RESEARCH REPORTS
Clinical

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ABSTRACT

Chronic periodontitis is a silent infectious disease prevalent worldwide and affects lifestyle-related diseases. Therefore, efficient screening of patients is essential for general health. This study was performed to evaluate prospectively the diagnostic utility of a blood IgG antibody titer test against periodontal pathogens. Oral examination was performed, and IgG titers against periodontal pathogens were measured by ELISA in 1,387 individuals. The cut-off value of the IgG titer was determined in receiver operating characteristic curve analysis, and changes in periodontal clinical parameters and IgG titers by periodontal treatment were evaluated. The relationships between IgG titers and severity of periodontitis were analyzed. The best cut-off value of IgG titer against *Porphyromonas gingivalis* for screening periodontitis was 1.682. Both clinical parameters and IgG titers decreased significantly under periodontal treatment. IgG titers of periodontitis patients were significantly higher than those of healthy controls, especially in those with sites of probing pocket depth over 4 mm. Multiplied cut-off values were useful to select patients with severe periodontitis. A blood IgG antibody titer test for *Porphyromonas gingivalis* is useful to screen hitherto chronic periodontitis patients (ClinicalTrials.gov number NCT01658475).

KEY WORDS: periodontopathic bacteria, *Porphyromonas gingivalis*, fingertip blood, screening test, cut-off value, multicenter trials.

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Assessment of the Plasma/Serum IgG Test to Screen for Periodontitis

INTRODUCTION

Periodontitis is an infectious disease of the tissues surrounding the teeth and is a well-known silent infectious disease worldwide. It was reported that 50% of the population has bleeding gums, and the incidence rate of periodontitis is 35% in the USA (Albandar, 2002). According to recent studies, chronic periodontitis (persistent low-grade infection of periodontal pockets by Gram-negative bacteria) is associated with increased atherosclerosis, heart disease, diabetes mellitus, and other systemic diseases (Beck *et al.*, 2005; Michalowicz *et al.*, 2006; Tonetti *et al.*, 2007). Poor oral health may have a profound effect on general health. Therefore, efficient screening of periodontitis patients is essential for the maintenance of general health.

Generally, diagnosis of periodontitis is made by the examination of the periodontal condition, such as the prevalence of periodontal pockets, mobility of teeth, degree of tooth loss (Hefti, 1997), and behavioral factors such as smoking (Ryder, 2007). Since periodontitis is a polymicrobial infectious disease (Walker and Sedlacek, 2007), it is recognized that infection with periodontal bacteria leads to humoral immunological responses and elevates the serum IgG antibody levels against pathogens (Murayama *et al.*, 1988). Additionally, it has been reported that the serum IgG antibody titer against *Porphyromonas gingivalis* (*P. gingivalis*) decreased corresponding to the decrease in the bacterial count in periodontal pockets by periodontal treatment (Horibe *et al.*, 1995). Although the usefulness of the IgG antibody test for understanding periodontitis is recognized, this examination has not been widely adopted worldwide.

In this study, we first analyzed data from individuals with or without periodontitis, to evaluate the clinical usefulness of the blood IgG antibody titer test against periodontal pathogens for periodontitis screening, by determining the cut-off value of the titer using the receiver operating characteristic (ROC) curve. Second, the clinical usefulness of the IgG antibody test was evaluated in a nationwide clinical study on chronic periodontitis patients.

MATERIALS & METHODS

Study Design

An overview of the study is shown in Fig. 1. In cooperation with 11 university hospitals in Japan, 618 chronic periodontitis patients without systemic disease were registered between January 2007 and November 2009. From these, 536 patients (mean ages: 51.8 ± 13.9 yrs) were diagnosed according to the Guidelines of the American Academy of Periodontology (Krebs and Clem, 2006), and were enrolled in this study. At a company facility in Japan, 769 employees were

registered between September 2008 and November 2009. The oral conditions of 745 employees (mean age: 44.0 ± 9.1 yrs) were examined at work-related physical examinations, with the Community Periodontal Index (CPI; WHO, 1997). Of these, 629 employees with one of the following criteria (CPI of 1 to 4, gingivitis-positive, or over 40 yrs old) were excluded, and 116 employees were selected as the "Health" group (without periodontitis).

Pocket probing depth (PPD) was used as an index in oral examinations at university hospitals, while CPI was used at the company facility. Hence, it was not possible to unify the indices of periodontitis severity. To evaluate the effects of periodontal treatment, we examined periodontal conditions and IgG titers. The "Periodontitis" group was categorized by clinical parameters: ratio of gingival bleeding on probing (BOP) {the number of sites with BOP divided by the total number of sites *per* mouth (%): < 25, 25 – 50, > 50}; the score of periodontal lesions {the number of sites with periodontal lesions (PPD ≥ 4 mm) divided by the total number of sites *per* mouth (%): < 10, 10 – 30, > 30}. The severity of periodontitis was categorized into 2 groups by the presence or absence of sites of PPD over 4 mm, and the relation between periodontitis and blood IgG titer against *P. gingivalis* was analyzed with multiple cut-off values, which were set to 2×, 3×, 4×, and 5× on the basis of the cut-off value of IgG titer obtained in this study. Furthermore, 536 chronic periodontitis patients received periodontal treatment, including scaling and root planing (SRP), and instruction in proper home care techniques. Individual periodontal clinical parameters and IgG titers against periodontal pathogens for 312 chronic periodontitis patients were compared between 'before and after' periodontal treatment.

Informed consent was obtained from each participant, the protocol having been approved by the institutional review board at each institution.

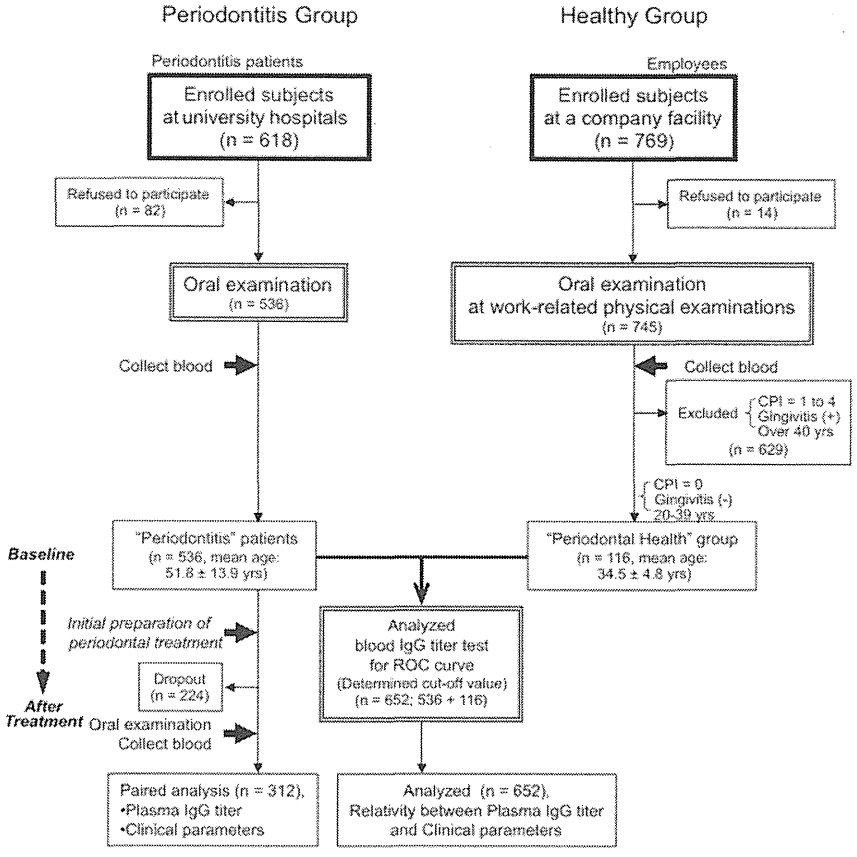
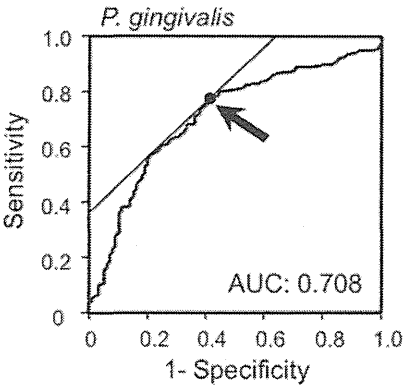


Figure 1. Flow chart of participant selection. Periodontal data were recorded by trained dental examiners at 11 university hospitals in Japan. The average number of periodontal lesions (PPD ≥ 4 mm), the score of BOP, and the score for mobile teeth were calculated for each patient. In contrast, in physical examinations at a company facility, the oral condition of employees was examined by assessment of CPI. Employees (CPI = 0, gingivitis-negative, and 20-39 yrs) were categorized as the "Periodontal Health" group for ROC curve analysis.



		Diagnostic test result		Total
		Periodontitis	Health	
plasma IgG Ab titer	>1.68	415	48	463
	<1.68	121	68	189
		536	116	652

Cut-off value	1.682
Sensitivity	0.774
Specificity	0.586

Figure 2. Setting cut-off value of the blood IgG antibody titer test against *P. gingivalis*. ROC curve of blood IgG antibody titer against *P. gingivalis* for the diagnosis of periodontitis. AUC, area under the curve.

Blood Sampling

In physical examinations at a company facility, serum samples were aliquoted and stored at -30°C. In university hospitals, the plasma from middle-finger fingertip capillary blood was obtained. From fingertip blood, a 50-μL quantity of whole blood was sampled, and device-treated plasma was obtained according to the procedures prescribed by Leisure, Inc. (Tokyo, Japan).

Measurement of IgG Titers against Periodontal Bacteria

Blood (serum or plasma) IgG antibody titers against periodontal pathogens were determined by Leisure with the enzyme-linked immunosorbent assay (ELISA) (Murayama *et al.*, 1988). As bacterial antigens, sonicated preparations of *P. gingivalis* FDC381, *Prevotella intermedia* (*P. intermedia*) ATCC25611, *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) ATCC29523, and *Eikenella corrodens* (*E. corrodens*) FDC1073 were used. The sera from 10 healthy participants without periodontitis (ages 20-29 yrs) were pooled and used to calibrate the analyses. With serial dilutions of this pooled control serum, standard titration curves were prepared. The absorbance of each sample after reaction was defined as an ELISA unit (EU), so that 100 EU corresponds to 1:3,200 dilution of the calibrator sample (Appendix Table 1). According to the formula for clinical use, the mean ± 2 SD of the controls, based on the reported dataset of IgG titers to individual pathogens among 10 healthy individuals, was defined as 1 of the standard value.

Statistical Analysis

Statistical analysis was performed with JMP 9 (SAS Institute Inc., Cary, NC, USA). Wilcoxon's signed-rank test was used to evaluate individual treatment effects. The differences in levels of IgG against periodontal pathogens among each group with severity were analyzed by the Kruskal-Wallis and Steel-Dwass tests. The cut-off value of IgG titer was obtained from the ROC curve. Diagnostic efficacy evaluation was calculated and represented as sensitivity and specificity. The relation between periodontitis and IgG titer was analyzed by the Cochran-Mantel-Haenszel χ^2 test, adjusted for age, with categorization by cut-off value.

