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Antimicrobial photodynamic therapy using a diode laser with a potential new photosensitizer, indocyanine green-loaded nanospheres, may be effective for the clearance of *Porphyromonas gingivalis*

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Background: Antimicrobial photodynamic therapy (aPDT) is a new treatment method for the removal of infectious pathogens using a photosensitizer and light of a specific wavelength, e.g., toluidine blue with a wavelength of about 600 nm. We explored a new photosensitizer and focused on indocyanine green (ICG), which has high absorption at a wavelength of 800–805 nm. We investigated the bactericidal effect of PDT on *Porphyromonas gingivalis* using a new photosensitizer, ICG-loaded nanospheres with an 805 nm wavelength low-level diode laser irradiation.

Methods: We designed ICG-loaded nanospheres coated with chitosan (ICG-Nano/c) as a photosensitizer. A solution containing *Porphyromonas gingivalis* (10^8 CFU/mL) with or without ICG-Nano/c (or ICG) was prepared and irradiated with a diode laser or without laser irradiation as a negative control. The irradiation settings were 0.5 W with a duty ratio of 10%, for 3–100 ms in repeated pulse (RPT) or continuous wave mode. CFU were counted after 7 d of anaerobic culture.

Results: We observed that ICG-Nano/c could adhere to the surface of *P. gingivalis*. When ICG-Nano/c was used for aPDT, irradiation with RPT 100 ms mode gave the lowest increase in temperature. Laser irradiation with ICG-Nano/c significantly reduced the number of *P. gingivalis* (i.e., approximately 2-$\log_{10}$ bacterial killing). The greatest bactericidal effect was found in the RPT 100 ms group. However, laser irradiation (RPT 100 ms) with ICG, as well as without photosensitizer, had no effect on the number of bacteria.

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Conclusions: Within the limits of this study, ICG-Nano/c with low-level diode laser (0.5 W; 805 nm) irradiation showed an aPDT-like effect, which might be useful for a potential photodynamic periodontal therapy.

Porphyromonas gingivalis is widely isolated from subgingival plaques in patients with chronic periodontitis and significantly increases with the progression of periodontitis. *P. gingivalis* is a significant pathogen in chronic periodontitis and is closely related to the development of periodontal disease. Therefore, many studies on periodontal diseases have examined *P. gingivalis* (1–3). The removal of periodontal pathogens is essential for periodontal treatment. However, it is very difficult to completely remove biofilm with conventional hand instruments and chemotherapy has only limited effects because the antibiotics do not readily penetrate biofilm. Moreover, the effects of antibiotics have decreased due to the emergence of drug-resistant bacteria (4–9). The number of patients with systemic diseases has recently increased in step with aging of the population, and thus new non-invasive/nonsurgical methods, in addition to the current treatment methods, are needed.

Photodynamic therapy (PDT) has recently emerged as a treatment option. Photosensitizers are excited by light irradiation at their maximum absorption wavelength and generate singlet oxygen through energy exchange with ambient oxygen (10). The efficacy of PDT is based on the cell- or tissue-injuring properties of this singlet oxygen. However, singlet oxygen is present for only a short duration and its range of action is narrow. If a photosensitizer could be introduced specifically into target tissue, it should be able to selectively destroy it without influencing the surrounding normal tissue. PDT that targets bacteria is called antimicrobial PDT (aPDT). aPDT is a new treatment method for destroying infectious pathogens; bacteria are stained with a photosensitizer and then irradiated with an appropriate wavelength and low-output energy density. Pfitzner *et al.* (11) reported that aPDT using chlorin e6 and BLC 1010 as photosensitizers completely inactivated periodontal pathogens, *P. gingivalis*, *Fusobacterium nucleatum* and *Capnocytophaga gingivalis*, *in vitro*.

Indocyanine green (ICG) is currently used for liver function tests and for funduscopy by intravenous infusion. It is also a photosensitizer with a wide optical absorption band from 600 to over 800 nm, and the main peak of the absorption spectrum is at 800–805 nm (12). An ideal photosensitizer for PDT/aPDT should have specific properties. First, the absorption band should be at a wavelength longer than 600 nm so that it is easily distinguished from biological substances, such as hemoglobin (12,13). Second, the molecular extinction coefficient of the absorption wavelength should be large and produce sufficient singlet oxygen upon light-induced excitation (14). Third, the substance should have a high affinity for the target, distribute homogeneously, and be rapidly excreted from normal tissue (15,16). ICG binds to plasma protein, is not chemically altered in the body, exists in a stable state and is rapidly excreted in bile (17). Thus, ICG has already been shown to satisfy most of the requirements for an ideal photosensitizer (12–17).

