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Specific-wavelength visible light irradiation inhibits bacterial growth of *Porphyromonas gingivalis*

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Background and Objective: The effects of laser irradiation on *Porphyromonas gingivalis* have been reported, but the results are still controversial regarding the efficiency because of the differences of the light sources and irradiation conditions. The aim of this study was to determine the wavelength and irradiation conditions under which the most effective inhibitory effect on *P. gingivalis* growth was seen without any photosensitizers.

Material and Methods: Using an Okazaki large spectrograph, monochromatic light spectra ranging from 400 to 700 nm were evaluated to determine which spectra effectively inhibited bacterial growth. Moreover, using a monochromatic 405-nm irradiating device, the effects of various irradiating conditions on *P. gingivalis* growth were examined.

Results: Growth of bacteria irradiated at 400 nm and 410 nm was significantly suppressed compared with a nonirradiated control, whereas wavelengths of 430 nm and longer produced no significant inhibition. A constant energy density of 15 J/cm^2 was found to be enough to show an inhibitory effect. Significant inhibition of bacterial growth was found after only 1 min at 50 mW/cm^2 irradiation.

Conclusion: These results indicate that *P. gingivalis* growth is specifically suppressed by 405-nm light irradiation, suggesting that visible blue light irradiation is a promising means for eradicating periodontopathogenic bacteria from periodontal lesions.

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Periodontal disease is very common in the adult population, and the most common form is chronic marginal periodontitis (1,2). It is widely recognized that periodontitis is caused by a mixed bacterial infection and that some specific gram-negative bacteria play crucial roles in the etiology of adult periodontitis (3–5). Although many virulence factors of periodontopathogenic bacteria have been identified in the last two decades, the main clinical means for infection control still depend on conventional mechanical debridement (i.e. scaling and root planing) and/or antibacterial agents. Sbordone *et al.* have reported the limitations of conventional mechanical debridement to restructure the subgingival microflora (3).

Photodynamic therapy using a photosensitizer was originally developed to target tumor cells (6). A large number of microorganisms have also been targeted by photodynamic therapy in a number of studies (7–9). Among them, *Propionibacterium acnes* has been reported to be susceptible to photosensitization through targeting of its endogenously produced porphyrins (10). *Porphyromonas gingivalis* is one of the black-pigmented anaerobes implicated as a pathogen associated

with the initiation and progression of periodontitis. *P. gingivalis* is also known to produce protoporphyrin by degrading haemoglobin for its growth (11), which could make this bacterium photosensitive by acting as an endogenous photosensitizer. In fact, some studies have reported the effects of laser irradiation towards periodontopathogenic bacteria, including *P. gingivalis*, but these reports are still controversial as to the efficiency of the laser irradiation because of the differences of the light sources and irradiation conditions (12).

In this study, we attempted to determine the most effective wavelength for inhibiting *P. gingivalis* growth, even in the absence of an exogenous photosensitizer, using an Okazaki large spectrograph, which can produce monochromatic light over a broad range of wavelengths. Using a monochromatic 405-nm light-emitting device, we also attempted to clarify irradiation conditions under which the most effective inhibition of bacterial growth was found. Besides, we tried to clarify that the bacterial growth inhibition was attributed to bacteriostatic and/or bactericidal activities of irradiation.

Material and methods

Microorganisms and culture

The bacterial strain used in this study was *P. gingivalis* ATCC 33277. The bacterium was maintained by weekly subculture in anaerobic basal medium containing 5% sheep blood. Bacteria were grown for $\approx$24 h to early stationary phase in anaerobic brain–heart infusion broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% yeast extract, 0.05% L-cysteine, 0.0005% hemin, 0.0001% vitamin $K_{1\ and}$ 0.025% resazulin at 37°C in an anaerobic cabinet with an atmosphere of 80% N_2, 10% CO_2 and 10% H_2. To prepare bacterial colony samples for the light-exposure experiment using the large spectrogragh, 1 μL of 24-h bacterial culture was applied to trypticase soy agar (Becton Dickinson, Sparks, MD, USA) supplemented with 0.1% yeast extract, 0.0005% hemin, 0.0001% vitamin $K_{1\ and}$ 20% hemolyzed sheep blood, and cultured at 37°C for 30 h anaerobically until small colonies became visible.

Light exposure using an Okazaki large spectrograph

To determine the most effective wavelength for bacterial growth inhibition, bacterial colonies were exposed to monochromatic light using an Okazaki large spectrograph at the National Institute for Basic Biology of Japan (13). The wavelengths examined were every 10–20 nm from 400 to 700 nm. The fluence rate of the light was measured by a power meter at the position of each sample to fix the actinic effect of the different wavelengths. Total energy density was fixed to 18 J/cm^2 by adjusting the exposure time.