RESULTS

Screening Power of the IgG Titer Test for Periodontal Disease

Chronic periodontitis patients (n = 536) and periodontally healthy individuals (n = 116) were evaluated. Using ROC curves, we found that the area under the curve (AUC) of the IgG titers against *P. gingivalis* was the largest among those of other periodontal pathogens (*P. gingivalis*, 0.708; *A. actinomycetemcomitans*, 0.601; *E. corrodens*, 0.583; *P. intermedia*, 0.525). Therefore, we focused on *P. gingivalis* for further analysis, based on the literature (Fischer *et al.*, 2003; Akobeng, 2007) (Fig. 2). The optimal cut-off value for the test against *P. gingivalis* was 1.682. The sensitivity was 0.774, and the specificity was 0.586 at this value. We focused on *P. gingivalis* and *A. actinomycetemcomitans* for further analysis according to their AUC significance.

Change of IgG Titer in Response to Treatment

We examined the change of plasma IgG titer against *P. gingivalis* and *A. actinomycetemcomitans* with the initial preparation of periodontal treatment (n = 312 after treatment). Corresponding to the improvement of clinical parameters (% of PPD ≥ 4 mm, average of PPD, % of BOP, and % of mobile teeth were decreased: Fig. 3A) after intensive initial preparation, the plasma IgG titers against periodontal pathogens also decreased significantly (Fig. 3B).

IgG Titer and Severity of Periodontitis

We further evaluated whether the plasma IgG titer reflects the severity of periodontitis by combining data obtained from chronic periodontitis patients (n = 536) and periodontally healthy individuals (n = 116). The variances between categories (% of BOP and % of PPD ≥ 4mm) were analyzed. There were significant differences in the IgG titers against *A. actinomycetemcomitans* and *P. gingivalis* between BOP and PPD categories (Figs. 3C, 3D). In chronic periodontitis patients, IgG antibody titer to *A. actinomycetemcomitans* was significantly higher than that of healthy control individuals, but there was no significant increase for severity. In contrast, the titer to *P. gingivalis* increased significantly corresponding to the severity of periodontitis.

IgG Titer against *P. gingivalis* in Individuals with and without Sites of PPD ≥ 4 mm

Finally, we evaluated the relationship between the presence of sites of PPD ≥ 4mm (Table) and cut-off values of IgG titer against *P. gingivalis* by combining data obtained from chronic periodontitis patients and periodontally healthy individuals. IgG titers larger than each cut-off value were defined as positive. Individuals with and without sites of PPD ≥ 4 mm were more frequently associated with positive groups of IgG titer against *P. gingivalis* ($P < 0.0001$). Individuals with sites of PPD ≥ 4 mm were at least 3.54 times more likely to be positive (crude odds ratio at titer 3.36) in the IgG antibody test for periodontitis compared with those without sites of PPD ≥ 4 mm. When the cut-off value was raised, the sensitivity decreased and the specificity increased. At a cut-off value of 6.72, the sensitivity was 0.493 and the specificity was 0.810. Conversely, when the cut-off value was lowered to 1.0, the sensitivity increased to 0.828 and the specificity decreased to 0.490 (Table).

DISCUSSION

A method for the measurement of blood IgG antibody levels to periodontal pathogens has been developed for the diagnosis of periodontitis. However, this test has not been popularized in general dentistry. The reasons for this are likely as follows: (1) lack of standardized values for evaluating periodontitis, (2) psychological pressure associated with the taking of blood for both dentists and patients, and (3) lack of clinical recognition of the benefits. In this study, we used a commercial device for painless self-collecting of fingertip plasma from chronic periodontitis patients, instead of regular venous blood.

Based on the results of IgG titer against *P. gingivalis* in each participant, the ROC curve was drawn to determine the cut-off value. The AUC was moderately accurate for predicting

periodontitis. The optimal cut-off value for the test against *P. gingivalis* was 1.682. The sensitivity was satisfactory, whereas the specificity was low. The AUC for others tested showed low accuracy (below 0.700), suggesting that *P. gingivalis* is suitable for periodontitis screening. The age of the healthy control population in this study was set at 20 to 39 yrs. Thus, similar investigations among healthy people older than 40 yrs would be interesting for comparison.

Next, we examined whether the severity of periodontitis can be determined by this test, with *P. gingivalis* and *A. actinomycetemcomitans* selected according to their AUC accuracy (over 0.600). Clinical attachment level (CAL) has been more frequently used than PPD to evaluate the effect of periodontal treatment. However, even if teeth have high CAL, their PPD may be less than 3 mm. IgG titer levels must be influenced by the size of the area of infection, not by the history of tissue destruction. Therefore, we preferred PPD to CAL as a measure of periodontal severity. As expected based on previous reports (Alexander *et al.*, 1996; Behle *et al.*, 2009), the IgG titers against both bacteria were significantly decreased by periodontal treatment, corresponding to improvement in periodontal condition. The results suggested that this test is useful for evaluating treatment effects from the perspective of infection levels, and the test would be useful as a self-evaluation system for the effects of periodontal treatment. Furthermore, we found that the titer to *P. gingivalis*, not to *A. actinomycetemcomitans*, increased significantly corresponding to the severity of periodontitis. It has been reported that the correlation between the number of periodontal pockets and the antibody levels to *P. gingivalis* was stronger than that for *A. actinomycetemcomitans* (Pussinen *et al.*, 2011). These results are reasonable, since chronic periodontitis is caused mainly by obligate anaerobic bacteria, *P. gingivalis*.

Finally, the relation between periodontitis and the IgG titer against *P. gingivalis* was analyzed with multiple cut-off values. No account was taken of smoking or sex during this study. There

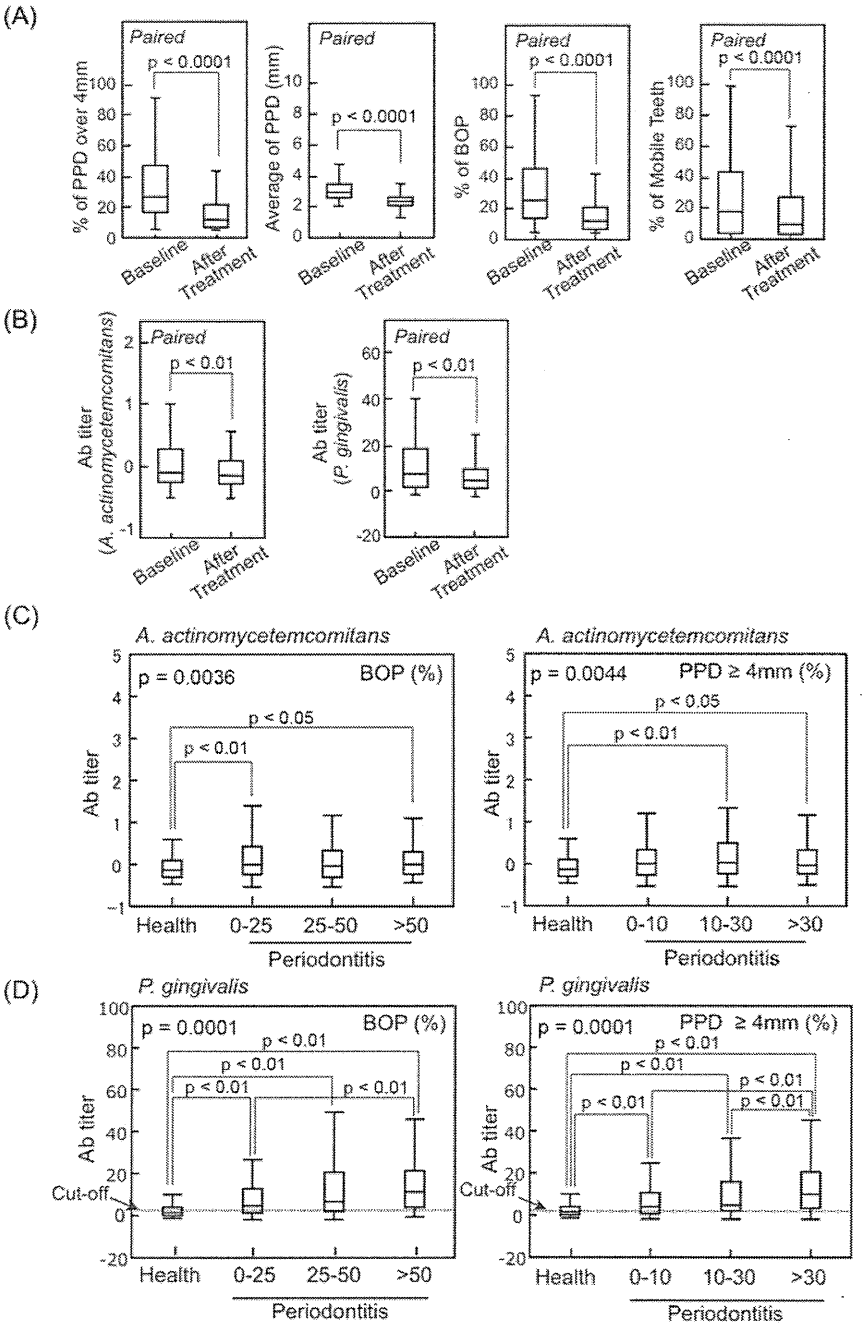


Figure 3. Blood IgG antibody levels reflecting periodontal treatment and severity of periodontitis. Individual periodontal clinical parameters **(A)** and plasma IgG antibody levels against periodontal pathogens **(B)** of 312 chronic periodontitis patients were compared between baseline and after intensive periodontal treatment. The periodontal clinical parameters are % of PPD ≥ 4 mm, average of PPD, % of BOP, and % of mobile teeth. The periodontal pathogens were *A. actinomycetemcomitans* and *P. gingivalis*. These data were analyzed by Wilcoxon's signed-rank test for paired samples. Box plot shows median, the lower and upper quartiles, and the minimum and maximum of all the data. Plasma IgG antibody levels against *A. actinomycetemcomitans* **(C)** and *P. gingivalis* **(D)** were compared among categorized groups (% of BOP, Healthy: n = 116, 0-25: n = 307, 25-50: n = 124, > 50: n = 105; % of PPD ≥ 4 mm, Healthy: n = 116, 0-10: n = 154, 10-30: n = 186, > 30: n = 196). These data were analyzed by the Kruskal-Wallis test for overall group differences and the Steel-Dwass test for between-group differences. Cut-off, IgG titer = 1.86 for *P. gingivalis*.