Recently, attention has been paid, particularly to biodegradable polymeric nanospheres, because of their high stability and ability to target specific tissues/organs either by adsorption or by binding ligands attached to the particle surface (18–22). Among these biodegradable polymers, poly (lactide) and poly(D,L-lactide-co-glycolide) (PLGA) have been approved by the Food and Drug Administration for certain human clinical uses due to their biocompatibility and ability to degrade in the body through natural pathways (23–26). In addition, PLGA nanospheres have been used successfully in the drug delivery of photosensitizers (27,28). More recently, the use of PLGA nanospheres as carriers of methylene blue in aPDT has been reported (29). There is strong evidence that the positive charge of a photosensitizer enhances its uptake and phototoxicity on bacterial species (30–32). Previously, we successfully developed mucoadhesive PLGA nanospheres by surface modification with chitosan, which gave a positive charge on PLGA nanospheres, for oral peptide delivery (19).

To use ICG for periodontal aPDT, we developed ICG-loaded nanospheres with a chitosan coating (ICG-Nano/c) that can efficiently adhere to bacterial walls. Before the application of aPDT for periodontal treatment, we investigated the photodynamic influence of low-output laser irradiation with our developed photosensitizer on a major periodontal pathogen, *P. gingivalis* (33).

Material and methods

Reagents for nanosphere manufacture

A PLGA with an average molecular weight of 20,000 and a copolymer ratio of lactide to glycolide of 75 : 25 was used as a wall material for the nanospheres. Chitosan (MW 50,000; deacetylation degree 80%) was used as a mucoadhesive polymer to coat the surface of PLGA nanospheres. Polyvinylalcohol (PVA-403; Kuraray Co., Ltd., Osaka, Japan) was used as a dispersing agent. Caprylate and caprate triglyceride (Triester F-810; Nikko Chemicals Co., Ltd., Tokyo, Japan) were used as a nontoxic oil-dispersing medium because of their good biocompatibility and low viscosity. Hexaglycerin-condensed ricinoleate (Hexaglyn PR-15; Nikko Chemicals Co., Ltd.)

was used as a nontoxic emulsifier for pulmonary administration. All materials in the present study were used as received.

Preparation of photosensitizer-loaded nanospheres

ICG (Ophthagreen; Santen Pharmaceutical Co., Ltd., Osaka, Japan)-Nano was prepared by the previously reported emulsion solvent diffusion method in oil (34). Briefly, PLGA (100 mg), ICG (5 mg) and span80 (100 mg) dissolved in a mixed solution of acetone (2 mL) and methanol (1 mL) were poured into an out phase (60 mL of 2% hexaglycerin-condensed ricinoleate containing triglyceride and 40 mL of n-hexane) at 2 mL/min under stirring at a speed of 450 rpm using a propeller-type three-blade agitator (Heidon 600G; Shinto Scientific Co., Ltd., Tokyo, Japan) at room temperature. After the system was agitated for 3 h to remove n-hexane until the total volume of the suspension reached 20 mL, the resultant suspension was centrifuged at 26,690 ***g*** for 10 min at 4°C. The sediment was dispersed in 20 mL of n-hexane and centrifuged to wash oil on the nanosphere surface. The nanospheres were then dispersed in 100 mL 2% PVA solution to which ICG-Nano had been added, and then centrifuged at 26,690 ***g*** for 10 min at 4°C. The PLGA nanosphere suspension was combined with distilled water with mannitol (500 mL; Mannitol; Towa Chemical Industry Co., Ltd., Tokyo, Japan) as a dispersing agent. Finally, the ICG-containing suspension was frozen at −45°C and lyophilized using a freeze-dryer (EYELA FD-81; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) for 48 h under ambient conditions. In this study, the developed ICG-Nano that contained 5 mg/g ICG was used at a concentration of 10 mg/mL. In some procedures, a mixed solution of 2% PVA/0.5% chitosan was used instead of the 2% PVA solution to coat the surface of ICG-Nano with chitosan, which is a mucoadhesive polymer (as shown in Fig. 1A). As a control, ICG alone was dissolved in distilled water to obtain a final concentration of 0.05 mg/mL, which was consistent with the amount of ICG in the ICG-Nano solution. In some experiments, we also prepared coumarin-loaded nanospheres, by using coumarin instead of ICG, as described above.

