significant difference between two values ($P < 0.05$).

Alteration of bacterial occurrence following ultrasonic scaling

To determine if the above five species (Aa, Td, Pi, Pn, and Pg with type II *fimA*) were involved in the pathogenesis of phenytoin-induced GO, we assessed the association of their occurrences with alteration of disease status following periodontal treatment. All of the epileptic subjects were complicated with mental retardation, and complete periodontal debridement was quite difficult to perform for all subjects. Therefore, we were able to obtain only 15 subjects for the treatment with ultrasonic scaling. The occurrences of the five species were analyzed just prior to and 1 month after the treatment (Table 2). At the improved sites, the occurrences of Aa, Tf, Td, and Pn were markedly reduced following the treatment, whereas Pi and Pg occurred with the same frequency as before. Among the *fimA* types, the occurrence of type I organisms was slightly decreased, whereas all of the other types persisted.

At the unchanged sites, there were no significant decreases observed in the occurrences of a majority of the bacterial species, including Pg variants, however, the occurrences of Aa and Pn were significantly decreased. These results suggest that Td is an agent related to GO, but Aa and Pn are opportunistic factors. In addition, it was also likely that Pg and Pi were hard to be completely removed just by ultrasonic scaling.

Quantitative alteration of bacterial species by bacterial elimination with Er:YAG laser

The occurrence of Pg and Pi was not reduced by ultrasonic scaling. The present subjects were not able to endure the pain that is caused by local anesthesia and an ordinary method of debridement with curettes. Therefore, an Er:YAG laser, which enables painless treatment without local anesthesia, was used for the bacterial elimination. Its effect against Pg and Pi was evaluated

Table 2 Alteration of bacterial occurrence following professional plaque removal

	Bacterial occurrence (%)			
	Clinically improved sites (n = 58)		*Clinically unchanged sites (n = 32)*	
Bacterial species	*Before treatment*	*After treatment*	*Before treatment*	*After treatment*
P. gingivalis (total)	93.1	82.8	96.9	87.5
flmA type				
I	41.4	31.0	34.4	31.3
II	13.8	13.8	31.3	31.3
III	20.7	20.7	0.0	0.0
IV	15.5	15.5	15.6	15.6
V	0.0	0.0	0.0	0.0
Ib	0.0	0.0	0.0	0.0
Unknown	1.7	1.7	15.6	9.4
A. actinomycetemcomitans	75.9*	39.7	71.9*	37.5
T. denticola	70.7*	37.9	65.6	62.5
P. intermedia	48.3	44.8	59.4	50.0
P. nigrescens	79.3*	60.3	71.9*	50.0

*Significant differecnce between before and after ($P < 0.05$).