Irradiation at a wavelength of 405 nm

The light-emitting device equipped with monochromatic wavelength of 405 nm was developed by Ushio Inc. (Tokyo, Japan). A 24-h bacterial culture grown in the supplemented brain–heart infusion broth was diluted 1 : 100, and 200 μL of suspension was applied to each well of 96-well culture plates. After light exposure, the plates were incubated in an anaerobic cabinet, and the optical density at 655 nm of each well, which reflects bacterial growth, was measured at intervals of 12 or 24 h. The percentage inhibition reported was calculated from the 36-h bacterial growth data. Irradiation conditions (i.e. energy density, exposure time and output power density), were evaluated using combinations of these factors as follows. A total of 15 J/cm^2 of constant-energy-density irradiation (50 mW/cm^2 for 300 s, 200 mW/cm^2 for 75 s or 400 mW/cm^2 for 38 s) was performed to investigate the effects of output power density and/or exposure time. Under constant-output power (50 mW/cm^2), 1–5 min of irradiation was performed to examine the effect of exposure time. Under the constant exposure time (5 min), 30, 50 or 100 mW/cm^2 of irradiation was performed to examine the effect of output power density. The average output power density was confirmed using a power meter (Ophir, Jerusalem, Israel) in each experiment.

Viable colony count

Following exposure of the bacterial suspension to light, as described above, samples were diluted 1 : 10 four or five consecutive times in sterile broth. Then, 50-μL suspensions were applied to the agar plates. Survival of these bacteria was determined by counting colony-forming units following incubation in an anaerobic cabinet.

Statistical analysis

One-way analysis of variance was performed to determine significant differences between the test sample and the control. The significance of individual differences was evaluated by the Scheffe's *F*-test in cases where a significant difference was detected by analysis of variance.

Results

Significant inhibition of *P. gingivalis* growth was observed upon exposure to 400 and 410 nm blue light (Fig. 1), whereas no significant growth inhibition was observed when exposed to light at wavelengths longer than 500 nm (data not shown). This result clearly indicated that selective monochromatic visible blue light (400–410 nm) can inhibit *P. gingivalis* growth without any exogenous photosensitizer. Monochromatic light exposure at 405 nm with 15 J/cm^2 of constant energy density produced significant inhibition (more than 75% inhibition compared with the nonirradiated control) under all irradiation conditions of 50 mW/cm^2 for 300 s, 200 mW/cm^2 for 75 s or 400 mW/cm^2 for 38 s (Fig. 2).

The effect of exposure time was examined under constant-output power (50 mW/cm^2). As shown in Fig. 3, 1 min of irradiation inhibited bacterial growth to some extent (45% inhibition). When exposure time was increased under constant-output power, the inhibitory effect seemed to be stronger.

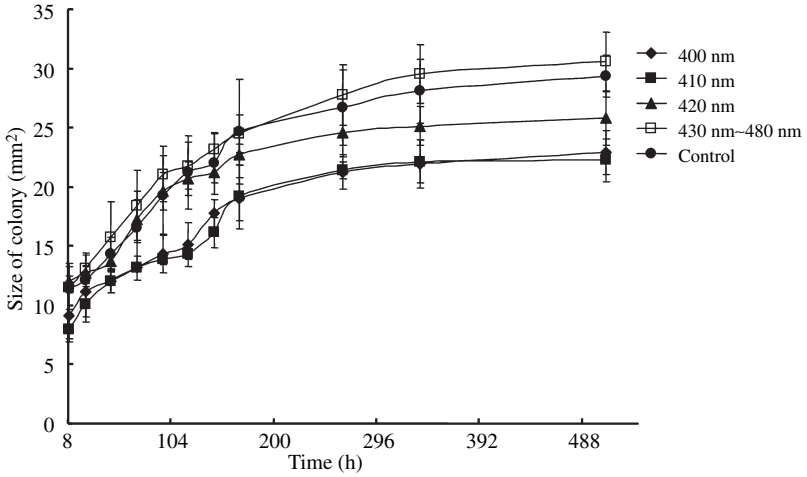

Fig. 1. Effect of monochromatic irradiation on the growth of *Porphyromonas gingivalis*. Significant inhibition of *P. gingivalis* growth was observed when exposed to 400- and 410-nm blue light. This result clearly indicates that selective monochromatic visible blue light (400–410 nm) can inhibit *P. gingivalis* growth without any exogenous photosensitizer.