Table. Relation between Pocket Depth and Cut-off Value of Blood IgG Antibody Level against *P. gingivalis*

	Titer	1.00		1.68		3.36		5.04		6.72		8.40	
Cut-off value of IgG titer	n/p (%)	n 24	p 76	n 29	p 71	n 41	p 59	n 51	p 49	n 58	p 42	n 62	p 38
Sites of PPD ≥ 4 mm (n = 652)	Absence (%)	11	12	13	10	15	8	17	5	19	4	19	4
	Presence (%)	13	64	16	61	26	51	34	44	39	38	43	34
Statistical analysis	Sensitivity	0.828		0.790		0.659		0.560		0.493		0.440	
	Specificity	0.490		0.565		0.646		0.769		0.810		0.837	
	Crude OR	4.61		4.48		3.54		4.24		4.13		4.02	
	95%CI	3.10 – 6.86		3.30 – 7.21		2.41 – 5.20		2.78 – 6.46		2.64 – 6.46		2.51 – 6.44	
	p value	< 0.0001		< 0.0001		< 0.0001		< 0.0001		< 0.0001		< 0.0001	

The numbers of negative and positive (n/p) individuals were converted to percentages. Severity of periodontitis was categorized into 2 groups based on the presence or absence of sites of PPD ≥ 4 mm, and numbers of participants were converted to percentages. Total number of participants = 652. Because percentage was rounded off, sample number of "sites of PPD ≥ 4 mm" was different from a real number (n = 147). Statistical comparisons of the categorical variables were conducted by the Cochran-Mantel-Haenszel χ^2 test adjusted for age. Crude OR, Crude odds ratio; 95%CI, 95% confidence interval; *p < 0.0001.

were significantly more females than males in this study (Appendix Table 2). At the university hospitals, female outpatients were usually more common than males. Additionally, females may be more health-conscious than males. Nonetheless, analysis of our data indicated that the crude ORs were high, at more than 3.54. Notably, the more severe the periodontitis, the higher the crude OR. Some suggest that odds ratios greater than 4 in case-control studies provide strong support for causation (Grimes and Schulz, 2002). Accordingly, these results indicate the possibility that the IgG titer against *P. gingivalis* is associated with periodontal severity. From investigation of adults aged over 40 yrs in parsimonious models including demographic data, smoking, and diagnosed diabetes, Dye *et al.* (2009) showed that high IgG titers to *P. gingivalis* were most strongly associated with periodontitis across all definitions (OR, 2.07 to 2.74) in unadjusted models. Future investigation controlling these factors may provide greater insights into this relationship.

Screening the chronic periodontitis patients with the various cut-off titers of this test may be able to detect those patients highly sensitized with *P. gingivalis*. This offers a clear advantage in selecting periodontitis patients at risk for lifestyle-related diseases associated with periodontitis. In contrast, there is a disadvantage in filtering out periodontitis patients less sensitized with *P. gingivalis* but with clinical symptoms. They may refrain from visiting the dental clinic, and therefore the periodontitis would remain untreated. To prevent this, it is important for screening tests to increase the sensitivity by reducing the cut-off value, assuming a decrease in specificity. Consequently, we consider that a cut-off value of 1.0 is the most suitable for screening. IgG titer reflects the existence of host immune-response against pathogens, thus showing the history of infection. Since response is delayed by the infection, sometimes the pathogen itself is not detected. There is a report describing the presence of *P. gingivalis* as the strongest determinant of the systemic antibody response to these pathogens, and contending that the extent of periodontitis has, at most, a modest modifying effect (Pussinen *et al.*, 2011). The practical use of this IgG titer test may lead to early detection of chronic periodontitis. In addition, caution must be taken for periodontal patients with unusual immune-responses, such as aggressive forms of periodontitis. Further studies are needed for expanded indications.

In conclusion, the results of this study supported the blood IgG antibody titer test as useful for the screening of latent periodontitis patients with high IgG titers to periodontal pathogens without subjective symptoms (Ohyama *et al.*, 2001), and for the prognostication of periodontitis recurrence in individuals without symptoms during supportive periodontal therapy (Sugi *et al.*, 2011). In addition, chronic low-grade inflammation is believed to have an effect on long-term health and on chronic lifestyle-related diseases. Thus, it is important to establish a test system for efficient evaluation of periodontal infection for physicians. It is possible to send plasma samples to a clinical laboratory by regular mail or courier. Therefore, it would be convenient for users if an online system for ordering the kit and retrieving test results could be established. This system facilitates periodontal examination for people with no time to visit a dental clinic.

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RESEARCH REPORTS
Biological

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Smad2 Decelerates Re-epithelialization during Gingival Wound Healing

ABSTRACT

During periodontal regeneration, inhibition of gingival downgrowth is necessary to promote migration of mesenchymal cells into the defects. Transforming growth factor (TGF)-β is a pleiotropic cytokine that has numerous cell functions, including regulation of epithelial growth. Recent studies have shown that Smad2, a downstream transcription factor of TGF-β, plays crucial roles in wound healing in the epithelia. Therefore, we investigated the effects of Smad2 overexpression on re-epithelialization of gingival wounds. Transgenic mice overexpressing *smad2* driven by the *keratin 14* promoter (*k14-smad2*) were confirmed to have significant Smad2 phosphorylation in gingival basal epithelia. Punch wounds were made in the palatal gingiva, and wound healing was assessed histologically for 7 days. Re-epithelialization was significantly retarded on day 2, while collagen deposition was enhanced on day 7 in *k14-smad2* compared with wild-type mice. Moreover, expression of keratin 16 (K16), an indicator of keratinocyte migration, was significantly inhibited in wound-edge keratinocytes in *k14-smad2*. The inhibition of K16 coincided with the induction of Smad2 in the corresponding epithelia, while BrdU incorporation was unaffected. These results indicated that Smad2 has inhibitory effects in regulating keratinocyte migration during gingival wound healing. TGF-β/Smad2 signaling mediating alteration of K16 expression must be tightly regulated during periodontal regeneration.

KEY WORDS: Smad2, gingival downgrowth, wound healing, re-epithelialization, keratinocyte migration, keratin 16.

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INTRODUCTION

Periodontal regeneration can be achieved through synergistic interactions of different cell populations of ectodermal and mesenchymal origin. Because of differences in cell proliferation rates of each periodontal component during wound healing, it is necessary to temporarily prevent gingival epithelial downgrowth along the root surface, allowing the intrabony defects to be repopulated by mesenchymal cells (Wikesjö and Selvig, 1999; Cho and Garant, 2000). The guided tissue regeneration (GTR) protocol (Nyman *et al.*, 1982) is the only periodontal regenerative treatment approach that aims to physically prevent epithelial downgrowth, but it is problematic because of susceptibility to infection (Tempro and Nalbandian, 1993; Bratthall *et al.*, 1998). Thus, other methods should be explored. Here, we developed a strategy to improve periodontal wound healing by exogenous biological molecules that can prevent gingival downgrowth.

Epithelial wound healing involves coordination between migration and proliferation of keratinocytes, extracellular matrix deposition, and re-epithelialization (Clark, 1996). Re-epithelialization begins with keratinocyte migration, which occurs in a series of sequential steps: adhesion formation by integrins at the leading edge, translocation of the cell body, adhesion disassembly at the cell rear by regulators of adhesion turnover, such as focal adhesion kinase (FAK), and rear retraction (Ridley *et al.*, 2003). Moreover, when injury occurs, strong induction of keratin 16 (K16) and K6 occurs in post-mitotic cells at the wound edge, which is a crucial event that precedes the onset of keratinocyte migration into the wound site (Paladini *et al.*, 1996). Although K16 is not present in the normal epidermis, it is constitutively expressed in some other stratified epithelia, including the palate, gingiva, and junctional epithelium (Presland and Dale, 2000). Thus, K16 appears to be involved in alteration of the adhesion molecules at the wound edge, affecting keratinocyte migration (Freedberg *et al.*, 2001).

Transforming growth factor (TGF)-β1 is important in multiple aspects of wound healing, including epithelial growth and collagen deposition (Werner and Grose, 2003). Signal transduction from TGF-β1 depends on phosphorylation of Smad2 and Smad3 proteins, which then translocate to the nucleus to initiate transcription of target genes (Heldin *et al.*, 1997; Massagué *et al.*, 2000). Although each Smad is phosphorylated directly by the TGF-β type I receptor, all may have distinct roles in the cellular and tissue responses to wounding (Massagué, 1999). *Smad2*-knockout mice are embryonic-lethal (Nomura and Li, 1998), while *smad3*-knockout mice die from immune defects after birth (Yang *et al.*, 1999). Importantly, loss of *smad3* markedly enhances the rate of wound re-epithelialization (Ashcroft *et al.*, 1999; Jinno *et al.*, 2009), whereas the role of Smad2 has not been fully explored.

Previously, transgenic mice overexpressing *smad2* driven by the *keratin 14* promoter were established (Ito *et al.*, 2001). These mice decelerate cutaneous wound healing because of impaired epithelial migration (Hosokawa *et al.*, 2005). Thus, we hypothesized that Smad2 is a feasible candidate molecule for inhibition of epithelial downgrowth. Although healing of both oral and cutaneous wounds proceeds through the same sequential phases, oral wounds are distinguished by rapid healing and reduced scar formation compared with cutaneous wounds (Szpaderska *et al.*, 2003). Moreover, gingival keratinocytes migrate directly through the fibrin clot, which is different from cutaneous keratinocytes (Hakkinen *et al.*, 2000). Thus, it is necessary to investigate the effects of Smad2 in gingival wounds for possible clinical applications. Here, we investigated gingival re-epithelialization from a histological and molecular biological viewpoint, with emphasis on the relevance of keratinocyte migration.

MATERIALS & METHODS

Mice and Genotyping

Ten-week-old transgenic mice expressing *smad2* by *k14* promoter (*k14-smad2*: TG) and their wild-type littermates (WT) were used for the experiments. Genotyping was performed as described previously (Ito *et al.*, 2001). All comparisons were carried out double-blind on at least 3 samples *per* genotype, *per* time-point; *n* is the number of mice used for each experiment. This study was conducted in accordance with the Guidelines for the Treatment of Experimental Animals and was approved by the Animal Research Control Committee of Okayama University (#OKU-2007189).

Wound-healing Model

Full-thickness wounds were made in the palatal gingiva near the first molar by means of a 1-mm biopsy punch (BIOPSY PUNCH®; Kai Industries, Tokyo, Japan). The wounds were assessed for 7 days post-wounding ($n = 3$ from each wound time-point for both genotypes). The wounds were photographed by stereomicroscopy (Stemi200CS; Carl Zeiss, Oberkochen, Germany). Quantitative wound-closure measurement was performed histologically as described previously (Graves *et al.*, 2001; Jinno *et al.*, 2009; Martinez-Ferrer *et al.*, 2010), with minor modifications. Briefly, the specimens were fixed in 4% paraformaldehyde-PBS for paraffin embedding. Cross-sections, measuring 5 μm, were taken from the wound gap, and an area measuring approximately 200 μm in the central portion was stained with H&E to determine the wound centers, visualized by microscopy (BX50, DP70; Olympus, Tokyo, Japan) at 40 × magnification. The wound width was measured with DP Controller (Ver.1.2.1.108) (Olympus). The re-epithelialization rates were calculated as the distance of epithelial migration divided by the distance between original wound margins, visible where the fibrous connective tissues and basal keratinocytes were disrupted.

Every 5 sections at the central portion of each wound were subjected to assessment of the organization and the amounts of collagen and non-collagenous proteins, with the use of the Sirius Red/Fast Green Collagen Staining Kit (Chondrex, Inc., Redmond, WA, USA) as *per* the manufacturer's instructions. To quantify collagen deposition in the specimens with exemption of bone and tooth, we analyzed microscopic images of the staining sections using ImageJ software as documented (http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html).