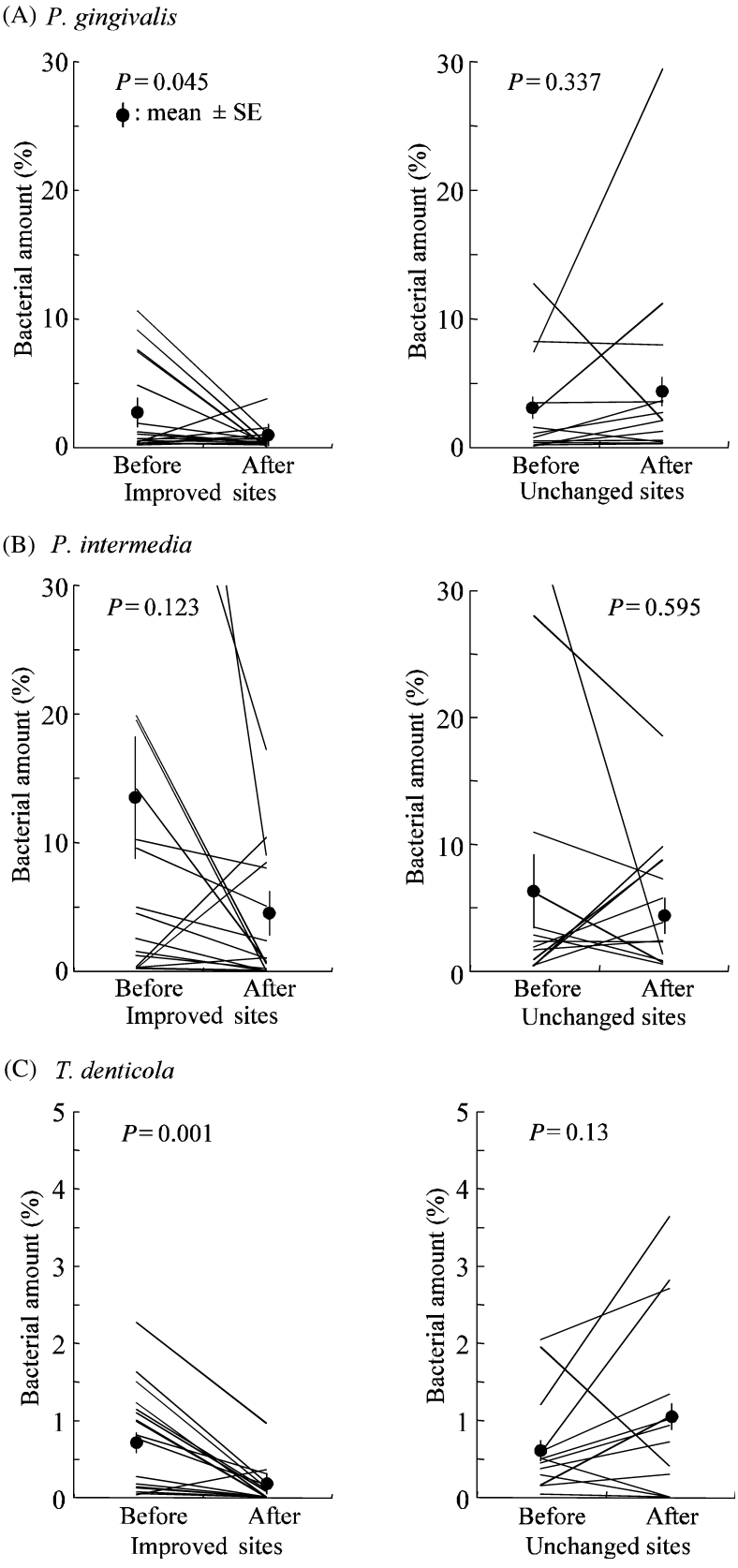

Figure 1 Quantitative alteration of bacterial species by root-planing using Er:YAG laser. Subgingival plaque specimens were collected from five subjects (30 sites), just prior to and 1 month after an Er:YAG laser treatment. Bacterial ratios of Pg, Pi, and Td were analyzed using real-time PCR. The quantity of each species is expressed as a ratio of the total amount of microorganisms, which was obtained using a universal primer set

using a quantitative detection method (real-time PCR). Td was employed as a positive control, as the occurrence of this bacterium was significantly reduced associated with clinical improvement, as shown in Table 2. Because of the operation time required for the treatment, we selected subjects who were able to accept prolonged

sitting and immobility during the treatment. As a result, five subjects harboring Pg, Pi and Td were available for this trial. They carried different Pg *fimA* types, as one subject had type I, two had type II, and two had type IV. One month after the treatment, we found 17 improved and 13 unchanged sites. Quantitative analysis revealed that ratio of Pg was significantly decreased in the improved sites in a manner similar to the control (Td), however, not in the unchanged sites (Figure 1). No statistical tendency was seen for the quantitative alteration of different *fimA* types. Pi was not quantitatively related to gingival change, unlike Pg and Td. These findings suggested that Pg is associated with phenytoin-induced GO, while Pi is independent from its pathogenesis.

Discussion

Dental plaque deposition is considered to be an essential cofactor influencing phenytoin-induced GO, as noted above. However, there are only two known reports regarding the bacterial factors related to phenytoin-induced GO. One of those studies used a culture method and the results suggested that black-pigmented rods, particularly *P. intermedia*, could be inhabitants in a GO environment (Takada *et al*, 2003). In the other report, which used an immunological assay (Yamada *et al*, 2001), no differences were found in the profiles of 13 periodontal bacteria between GO and non-GO groups in an epileptic population taking phenytoin. To compensate for this limited available data, we examined microbial agents associated with phenytoin-induced GO, by employing patients taking phenytoin without GO as a control group. The occurrence of four bacterial species (Aa, Td, Pi, and Pn) was significantly associated with phenytoin-induced GO in the present subjects (Table 1). In addition, it should be noted that the occurrence of type II *fimA* organisms showed a clear relationship with disease-associated deterioration (Table 1).

Several studies have demonstrated the efficacy of scaling, root-planing, and anti-plaque agents for the prevention of phenytoin-induced GO (O'Neil and Figures, 1982; Modeer and Dahllof, 1987; Brown *et al*, 1991), and their results demonstrated that the occurrences of GO-related bacteria are altered depending on gingival status. An improvement of gingival conditions following periodontal treatment using ultrasonic scaling was found to be associated with a reduced occurrence of Td (Table 2), whereas Aa and Pn, which occurred with decreased frequency in both improved and unchanged sites, did not seem to be disease-associated factors. Thus, Td is considered to be a causative bacterial agent of GO, while Aa and Pn are not.

Pg and Pi are known to demonstrate a strong resistance against periodontal therapy (Williams and Offenbacher, 2000). Thus, we evaluated their quantitative changes following an Er:YAG laser treatment. After the treatment, the quantitative reduction of Pg including type II organisms was as significant as that of Td. On the other hand, Pi was not quantitatively associated with gingival change. These findings suggest that Pg with type II *fimA* and Td are causative agents of phenytoin-induced GO, whereas Pi seemed to be an independent factor. Interestingly, Pi and Td were completely removed in some of the improved sites (four and eight sites, respectively) following the treatment, whereas Pg was found to persist in all of the treated sites. These findings may indicate that Pg has a strong resistance against periodontal therapy.

We previously found that a majority of periodontitis patients carried type II *fimA* organisms, followed by type IV, and the occurrence of type II *fimA* organisms was significantly increased with the more severe forms of periodontitis (Amano *et al*, 1999, 2000b). In contrast, the most prevalent *fimA* type of Pg in adults with healthy gingivae was found to be type I. Similar findings were observed in Down syndrome patients, who are congenitally susceptible to periodontal diseases (Amano *et al*, 2001). Further, several other studies conducted in different countries also support our findings that type II *fimA* organisms are strongly related with the development of periodontitis (Beikler *et al*, 2003; Missailidis *et al*, 2004; van der Ploeg *et al*, 2004). The present findings strongly suggest that Pg with type II *fimA* is a causative factor of phenytoin-induced GO, similar to its effect towards adult periodontitis.

In summary, we examined the relationships between bacterial occurrence and GO, as well as alterations of bacterial occurrence and amount associated with improvement of gingival conditions. The present results suggest that Td and Pg with type II *fimA* are causative agents of phenytoin-induced GO.

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