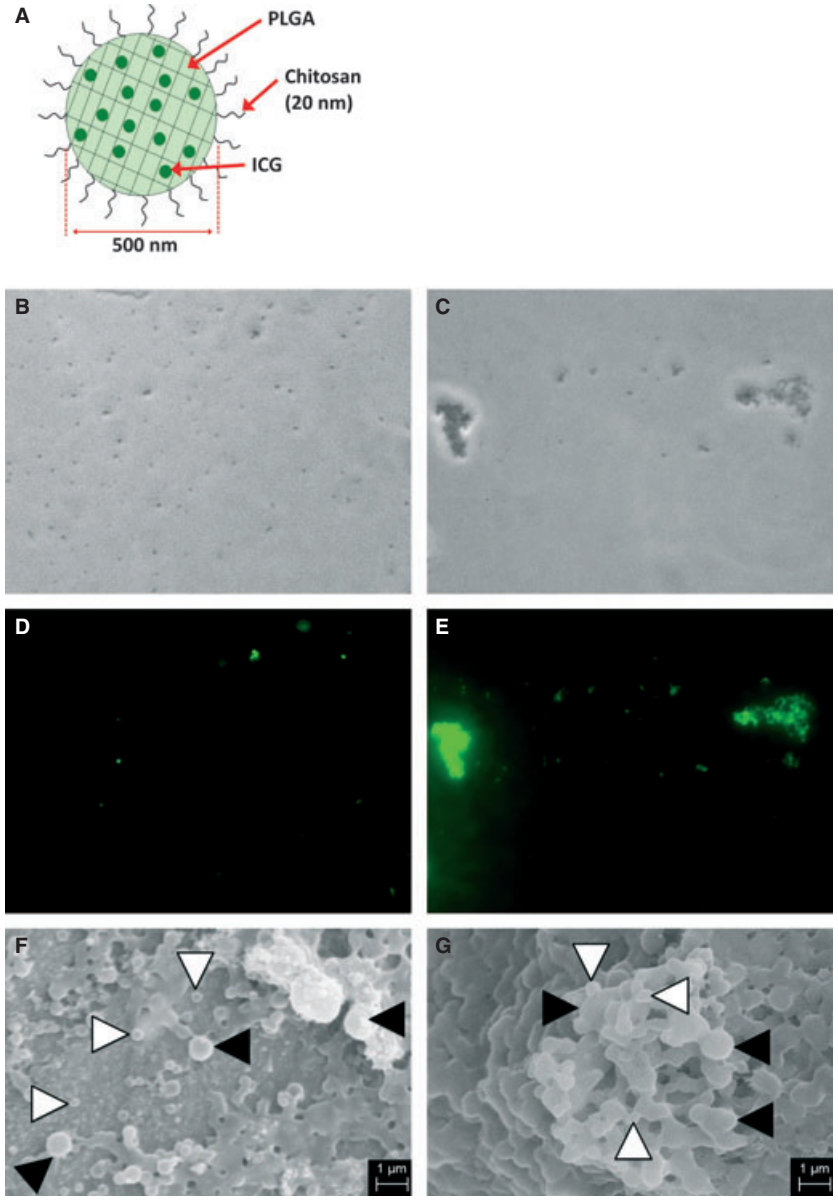

Fig. 1. (A) Schematic illustration of the structure of ICG-Nano/c. ICG-Nano is composed of PLGA and ICG, which form the matrix structure. Chitosan was used as a mucoadhesive polymer to coat the surface of nanospheres. (B–E) *P. gingivalis* and nanospheres coated with (B,D) or without (C,E) chitosan were mixed, and phase-contrast (B,C) or fluorescence (D,E) micrographs were taken. Nanospheres coated with chitosan (E) show efficient adhesion to *P. gingivalis* compared to those without coating (D). (F,G) Electron micrograph images (× 5000) that show the relations of *P. gingivalis* (closed arrowhead) and ICG-Nano (without chitosan coating) (F) (open arrowhead) or ICG-Nano/c (with chitosan coating) (G) (open arrowhead). While ICG-Nano failed to adhere to *P. gingivalis* (about 1 μm), ICG-Nano/c (about 500 nm) efficiently adhered to *P. gingivalis*. Typical results from three independent experiments are shown. ICG-Nano/c, ICG-loaded nanospheres coated with chitosan; PLGA, poly(D,L-lactide-co-glycolide).