Western Blotting Analysis

Wound tissues containing the punched area were collected on various days post-wounding ($n = 5$ from each wound time-point for both genotypes). Homogenized samples were used for blotting as described previously (Yamamoto *et al.*, 2003). The membranes were blocked for 1 hr with 5% skimmed milk (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in TBS and then incubated at 4°C for 12 hrs with antibodies against Smad2 (#610842, 1:1,000 dilution; BD Transduction Laboratories, San Diego, CA, USA), phospho-Smad2 (P-Smad2) (#3101, 1:1,000 dilution; Cell Signaling Technology, Beverly, MA, USA), TGF-β1 (#MAB240, 1:1,000 dilution; R&D Systems, Minneapolis, MN, USA), integrin α3 (#V76720, 1:250 dilution; BD Transduction Laboratories), focal adhesion kinase (FAK) (#3285, 1:1,000 dilution; Cell Signaling Technology), or keratin16 (K16) (#LS-C50087, 1:5,000 dilution; Lifespan Biosciences, Seattle, WA, USA), and GAPDH (#MAB374, 1:1,000 dilution; Chemicon International, Temecula, CA, USA) as a loading control. Immunoreactive proteins were detected with Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The signal intensities were quantified by ImageJ.

Immunofluorescence

Sections were blocked for 1 hr with 10% goat serum (Invitrogen, Carlsbad, CA, USA) in PBS, and then incubated at room temperature for 12 hrs with 1:100 dilution of antibodies against Smad2, P-Smad2, or K16 (as described above) followed by addition of either Alexa Fluor® 488 or Alexa Fluor® 594-labeled secondary antibody (Invitrogen). Nuclear staining was performed with DAPI (49,6-diamidino-2-phenylindole). Staining signals were visualized by fluorescence microscopy (BX50, DP70; Olympus). Staining was performed on 3 separate samples *per* genotype, *per* time-point. Non-specific staining was determined with the use of an appropriate isotype control antibody (Cell Signaling Technology) at the same concentration.

Cell Proliferation Assay

Mice were injected intraperitoneally with BrdU (5-bromo-2′-deoxyuridine: Sigma-Aldrich, St. Louis, MO, USA) solution (100 μg *per* g body weight) every 12 hrs, and sacrificed 1 hr after the last injection ($n = 3$ from both genotypes). BrdU incorporation was detected on paraffin sections with the use of a Zymed® BrdU Staining Kit (Invitrogen) according to the manufacturer's instructions. Twenty randomly selected sections from the center of each wound were used for the analysis. The number of BrdU-labeled cells was determined from wound-edge keratinocytes at 100 × and 200 × magnification.

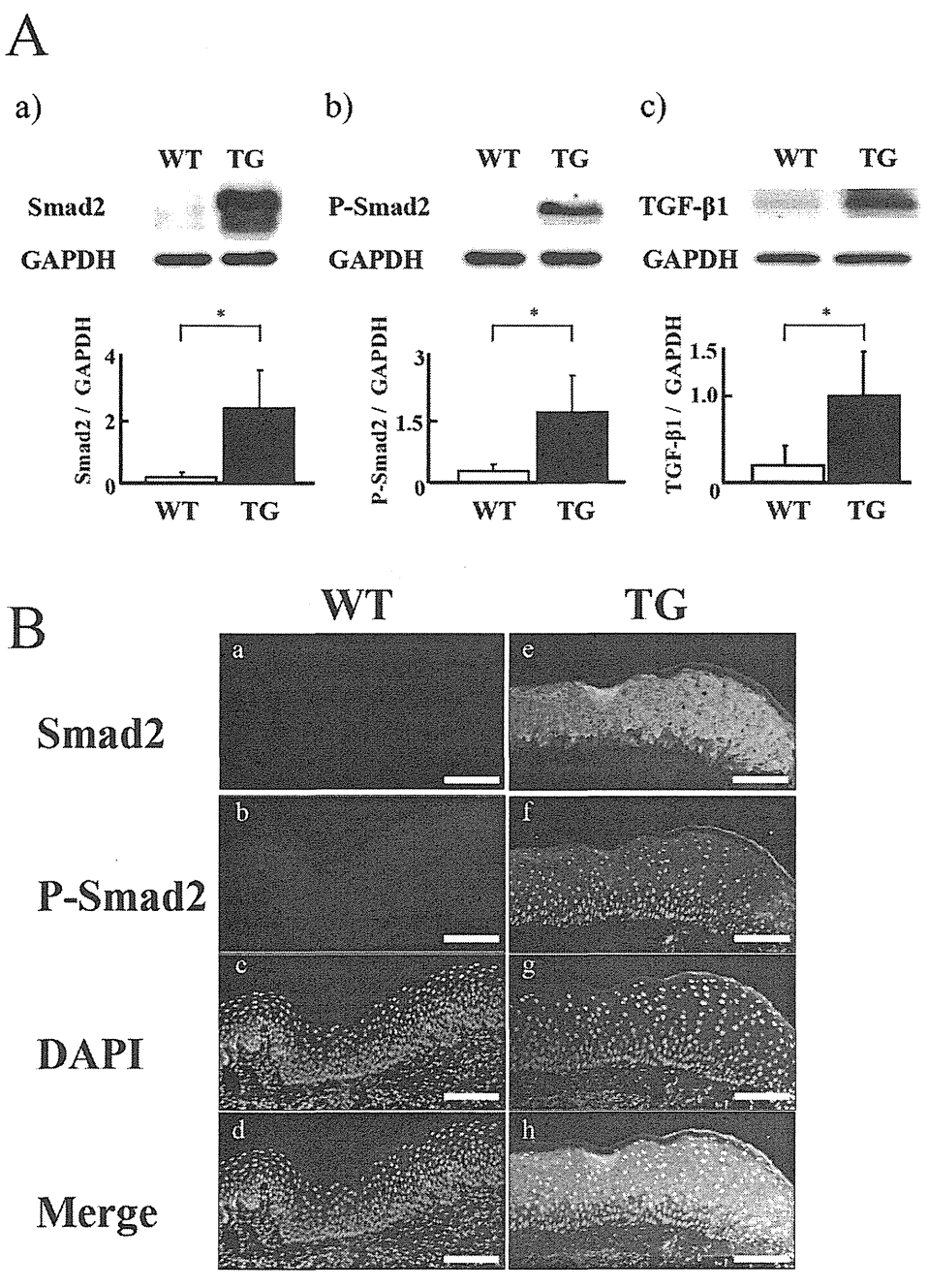

Figure 1. TGF-β/Smad2 signaling is elevated in *k14-smad2* transgenic gingiva. Intact palatal gingiva from *k14-smad2* transgenic mice (TG) and wild-type littermates (WT) were subjected to the following assays: **(A)** Western blotting analysis of protein extracts from WT and TG mice for Smad2 (a), P-Smad2 (b), and TGF-β1 (c). Bar charts represent densitometry results, means ± SD, $n = 5$ independent experiments. GAPDH was used as a loading control. *$p < 0.01$. Open bars, WT; solid bars, TG. **(B)** Immunofluorescence analysis of palatal gingiva from WT and TG mice for Smad2 (a, e), P-Smad2 (b, f), and DAPI (c, g). Overlay of the 3 images is shown (merge: d, h) with co-localization shown in yellow. The signals of both Smad2 and P-Smad2 were more intense in TG mice than in WT mice. Scale bar: 100 μm.

Statistical Analysis

The data were presented as means ± SD and analyzed for statistically significant differences by ANOVA/Scheffé's (for assessment of re-epithelialization rates) or two-tailed paired Student's *t* test. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

RESULTS

Induction of TGF-β/Smad2 Signaling in Smad2-overexpressing Gingiva

To verify the effects of *smad2* overexpression on TGF-β/Smad2 signaling, we performed Western blotting with intact gingiva. The levels of Smad2 and P-Smad2 were 5- to 8-fold higher in TG than in WT mice (Figs. 1Aa, 1Ab). Moreover, TG mice had a significant increase in TGF-β1 compared with WT mice (Fig. 1Ac). Immunofluorescence analysis indicated that Smad2 was expressed throughout all layers of epithelia except the basal layer (Fig. 1Be), while P-Smad2 was highly expressed in the basal layer (Fig. 1Bf). In WT, Smad2 immunofluorescence was virtually undetectable, and there was a non-specific background of staining only for P-Smad2 in WT (Figs. 1Ba, 1Bb, Appendix Fig. 1).

Retarded Wound Re-epithelialization in Smad2-overexpressing Gingiva

To investigate the biological function of Smad2 in regulating epithelial wound healing, we examined the extent of wound closure. The wound area was visually larger in TG mice than in WT mice on day 2 (Figs. 2Ab, 2Af); however, TG and WT wounds became similar after day 3 (Figs. 2Ac, 2Ad, 2Ag, 2Ah). Histological examination demonstrated that, on day 2, Smad2 overexpression clearly decelerated gingival wound healing, and TG wounds were 18 % re-epithelialized ($p < 0.05$), compared with 54 % in WT (Figs. 2B, 2C). Collagen fiber was absent below the migrating keratinocytes in both TG and WT wound sites on day 2 (Appendix Fig. 3). Interestingly, on day 3, the wound healing in TG mice caught up to that in WT mice; however, epithelial thickness was thinner in TG mice than in WT mice (Appendix Fig. 2). On day 7, epidermal architecture in

TG mice was similar to that in WT mice; however, discontinuity of collagen was more frequently observed in the center area of WT wounds than in that of TG (Fig. 2D). Although the wound was completely re-epithelialized, the wound bed could be distinguished by the presence of a thin collagen layer and disrupted stratum corneum and basal stratum in TG mice (Fig. 2D). The first molar could also be an anatomic landmark for the punched region (left side of Fig. 2D). Quantification of collagen deposition showed that the intensity was significantly higher in TG mice than in WT mice (Fig. 2E).

Effects of Smad2 Overexpression on Proliferation in Gingival Epithelial Cells during Re-epithelialization

To examine the direct role of proliferation in the decelerated wound closure and re-epithelialization by Smad2 overexpression, we analyzed the incorporation of BrdU in the wound-edge keratinocytes on day 2. Keratinocyte proliferation was increased away from the wound edge in the basal layer cells (Figs. 3Aa, 3Ab). However, the incorporation of BrdU was uncommon in the epithelial edges of the wound in both WT and TG mice (Figs. 3Ac, 3Ad). We counted the number of BrdU-positive cells to determine the effect of Smad2 on wound-edge keratinocytes, and found no significant difference between genotypes on day 2 (Fig. 3B).