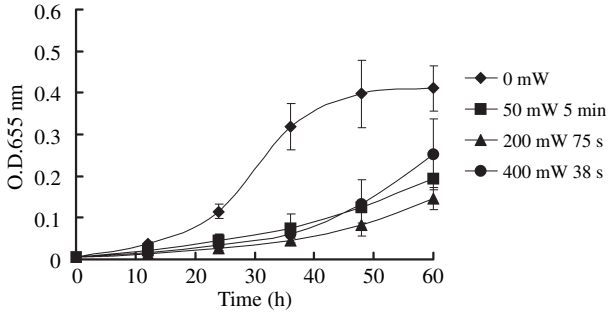

Fig. 2. Effect of monochromatic light exposure at 405 nm with 15 J/cm^2 of constant energy density. Significant inhibition (more than 75% inhibition after 36 h compared with the nonirradiated control) was observed under all the irradiation conditions (50 mW/cm^2 for 300 s, 200 mW/cm^2 for 75 s or 400 mW/cm^2 for 38 s).

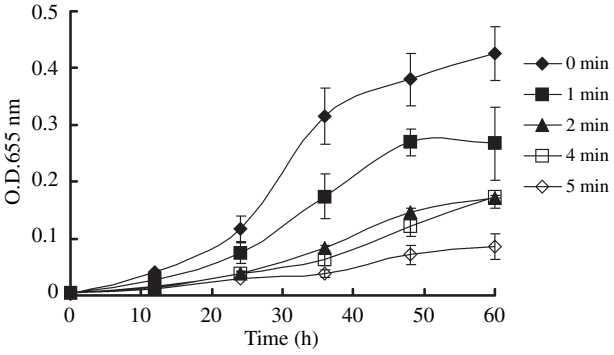

Fig. 3. Effect of exposure time under constant-output power (50 mW/cm^2). With constant-output density, irradiation for 1 min inhibited bacterial growth to some extent (45% inhibition) at the 36-h time point. A longer exposure time produced a stronger inhibitory effect.

The effect of output power density was examined with a constant exposure time (5 min). Whereas 30 mW/cm^2 of irradiation showed a significant inhibitory effect ($\approx$ 70% inhibition), 50 and 100 mW/cm^2 showed a much stronger effect ($\approx$ 90% inhibition) (Fig. 4).

Bacterial viability following exposure to 405-nm monochromatic light was expressed as percentage survival of bacteria in suspension (Table 1). Irradiation at 50 mW/cm^2 for 300 s showed significant bactericidal activity. The bactericidal effect of exposure time under constant-output power (50 mW/cm^2) was consistent with bacterial growth inhibition. As shown in Table 1, 1 min of irradiation showed bactericidal activity to some extent (71.3% survival). When exposure time was increased under constant-output power, the bactericidal activity seemed to be stronger. The output power density (30, 50 and 100 mW/cm^2) with a constant exposure time (5 min) showed a significant effect on bacterial survival ($\approx$ 60% survival) (Table 1).

Discussion

Periodontal disease is a mixed bacterial infection and its management still depends on conventional mechanical debridement, such as scaling and root planing, which often fails to yield a satisfactory outcome. Consequently, an alternative management of periodontitis, which would be easy to perform, less burdensome for patients and repeatable, has been sought for a long time. Photodynamic therapy can be defined as eradication of target cells by reactive oxygen species produced by means of a photosensitizing compound and light of an appropriate wavelength (14). Some studies have demonstrated that photodynamic therapy using an exogenous photosensitizer has the potential to kill *P. gingivalis*, which is considered to be a major pathogenic bacterium of periodontitis (9,15,16). However, some complications and problems of using exogenous photosensitizers have also been elucidated (17). From this point of view, photodynamic therapy without any photosensitizers must be developed as an alternative management of periodontitis. In the present study, we confirmed that photodynamic therapy by irradiation at 400–410 nm inhibited growth of *P. gingivalis*, even without

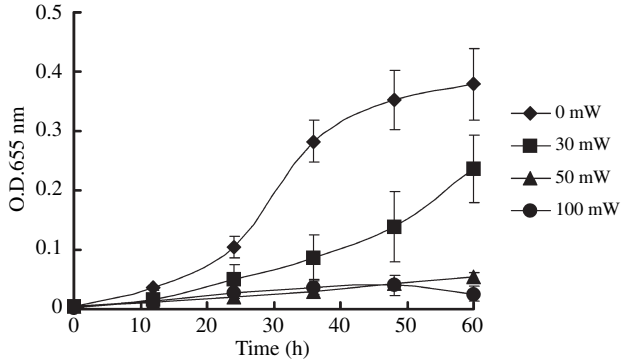

Fig. 4. Effect of output power under constant exposure time (5 min). While 30 mW/cm^2 showed a significant inhibitory effect (≈ 70% inhibition at 36 h), 50 and 100 mW/cm^2 showed a much stronger inhibitory effect (≈ 90% inhibition at 36 h).