Reduced Expression of Keratin16 in Smad2-overexpressing Gingiva

To address the molecular mechanism of retarded re-epithelialization regulated by Smad2 overexpression, we examined the expression of cell migration molecules integrin α3, FAK, and K16 on day 2. Densitometric analysis indicated that K16 levels were

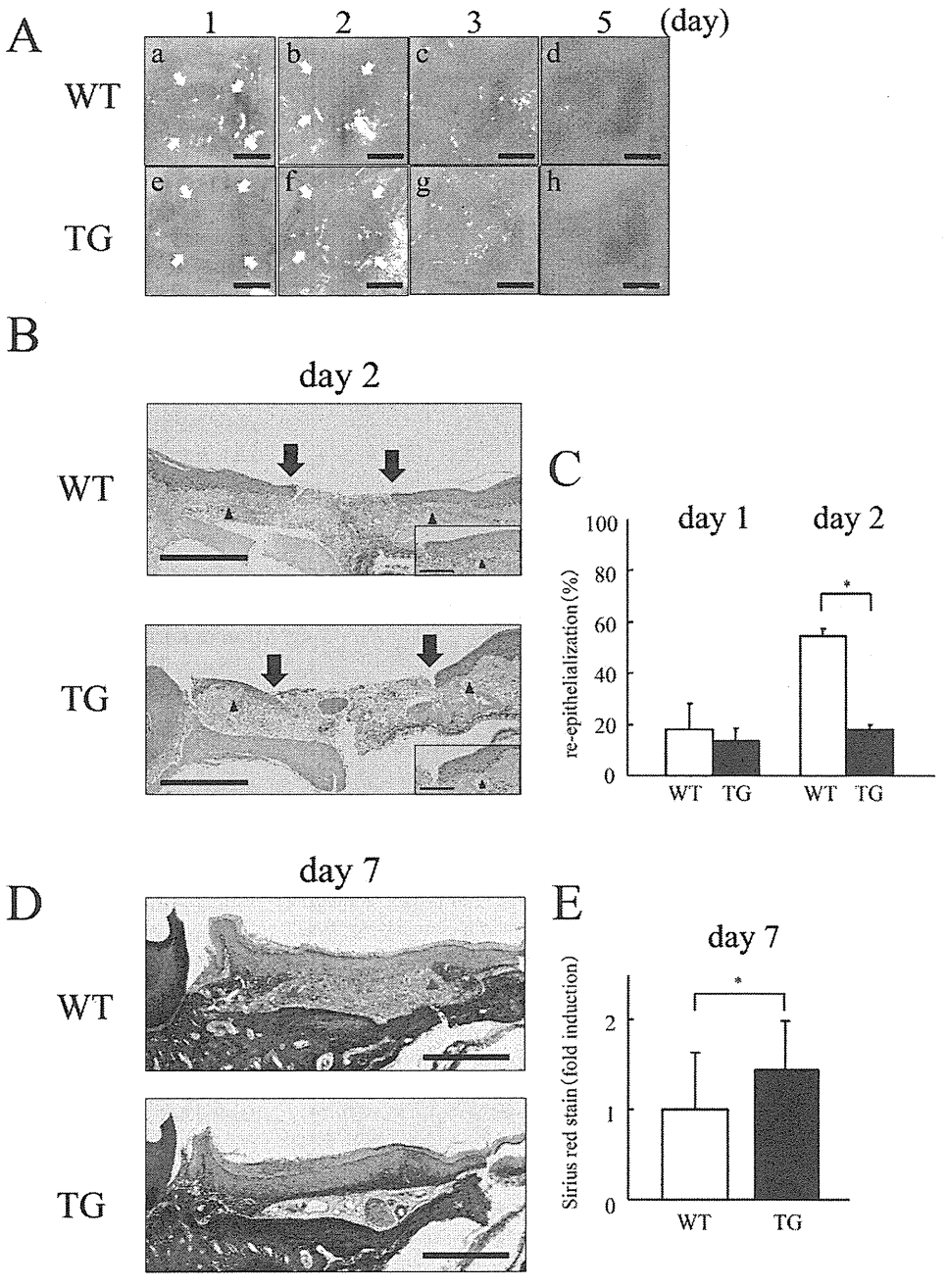

Figure 2. Re-epithelialization is decelerated in *k14-smad2* transgenic gingiva. **(A)** Full-thickness wounds were made in the palatal gingiva near the first molar by means of a 1-mm biopsy punch. Stereophotographs of wounded gingiva of wild-type (WT) (a-d) and *k14-smad2* transgenic (TG) (e-h) mice on day 1 (a, e), day 2 (b, f), day 3 (c, g), and day 5 (d, h) post-wounding. Arrows indicate the leading edges of the wound. Scale bar: 500 μm. **(B)** Sections of wounded gingiva from WT and TG mice were stained with H&E for examination of re-epithelialization on day 2. Arrows represent the leading edges of the wound. The insets show high magnification of original wound margins (arrowheads), where the fibrous connective tissues and basal keratinocytes were disrupted. Scale bar: 500 μm for low magnification; 20 μm for high magnification. **(C)** Quantification of wound closure (re-epithelialization rates) on day 1 and day 2 post-wounding. Results represent the means ± SD (n = 3) for each time-point and genotype. *$p < 0.01$. **(D)** Collagenous and non-collagenous content was visualized by Sirius Red and Fast Green staining in sections of wounded gingiva from WT and TG mice on day 7. The images indicate corresponding areas in the wound bed where the differences between the genotypes can best be appreciated. WT mice frequently had discontinuity of collagen in the centers of wounds (blue arrowheads), while TG mice had thin collagen fibers beginning to coalesce in the wound bed, covered with immature stratum corneum and basal stratum. Scale bar: 500 μm. **(E)** Quantitation of collagen (Sirius Red) staining by digital image analysis on day 7. Relative intensity was shown by fold induction to WT mice (= 1.0) on the y axis. Results represent the means ± SD (n = 3) for each time-point and genotype. *$p < 0.05$.

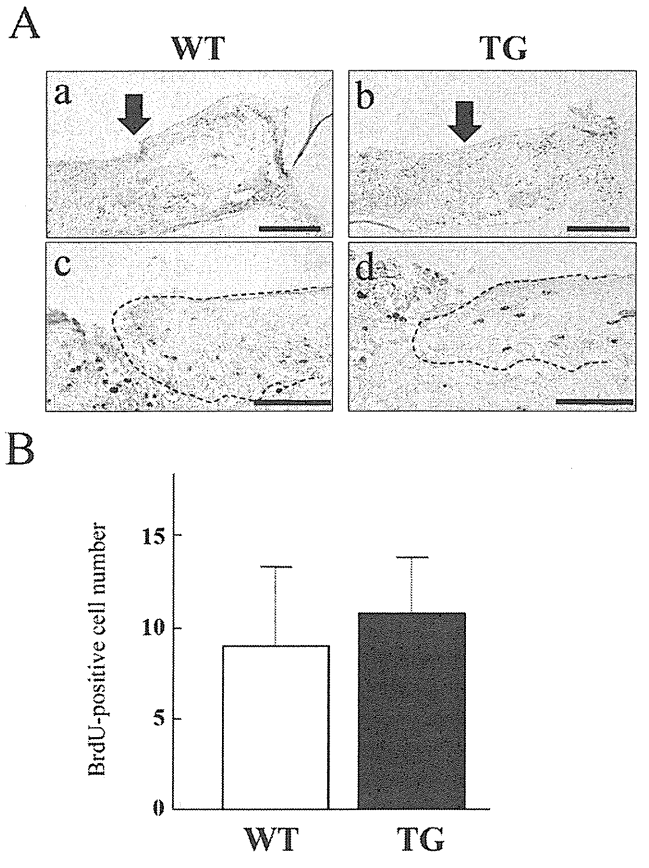

Figure 3. BrdU incorporation into wounded gingiva. **(A)** Mice were injected intraperitoneally with BrdU every 12 hrs. The brown staining represents the incorporation of BrdU in sections containing the epithelial wound edges from wild-type (WT) (a, c) and *k14-smad2* transgenic (TG) (b, d) mice on day 2. Arrows indicate the leading edges of the wound, and the wound area is on the left. Lower panels (c, d) indicate the wound-edge keratinocytes. Scale bar in a, b = 100 μm; in c, d = 50 μm. **(B)** Quantification of BrdU-positive cells in wound-edge keratinocytes on day 2 post-wounding. Typical images for quantification (Ac, Ad). Results represent the means ± SD (n = 3) for each genotype. Open bars, WT; solid bars, TG. There was no significant difference in the number of positive cells between WT and TG mice.

significantly lower in TG than in WT mice, whereas FAK and integrin α3 levels were similar (Fig. 4A). Immunofluorescence analysis showed that gingiva constitutively expressed K16 in the spinous cell layer, including the leading edges of the wound. The intensity of K16 in either wound or intact gingiva was significantly decreased in TG compared with that in WT mice. When compared between intact and wound sites, wound tissues had significantly lower intensity than intact tissues in TG mice, while sites in WT mice were similar to each other (Fig. 4B). Moreover, to determine the correlation with Smad2 overexpression, we examined the co-localization of Smad2 and K16 on day 2. Both molecules were mostly co-localized in the wound edges; however, TG mice showed reduced K16 expression in the corresponding location with enhanced Smad2 expression compared with WT mice (Figs. 4Ca, 4Cb, 4Ce, 4Cf).

DISCUSSION

During wound healing after periodontal regenerative therapy, it is critical to inhibit epithelial migration along the root surface in the early phase, and thereafter re-epithelialization must be completed as rapidly as possible to re-establish tissue integrity. In this study, re-epithelialization was significantly decelerated in TG compared with WT mice until day 2 (Figs. 2B, 2C); however, it became histologically similar between WT and TG mice after day 3 (Appendix Fig. 2). Analysis of these data indicated that Smad2 signaling effectively inhibits re-epithelialization only in the early stage after wounding, and subsequently there must be a compensatory signal that accelerates re-epithelialization. TG mice showed significantly high levels of TGF-β1 in addition to Smad2 and P-Smad2 expression in the gingiva (Fig. 1A), suggesting that there may be an autocrine loop of TGF-β1 ligand secretion (Van Obberghen-Schilling *et al.*, 1988) enhanced by Smad2 overexpression. Continuous activation of TGF-β receptors has been reported to lead to proteasome-mediated elimination of activated Smad2 (Lo and Massagué, 1999). The self-elimination signal may contribute to recuperation in normal wound healing in the late stage after wounding.

Since TGF-β signaling has been implicated in collagen deposition, Sirius Red analysis was performed (Figs. 2D, 2E). Although TG mice had a well-developed collagen compared with WT mice on day 7, there was no significant abnormality in collagen quality and quantity in TG mice. While TGF-β signaling is implicated in several aspects of fibrosis (Derynck and Akhurst, 2007), longitudinal wound analysis is needed for the assessment of gingival repair architecture. The acquisition of collagen maturation and decelerated re-epithelialization in TG mice reinforced the concept that TGF-β/Smad2 signaling is mitogenic for fibroblasts, but anti-proliferative for epithelial cells during wound healing. Keratinocyte migration precedes the proliferation process in open wounds, such as on day 2 in this study. Although individual processes are overlapping across the early phases of wound healing, it can be excluded that cell proliferation has a predominant role in decelerated re-epithelialization in TG mice, since there was no significant difference in BrdU incorporation between WT and TG mice on day 2 (Fig. 3); the decelerated re-epithelialization was mostly due to impaired gingival migration by Smad2 overexpression.

In response to epidermal injury, keratinocytes express a specific set of keratin proteins, distinct from the keratins in the healthy epidermis (Tomic-Canic *et al.*, 1998). Our results indicated that intact TG mice had a significantly lower K16 level than intact WT mice. Moreover, K16 in wounded TG mice was decreased compared with that in intact TG mice (Fig. 4). Since TGF-β1; is up-regulated during gingival wound healing (Hakkinen *et al.*, 2000), and also in TG mice (Fig. 1A), keratinocyte-specific overexpression of TGF-β1/Smad2 signaling may suppress K16 expression, resulting in decelerated re-epithelialization. It will be intriguing to investigate the physiological role of constitutively expressed K16 in normal gingiva, and the precise mechanism for down-regulation of K16 by Smad2 signaling during re-epithelialization.

In summary, our study clearly demonstrated that overexpression of Smad2 decelerates re-epithelialization in the early stage

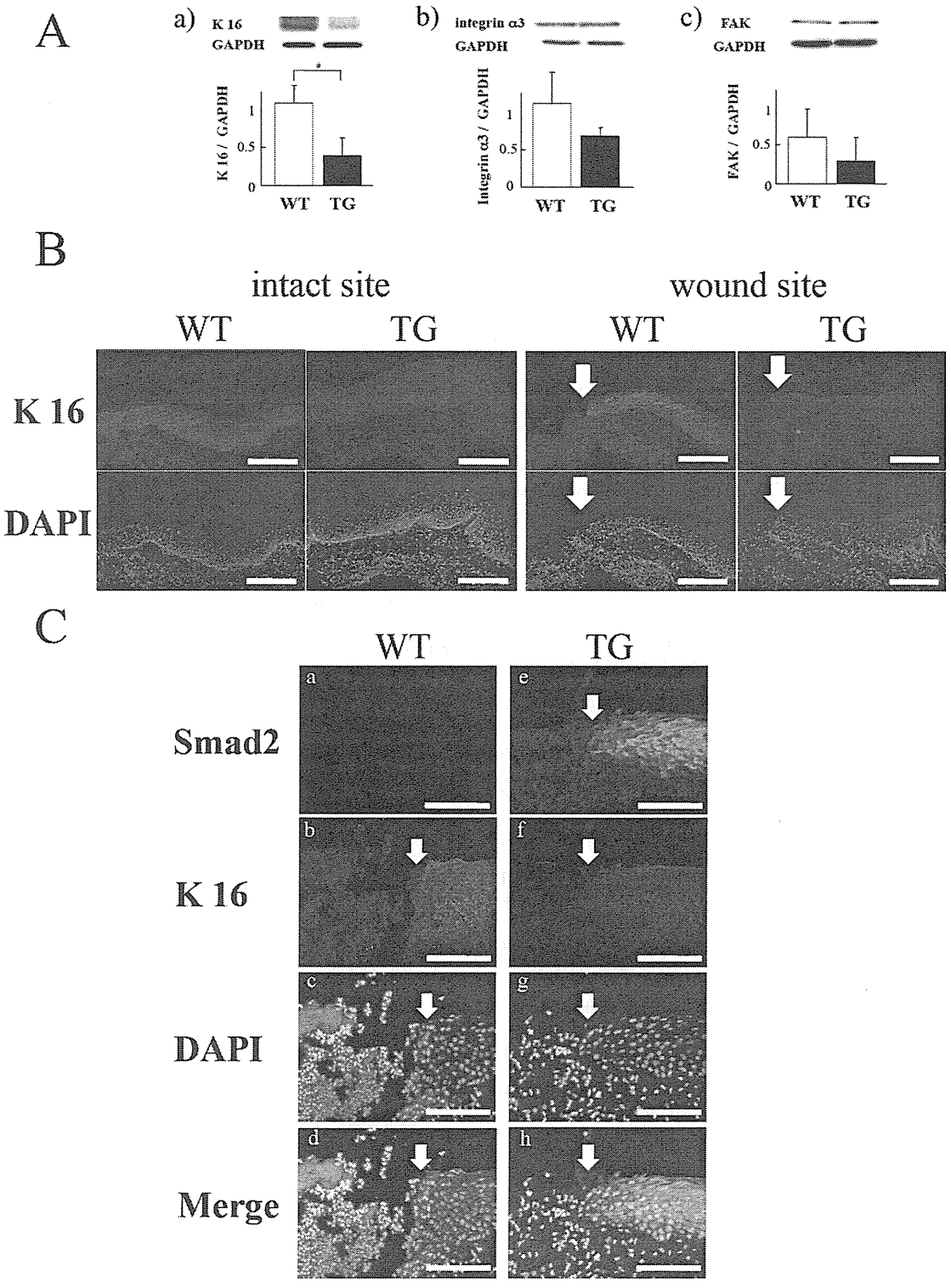

Figure 4. K16 protein levels were down-regulated in *k14-smad2* transgenic wounds. **(A)** Wounded palatal gingivae containing punched areas were collected on day 2 post-wounding. Western blotting analysis of protein extracts from wild-type (WT) and *k14-smad2* transgenic (TG) mice for K16 (a), integrin α3 (b), and FAK (c). Densitometry results were normalized relative to the expression of GAPDH (= 1.0), means ± SD, $n = 3$ independent experiments. *$p < 0.01$. Open bars, WT; solid bars, TG. **(B)** Immunofluorescence analysis of intact and wounded palatal gingiva on day 2 from WT and TG mice for K16 and DAPI. Arrows represent the leading edges of the wound. The signal for K16 was more intense in WT than in TG mice, and wound TG had significantly lower intensity than intact TG. Scale bar: 20 μm. **(C)** Double-immunofluorescence analysis of wounded palatal gingiva from WT and TG mice on day 2 for Smad2 (a, e), K16 (b, f), and DAPI (c, g). Overlay of the 3 images is shown (merge: d, h), with co-localization shown in yellow. Arrows represent the leading edges of the wound, and the wound area is on the left. TG mice showed enhanced Smad2 expression and reduced K16 expression compared with that in WT mice. Scale bar: 20 μm.

after wounding. The reduced expression of K16 may re-organize cytoskeletal components, affecting keratinocyte migration. We demonstrate that gingival downgrowth can be prevented by exogenous molecules *in vivo*. Since the inhibitory effect on gingival migration by overexpression of Smad2 is transient and does not affect tissue integrity, further investigation of the detailed mechanism, and manipulation of the duration and extent, of Smad2 signaling may be useful for molecular control of gingival downgrowth, leading to successful periodontal regeneration.

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CASE REPORT

Histological and immunohistochemical features of gingival enlargement in a patient with AML

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Abstract Here, we discuss the pathophysiology of leukemia-associated gingival enlargement based on a case of acute myelomonocytic leukemia (AML-M4) with typical gingival enlargement. Uniquely, this patient was well enough to allow full periodontal examination and incisional gingival biopsy to be performed both before and after chemotherapy. The patient was a 39-year-old Japanese woman with AML-M4 showing gingival enlargement. Histological and immunohistochemical features of gingiva and bacterial counts in the periodontal pockets were examined before and after chemotherapy. The results were as follows: (1) infiltration of myelomonocytic blasts in enlarged gingiva; (2) resolution of gingival enlargement with complete remission of AML by anticancer chemotherapy; and (3) the numbers of bacteria in the periodontal pockets were not high and were not altered before or after chemotherapy. In patients with AML-M4, remarkable mucosal enlargement is not generally observed in the body except in the gingiva. We hypothesized that antigens derived from periodontal bacteria, even if they are not present in large numbers, could act as chemoattractants for myelomonocytic leukemic cells.

Keywords Gingival enlargement · Acute myelomonocytic leukemia · Pathogenesis · Histology · Immunohistochemistry

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Introduction

Gingival enlargement is an extramedullary clinical manifestation of myeloblastic leukemia [1–3]. Among the various types of acute leukemia, gingival enlargement is particularly prevalent in French/American/British (FAB) classification of acute myelomonocytic leukemia (AML-M4) and acute monocytic leukemia (AML-M5) subtypes [1]. Dreizen et al. evaluated 1,076 leukemic patients and found gingival involvement in 66.7% of M5 patients and 18.5% of M4 patients [4].

Remarkable mucosal enlargement is not generally observed in the body except in the gingival tissues. This may be explained by the characteristic and unique anatomy of the periodontal tissues, which are continuously exposed to periodontal bacteria. However, in the majority of cases it is not possible to fully understand the pathology of leukemia-associated gingival enlargement because patients

are often so severely medically compromised and associated thrombocytopenia causes difficulty in hemostasis. Thus, it is difficult to perform detailed periodontal examination, including incisional biopsy and subsequent histological examination of tissue.

In our hospital, effective collaboration has been established between hematologists and periodontists. All of the leukemia patients admitted to our hospital are referred to the Department of Periodontology from the Department of Hematology. In daily clinical work, we encountered a typical case of AML-M4 with gingival enlargement. This patient was well enough to allow detailed periodontal examination and incisional gingival biopsy to be performed.

Here, we report the details of the gingival condition in this patient, and discuss the possible pathophysiology of gingival enlargement in leukemia patients.

Table 1 Laboratory data of the patient's blood at the first oral examination

	Patient	Normal range
White blood cell counts	19,590/μL	3,500–8,500/μL
Differential		
Segmented cell (mature neutrophil)	9.5%	29.0–70.0%
Stab cell (immature neutrophil)	1.5%	0.0–13.0%
Lymphocyte	13.0%	20.0–52.0%
Monocyte	36.0%	0.0–13.0%
Eosinophil	0.0%	0.0–11.0%
Basophil	0.0%	0.0–2.0%
Blast	36.5%	
Platelets	336,000/μL	150,000–350,000/μL
PT	69%	80–120%
APTT	33.8 s	25.0–36.0 s
CRP	1.76 mg/dL	0.0–0.3 mg/dL

Case description

The patient was a 39-year-old Japanese woman who had visited a local hospital because of high fever. Antibiotic treatment did not improve her general condition and leukemia was suspected based on the results of blood examination. Eight days after the initial visit to the local hospital, she was referred to the Department of Hematology, Okayama University Hospital, and a diagnosis of AML-M4 was made. On admission, she was referred to the Department of Periodontics and Endodontics for oral examination. Aside from her leukemia, her medical history was unremarkable.

The first oral examination was performed just prior to induction chemotherapy. The appearance of the gingival tissues is shown in the figure demonstrating marked gingival enlargement. Periodontal pockets with probing depths ranging from 4 to 6 mm as a result of hypertrophy were observed around the interdental gingiva; no periodontal bone resorption was observed by full-mouth dental X-ray radiographic examination.

The patient's blood data are shown in the Table 1. On the basis of these results it was considered safe to perform a biopsy of the patient's gingivae. We wished to determine whether the gingival enlargement was caused by inflammation or was the result of leukemic infiltration. Furthermore, a biopsy was indicated to exclude other gingival diseases that can cause gingival enlargement. An incisional biopsy sample was obtained from the interdental gingival region between 23 and 24.

Histological examination of hematoxylin and eosin (H&E)-stained sections showed diffuse infiltration of blasts. These cells had convoluted round or ovoid nuclei and abundant eosinophilic cytoplasm, and showed morphological features of myelomonocytes (Fig. 1). AML-M4 is defined as an acute leukemia with differentiation along both myeloid and monocytic lines. Therefore, we performed immunohistochemical analysis for the myeloid marker myeloperoxidase (MPO) (polyclonal rabbit anti-human antibody; 1:800 dilution; DAKO #A0398) and the monocytic marker CD68 (monoclonal mouse anti-human antibody; clone number: KP1; dilution 1:200; DAKO #M0814) (Fig. 1).