Table 1. Effect of the monochromatic light exposure at 405 nm on the viability of bacteria in a suspension of Porphyromonas gingivalis

Exposure condition Energy density (output power, time)	Viability (% of control) mean ± SD
15 J/cm^2 (50 mW/cm^2, 300 s)	57.2 ± 9.0
3 J/cm^2 (50 mW/cm^2, 60 s)	71.3 ± 5.5
6 J/cm^2 (50 mW/cm^2, 120 s)	65.3 ± 9.9
12 J/cm^2 (50 mW/cm^2, 240 s)	56.5 ± 9.2
9 J/cm^2 (30 mW/cm^2, 300 s)	60.0 ± 9.4
30 J/cm^2 (100 mW/cm^2, 300 s)	56.9 ± 7.2

any photosensitizers, which strongly suggested the possibility that photodynamic therapy could be a promising new management of periodontitis.

The mechanisms underlying the inhibitory effect of irradiation by light of specific wavelengths are still unclear in the present study. A study using a photosensitizer showed that irradiation inactivated the proteolytic activity of *P. gingivalis*, suggesting that the inactivation of proteolytic enzymes occurs through the oxidation of active-site thiol groups (9). Other studies, using black-pigmented bacteria, suggested that endogenously produced porphyrins might act as photosensitizers under light irradiation, leading to a reduction of bacterial growth and viability (18,19). The proteolytic enzymes, Arg-gingipain and Lys-gingipain, produced by *P. gingivalis*, are known to contribute to a variety of virulence factors of periodontopathogenic bacteria, such as efficient growth in human serum, hemagglutination activity, biofilm formation and cytotoxic activity (20). Arg-gingipain and Lys-gingipain are known to be necessary for bacteria to obtain iron by degrading host hemoglobin (21), in the process of which protoporphyrin is generated from heme (11). It is therefore probable that most pathogenic bacterial strains possess high levels of porphyrins that could function as endogenous photosensitizers. This fact also strongly suggests that photodynamic therapy without any exogenous photosensitizer would be a beneficial strategy for the continuing management of periodontitis. Although the effective spectra confirmed in this study were consistent with the strongest porphyrin photoexcitation band at 405–415 nm (19), further investigation will be necessary to clarify how light irradiation at this specific wavelength suppresses bacterial growth with no exogenous photosensitizer.

The determination of bacterial survival revealed that the growth inhibition caused by exposure to 405-nm light, in part was caused by bacteriostatic activity of the blue light. The bactericidal activity was not attributed to the side-effects of irradiated contents, including hemin-derived porphyrin, in the assay medium because the bacterial growth of *P. gingivalis* was not affected in irradiated assay medium and the bacterial growth of other bacteria, such as *Fusobacterium nucleatum* was not affected by the same irradiation (data not shown).

Periodontopathogenic bacteria cause not only periodontitis, but also halitosis, by producing volatile sulfate components after degrading proteins of host oral epithelial cells (22). Recently it has been reported that 400–500-nm blue light irradiation suppressed volatile sulfate component production in saliva samples and reduced the population of gram-negative bacteria (23). These studies revealed growth inhibition of periodontopathogenic bacteria, including *P. gingivalis*, by 405-nm light irradiation, suggesting that irradiation might affect bacterial metabolism as well as growth. *P. gingivalis* possesses strong proteolytic activity and degrades host proteins to small peptides, which are used as an energy source by other subgingival bacteria that have no, or weak, proteolytic activity (24). This means that inhibition of *P. gingivalis* growth subsequently leads to the suppression of other periodontopathoganic bacteria, such as *F. nucleatum* and *Peptostreptococcus micros*, and suggests that suppression of the growth and/or metabolism of *P. gingivalis* by 400–410-nm irradiation would be a great advantage in the management of periodontitis.

Recently, the existence of oral chronic inflammatory lesions caused by periodontopathogenic bacteria has been widely recognized as being associated with crucial systemic diseases, such as coronary heart disease (25,26). Additionally, *P. gingivalis* has been detected within atherosclerotic plaques by the polymerase chain reaction (27). Therefore, eradication of periodontopathogenic bacteria may lead to prevention of these systemic diseases. The elimination of periodontopathogenic bacteria by conventional mechanical debridement and/or antibacterial agents is limited because

of technical limitations and side-effects. We have already confirmed that irradiation with 15 J/cm^2 of blue light did not affect cell viability (data not shown). Even though the effect on host tissues must be carefully investigated in future studies, the present study clearly demonstrated that photodynamic therapy against periodontopathogenic bacteria by monochromatic irradiation is a promising and hopeful strategy for the alternative management of not only periodontal disease, but also systemic diseases.

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