Immunohistochemical staining was positive in blast cells and showed strong cytoplasmic positivity for MPO, indicating infiltration of the gingival tissue with myeloid cells. CD68-positive cells were also observed in the gingival tissue, indicating infiltration of monocytic cells. Generally, normal macrophages and histiocytes are positive for CD68, but their numbers were elevated. Local pathological diagnosis was extramedullary infiltration of acute myeloid leukemia, while a diagnosis of AML-M4 was made based on corroborating bone marrow biopsy observations.

Bacterial counts in the periodontal pockets in three regions (buccomedial of 21, buccomedial of 37, and palatomedial of 17) were examined. The methods used for isolation and detection of total periodontal bacteria by real-time PCR have been described previously [5]. A total bacterial count of about 10 [3–6] was detected. Our group reported a total bacterial count of about 10 [4–7] in patients with severe periodontitis by the same method [6]. The numbers of bacteria in the periodontal pockets were not high in this case.

After recovery from neutropenia induced by initial chemotherapy (idarubicin, 12 mg/m^2; cytarabine, 100 mg/m^2), the gingival enlargement resolved (Fig. 1) although no

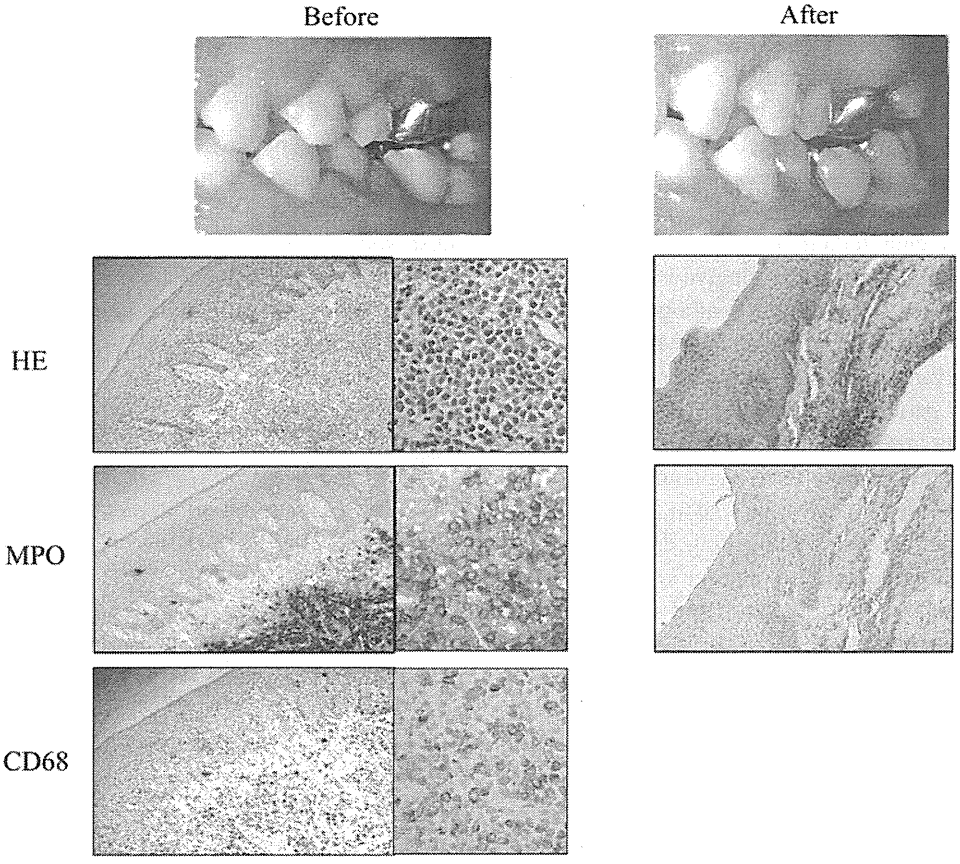

Fig. 1 Clinical and microscopic appearance at the interdental gingiva between maxillary left canine and first premolar before and after induction chemotherapy. *HE* Hematoxylin & eosin staining, *MPO* immunohistochemical staining for myeloperoxidase, *CD68* immunohistochemical staining for CD68. All immunohistochemistry was performed with a diaminobenzidine (DAB) chromogen and hematoxylin counterstaining. Before commencement of induction chemotherapy, gingival hyperplasia was remarkable. The histopathological findings on H&E staining showed many blasts with high N/C ratios in the lamina propria. The same region was strongly stained with MPO, indicating the presence of many myeloid cells. CD68 staining showed that the cells were monocytic in nature. Just after induction chemotherapy, gingival hyperplasia disappeared. A second biopsy of the same area showed that blasts with high N/C ratio disappeared from the lamina propria on H&E examination and immunohistochemistry showed numbers of MPO-positive cells were also markedly decreased. Thus, repeat immunohistochemistry for CD68 was not performed

periodontal treatment was performed. Periodontal pocket depth was within the healthy range (<3.0 mm).

A second gingival incisional biopsy was performed to evaluate the effects of chemotherapy on the gingiva. In this biopsy specimen, blast cells were not identified concomitant with the resolution of gingival enlargement (Fig. 1). MPO-positive cell numbers were also markedly decreased (Fig. 1). Bone marrow biopsy also showed complete remission.

Antibiotics were used for about 1 week (cefozopran) before the first examination, and no antibiotics were used within 2 weeks before the other examinations. The total bacterial counts showed no marked changes within the range of 10 [4, 5].

This patient received hematopoietic cell transplantation following a total of three cycles of chemotherapy. Bone marrow biopsy continued to show complete remission. Gingival enlargement as observed at the initial visit has not been seen after the initial round of chemotherapy.

Discussion

The main findings in this case were infiltration of myelomonocytic blasts in hypertrophied gingival tissue, resolution of gingival enlargement with complete remission of AML by anticancer chemotherapy, and the numbers of bacteria in the periodontal pockets were not high and showed no changes before or after chemotherapy.

These observations provide some interesting insights into the pathobiology of AML-associated gingival enlargement. The gingival changes observed in this patient were caused mainly by the infiltration and accumulation of myelomonocytic leukemia cells into the gingival connective tissue. In

AML-M4 and M5 subtypes, the leukemic cells are monocytic. Generally, monocytes have the ability to infiltrate tissues with strong chemoattractant ability. We suspect that original periodontitis was mild, but low-level antigens derived from periodontal bacteria acted as chemoattractants for myelomonocytic leukemic cells. Indeed, remarkable mucosal enlargement is not generally observed except in the gingiva. Gingival enlargement in leukemia may involve accumulation of blast cells in the gingiva by chemoattractants derived from periodontal pathogens. Gingival enlargement, which is often observed in AML-M4 and M5, could be more severe in patients with severe periodontitis. AML-M4 or M5 patients may suffer severe periodontitis, which would result in severe gingival swelling and bleeding, and thus prevent the patients from maintaining good oral hygiene. Poor oral hygiene may then lead to further gingival swelling and bleeding, thus establishing a vicious cycle. Further studies are required to determine the relations between clinical status, oral hygiene, and the severity of gingival swelling and bleeding.

In addition, further studies in larger numbers of cases as well as studies of specific bacterial antigens and receptors on AML cells are required. Some AML-M4 and M5 patients do not show gingival enlargement. The blasts in some patients may show deficiency of the ability of some receptors. The endothelium of gingival vessels and possibly antigen presenting cells, such as Langerhans cells, are also factors worthy of consideration.

The differential diagnosis for gingival hypertrophy should be considered and may include drug-influenced gingival enlargements and hereditary gingival fibromatosis, which are described in the classification system for periodontal diseases and conditions [7]. Based on the patient's responses to the initial questionnaire, these were not considered relevant in this case. Histological examination completely excluded these diagnoses, indicating that the gingival enlargement was caused by remarkable leukemia cell infiltration. Furthermore, the total bacterial count within the periodontal pockets was not high in this case. Based on these results, we could consider anticancer chemotherapy should be the matter of the highest priority.

As the oral cavity may be the primary site revealing clinical findings in leukemia patients, physicians and dentists should be aware of these potential changes and consider leukemia as a diagnosis along with more common inflammatory causes of gingival enlargement. Dentists should be aware that the main treatment for gingival enlargement in leukemia patients without remission of leukemic cells could in fact be anticancer chemotherapy by hematologists, rather than local periodontal treatment.

In conclusion, gingival enlargement observed in a patient with acute myelomonocytic leukemia was caused by remarkable infiltration of myelomonocytic blasts (leukemia cells) into the gingival connective tissue. The characteristic anatomy of periodontal lesions, which are continuously exposed to periodontal bacteria, may explain why remarkable mucosal enlargement is not generally observed in the body except in the gingiva. Antigens derived from periodontal bacteria, even if not present in large numbers, could act as chemoattractants for myelomonocytic leukemic cells.

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Conflict of interest We have no conflicts of interest to report.

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Relationship between Periodontitis-Related Antibody and Frequent Exacerbations in Chronic Obstructive Pulmonary Disease

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Abstract

Background: To identify patients with chronic obstructive pulmonary disease (COPD) who are susceptible to frequent exacerbations is important. Although periodontitis aggravated by poor oral hygiene might increase the risk of lower respiratory tract infection, the relationship between periodontitis and COPD exacerbations remains unknown. This prospective cohort study investigates the relationship between periodontitis-related antibody and exacerbation frequency over a one-year period.

Methods: We assessed an IgG antibody titer against *Porphyromonas gingivalis,* which is a major pathogen of periodontitis, and then prospectively followed up 93 individuals over one year to detect exacerbations.

Results: The numbers of exacerbations and the rate of individuals with frequent exacerbations (at least two per year) were significantly lower in patients with higher IgG titer than those with normal IgG titer (0.8 vs. 1.2 per year, $p = 0.045$ and 14.3 vs. 38.6%, $p = 0.009$, respectively). Multivariate logistic regression analysis showed that being normal-IgG titer for periodontitis-related antibody significantly increased the risk of frequent exacerbations (relative risk, 5.27, 95% confidence interval, 1.30–25.7; $p = 0.019$) after adjusting for other possible confounders, such as a history of exacerbations in the past year, disease severity, COPD medication and smoking status.

Conclusions: Normal-IgG titer for periodontitis-related antibody can be an independent predictor of frequent exacerbations. Measuring periodontitis-related antibody titers might be useful to identify patients with susceptibility to frequent exacerbations so that an aggressive prevention strategy can be designed.

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Introduction

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death worldwide, and it is associated with an increasing economic cost and social burden [1]. The natural history of COPD is punctuated by exacerbations, which consist of acute episodes of worsening symptoms that might warrant changes in regular medications. These exacerbations negatively impact lung function, health-related quality of life, prognosis and socioeconomic burden [1]. Thus, exploring predictors of exacerbations and identifying patients with susceptibility to frequent exacerbations are important to design an efficient preventive strategy.

Factors associated with exacerbation include disease severity [1], a history of exacerbations [2], smoking [3], chronic inflammation [4], bacterial colonization [5] and gastro-esophageal reflux disease [6]. In addition, we have reported an association between an impaired swallowing reflex and bacterial colonization, systemic inflammation and an increased risk of exacerbations [7]. Since an impaired swallowing reflex might cause the aspiration of oral bacteria leading to lower respiratory tract infection, and poor oral hygiene itself is involved in the risk of aspiration pneumonia [8–10], we speculated that poor oral hygiene would increase the frequency of exacerbations.

Periodontitis is a common oral infectious disease that is associated with poor oral hygiene among the general population. It is characterized by inflammation of the periodontium induced

by subgingival plaque bacteria such as anaerobic gram-negative rods [11], that can also be associated with COPD exacerbation [12]. Chronic marginal periodontitis is more prevalent among patients with severe COPD than in other equally severe respiratory diseases [13] and the prevalence of periodontitis increases together with COPD severity [14]. In addition, serum antibody to *Porphyromonas gingivalis* (*P. gingivalis*), which is a frequently isolated pathogen, can be involved in systemic diseases such as cardiovascular disease [15–17]. However, the relationship between periodontitis and COPD exacerbation remains unclear.

We postulated that periodontitis is associated with COPD exacerbations. This prospective cohort study investigated the impact of baseline antibody titers for periodontal antigen (an index of periodontitis) on COPD exacerbation frequency for over one year. We also investigated the relationship between elevated-IgG titer for periodontitis-related antibody and inflammatory cytokines.

Methods

Ethics Statement

The study was approved by the ethics committee of Kyoto University (approval No. E182), and written informed consent was obtained from all participants.

Protocol and Study Participants

We recruited 109 patients with COPD from an outpatient clinic at Kyoto University Hospital, Japan, between September 2006 and August 2008 for this study. All patients provided written informed consent to participate. Blood and induced sputum samples were collected under stable conditions (as defined below) at entry for subsequent assay. We excluded 16 patients based on the following criteria: female, Brinkman index <10 pack-years, respiratory diseases other than COPD, daily intake of systemic corticosteroids and complicated with malignant diseases within 5 years. Thus, 93 patients were prospectively followed up for over one year to detect exacerbations.

Exacerbation Criteria

Exacerbations and stable periods were prospectively identified using diary cards as in our previous study [6,7]. We adopted a modified version of the East London cohort study criteria to define COPD exacerbations [6] based on an increase in any two "major" symptoms (dyspnea, sputum purulence and sputum quantity) or an increase in one "major" and one "minor" symptom (wheeze, sore throat, cough and nasal congestion/discharge) for at least two consecutive days [18]. Stable condition was defined as an exacerbation-free interval of >4 weeks [6].

Clinical Examinations

Pulmonary function tests (Chestac-65V; Chest MI Corp.; Tokyo, Japan) were performed after inhaling short-acting bronchodilators (salbutamol and ipratropium) at entry into the study. Lung volumes and diffusion capacity were measured using helium dilation and the single-breath method, respectively. The British Medical Research Council dyspnea scale (MRC) and Charlson Commorbidities index were also assessed.

Venous blood at entry was collected on the entry day during the stable period and stored at −80°C. Serum levels of immunoglobulin G (IgG) against *P. gingivalis* were measured using an enzyme-linked immunosorbent assay (ELISA) based on validated method [15,19,20]. Since the FDC381 and Su63 strains of *P. gingivalis* are serologically different, we separately measured antibody titers of *Pg*FDC381 and *Pg*Su63. We defined the cut-off point as the mean +2 SD of the controls based on the reported dataset of IgG titers to *Pg*FDC381 and *Pg*SU63 among 10 control individuals [20]. We divided COPD patients into two groups: one is patients whose antibody titers were higher than mean +2SD (High-IgG titer group), and the other includes those whose titers were lower than mean +2SD (Normal-IgG titer group).

Serum C-reactive protein (CRP) and γ-globulin were measured using the High Sensitivity CRP assay (Behring Diagnostics, Westwood, MA, USA), and cellulose acetate electrophoresis, respectively. We assayed 27 cytokines (PDGF, IL-1b, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9,IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, FGF, G-CSF, GM-CSF, IFN-γ, IP-10,MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α and VEGF) in 62 patients using a multiplex bead-based immunoassay kit (Bioplex, Bio-Rad Laboratories, Life Science Research Group, Hercules, CA, USA).

Statistical Analysis

Data were statistically analyzed using the Mann-Whitney U-test and the χ^2 test with JMP 8.0 software (SAS Campus Drive, Cary, NC, USA) and all results are presented as medians and 25th-75th percentiles. Independent predictors of frequent exacerbations (defined as at least two exacerbations per year) were detected using multivariate logistic regression analysis. All p-values are two-sided and $p<0.05$ was considered significant.

Results

Baseline Characteristics of Study Patients

Table 1 shows the baseline characteristics of the 93 patients and the prevalence of elevated IgG antibody titer against *P. gingivalis*. The distribution of standardized IgG values were 1.29 (−0.05~3.75) for PgFDC 381 and 0.06 (−0.47~0.76) for Pg SU63. The rates of high IgG titer against *Pg*FDC381 and *Pg*Su63 were 52.7% and 23.7%, respectively. Table 2 shows that age, smoking status, smoking index, body mass index, forced expiratory volume in 1 second (FEV_1), Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage, and MRC did not differ between High-IgG titer group and Normal-IgG titer group. Comorbidities assessed by Charlson Comrbidity index [21] were similar between these two groups. The frequency of using inhaled corticosteroid (ICS) was lower and serum γ-globulin levels were higher in High-IgG titer group than in Normal-IgG titer group, whereas the frequency of using tiotropium and long-acting β2 agonists (LABA), and of serum levels of CRP did not differ (Table 3). None of the patients received salmeterol/fluticasone propionate combination therapy.

Relationship between Elevated IgG Antibody Titer against *P. gingivalis* and Exacerbation Frequency

We hypothesized that more periodontitis lead to more exacerbations, but in contrast to our hypothesis, exacerbations were less frequent in High-IgG titer group than in Normal-IgG titer group (0.8 vs. 1.2 per year, $p = 0.045$, Figure 1 and Table 4). Moreover, the rate of patients who experienced frequent exacerbations (at least two per year) was also lower in High-IgG titer group than in Normal-IgG titer group (14.3% vs. 38.6%, $p = 0.009$, Table 4).

Multivariate Analysis to Detect Independent Predictors of Frequent Exacerbation

We performed multivariate logistic regression analysis to determine whether being normal IgG antibody titer against *P. gingivalis* could be an independent risk factor for frequent

Table 1. Patients' baseline characteristics (n = 93).

Age (y)	73 (45–88)
Body mass index (kg/m^2)	21.7 (14.3–30.5)
Smoking index (pack-years)	57 (20–220)
FEV_1 (% predicted)	55.0 (19.8–107.1)
GOLD stage, I/II/III/IV	12/46/30/5
High-IgG titer, n (%)	
*Pg*FDC381	49 (52.7)
*Pg*Su63	22 (23.7)

FEV_1, forced expiratory volume in one second;
GOLD, Global Initiative for Chronic Obstructive Lung Disease;
"High-IgG titer" includes subjects whose titers against *Porphyromonas gingivalis* (*Pg*FDC381 and *Pg*Su63) are above mean+2SD of healthy subsets [20].
Pg, *Porphyromonas gingivalis*. Data are expressed as median (25th–75th percentiles).
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exacerbations (Table 5). This analysis included the occurrence of frequent exacerbations as a dependent categorical variable and higher IgG antibody titer against *P. gingivalis*, age, smoking status, body mass index, γ-globulin, CRP, use of ICS, tiotropium and LABA, FEV_1(%predicted) and a history of exacerbations in the year before entry as independent variables. We found that a history of exacerbation in the previous year and normal IgG antibody titer against *P. gingivalis* significantly increased the occurrence of exacerbations (Relative Risk [RR] 4.43, 95% confidence interval [95%CI] 1.20–19.6, $p = 0.025$; RR 5.27, 95%CI 1.30–25.7, $p = 0.019$, respectively).

Relationships between Elevated IgG Antibody Titer against *P. gingivalis* and Serum Inflammatory Cytokines: Subanalysis of Immune Status among 62 Patients

Tables S1 and S2 show the results of a comparison of 27 cytokines in 62 patients placed in groups that were higher-IgG titer or normal-IgG titer against *P. gingivalis* according to the multiplex bead-based immunoassay. Levels of serum IL-4 and IL-7 were significantly higher in Normal-IgG titer group than in High-IgG titer group, whereas the other cytokines did not significantly differ between them.

Discussion

We demonstrated that the frequency of exacerbations was higher among patients with COPD whose antibody titer against *P. gingivalis* was normal compared with those whose antibody titer against *P. gingivalis* was higher. Multivariate logistic regression analysis showed that being normal-IgG titer against *P. gingivalis* was independently associated with frequent exacerbations during the following year after adjustment for established exacerbation-related factors such as a history of exacerbation [2], FEV_1 [22,23] and the use of COPD medications [24,25]. These findings contradicted our hypothesis that periodontitis is a risk factor for COPD exacerbation. Nevertheless, the present findings are important because, to our knowledge, this is the first study to demonstrate a relationship between antibodies and COPD exacerbation, and to indicate the value of *P. gingivalis*-related antibodies as a predictor of exacerbation. There were no significant differences between High-IgG titer group and Normal-IgG titer group in age, FEV_1, GOLD stage, and exacerbation frequencies at the baseline characteristics.

It has been shown that a history of COPD exacerbations is a good predictor for the future exacerbation [2] and our result suggests that the possible mechanism for the frequent exacerbation, thus it seems apparently inconsistent that the frequencies of COPD exacerbation in the previous year are similar in the two groups in the baseline characteristics. However, it is also reported that considerable part of patients who experienced exacerbation in the previous year did not experience COPD exacerbation in the following year [2]. Moreover, we could not specify the timing that the antibody titers increased or decreased. It is possible that the severity of periodontitis, antibody titers or immune status might be different in the previous year and the actual prospective observational period. Further investigation is needed to verify these speculations.

Periodontitis is a disease that afflicts dentate people, so we checked only in cases with teeth. In the dentate patients group,

Table 2. Comparison of baseline characteristics between patients with normal and higher IgG titer against *Porphyromonas gingivalis*.

	Normal-IgG titer (n = 44)	High-IgG titer (n = 49)	*p* value
Age (y)	74 (65–78)	73 (66–78)	0.83
Smoking status (Former : Current)	37:7	43:6	0.77
Smoking index (pack-years)	60 (47–102)	53 (40–80)	0.07
Body mass index (kg/m^2)	21.3 (19.5–23.3)	22.0 (20.1–23.5)	0.46
FEV_1 (% predicted)	54.4 (39.5–66.7)	56.5 (42.4–72.4)	0.39
GOLD stage (I/II/III/IV)	5/23/13/3	7/23/17/2	0.94
Numbers of patients with frequent exacerbations in the previous year, n (%)	22 (50.0)	24 (49.0)	1.00
MRC	1 (1–2)	1 (1–2)	0.86
Charlson comorbidity index	2 (1–3)	2 (1–3)	0.76

High-IgG titer group includes subjects whose titers against *Porphyromonas gingivalis* (*Pg*FDC381 and/or *Pg*Su63) are above mean+2SD of healthy subsets [20].
"Frequent exacerbations" are defined as ≥2 exacerbations per year.
FEV_1, forced expiratory volume in one second; GOLD, Global Initiative for Chronic Obstructive Lung Disease.; MRC, British Medical Research Council. Data are expressed as medians (25th–75th percentiles).
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