Microorganisms

Porphyromonas gingivalis strain ATCC33277 was maintained by weekly subculture on blood agar plates (Poa Media; Eiken Chemical Co., Ltd., Tokyo, Japan) in an anaerobic chamber at 37°C. *P. gingivalis* was then inoculated into tryptic soy broth (BD Tryptic Soy Broth; Becton, Dickinson & Co., Cockeysville, MD, USA) supplemented with yeast extract (1 mg/mL), hemin (5 μg/mL) and menadione (1 μg/mL), and cultured anaerobically to the mid-log phase at 37°C. Cell numbers were measured in a spectrophotometer [wavelength, 600 nm; 0.1 optical density unit equals approximately 10^8 colony-forming units (CFU)/mL] in a 1 mL cuvette.

Phase-contrast and fluorescence microscopy

Coumarin (fluorescence wavelength, 490 nm; absorption wavelength, 520 nm)-loaded nanospheres with a chitosan coating were mixed with a suspension of *P. gingivalis* (10^8 CFU/mL in liquid culture medium) on a glass slide and observed using a fluorescence microscope system (Leica AF6000LX fluorescence microscope equipped with a Leica DFC 300FX digital camera; Leica, Heidelberg, Germany). Phase-contrast and a GFP filter (excitation filter 470/40; emission filter 525/50) were used for imaging.

Scanning electron microscopy

The ability of the newly developed photosensitizer to adhere to *P. gingivalis* was assessed by electron micrograph imaging. Briefly, 100 μL of ICG-Nano or ICG-Nano/c (10 mg/mL) was mixed with the same volume of *P. gingivalis* suspension (10^8 CFU/mL in liquid culture medium). After being allowed to rest for 5 min, the mixed suspensions were rinsed three times by centrifugation at 10,000 ***g*** for 15 min followed by the addition of distilled water. Next, 4% paraformaldehyde fixative was added to the harvested bacteria, which were then dried at room temperature. Samples were sputter-coated with gold to a thickness of 10 nm using an ion coater (JFC-1500; JEOL Ltd., Tokyo, Japan) and observed using a scanning electron microscope (EVO 40; ZEISS, Peabody, MA, USA) at 15 kV to examine the ability of ICG-Nano or ICG-Nano/c to adhere to *P. gingivalis*.

Laser application

The irradiation source used was a diode laser (P-Laser; Panasonic Dental Co., Ltd., Osaka, Japan) with a power output capacity of 20 W and a central wavelength of 805 ± 20 nm. The light was distributed by means of a fiber-optic applicator with a 400 μm cylindrical diffusing tip. The laser equipment has both a continuous wave (CW) mode and repeated pulse (RPT) mode with a variable pulse width (3, 5, 10, 20, 50, 100 and 200 ms) and a variable duty cycle (10%, 20%, 30% and 50%). In this study, the diode laser was used with four settings: (1) CW mode: 0.5 W (power output); (2) RPT mode: 3 ms (pulse width), 5 W (peak power output), 10% (duty cycle); (3) RPT mode: 10 ms (pulse width), 5 W (peak power output), 10% (duty cycle); and (4) RPT mode: 100 ms (pulse width), 5 W (peak power output), 10% (duty cycle), with the same total energy level. When used with a fiber probe tip of 400 μm (core diameter), the actual output at the distal portion of the tip was reduced to 95% of the indicated output [e.g., the actual output for RPT, 5 W (peak power output), 10% (duty cycle) in this study is calculated as follows: 5 W × 10% × 95% = 0.475 W].

Temperature measurement

Four hundred microliters of ICG liquid (0.05 mg/mL) or ICG-Nano/c liquid (10 mg/mL) at 26°C was added to an infrared transparent cuvette (10 × 10 × 45 mm). The light probe was set 10 mm above the solution in the cuvette. We monitored the change in temperature during laser irradiation at the four settings described above with ICG-Nano/c. The distributions of the increase in temperature were captured by thermography (TH7100; NEC Ltd., Tokyo, Japan).

Bacterial viability after the antimicrobial photodynamic therapy procedure

Two hundred microliters of the bacterial suspensions (10^8 CFU/mL in liquid culture medium) were mixed with an equal volume of respective photosensitizers in a 1.5 mL tube, and then irradiated with a diode laser that was moved horizontally at a distance of 10 mm from the liquid level using a scanning stage. In some experiments, no irradiation was applied to confirm that the effect was not the result of photosensitizer alone. After these procedures, the mixed suspension was diluted from 10^{-3} to 10^{-6} and plated on to blood agar plates. CFU were counted after 7 d of anaerobic culture.

Statistical analysis

The data were analyzed by normality tests (Shapiro–Wilk test and Kolmogorov–Smirnov test), and a normal distribution was confirmed. Thus, CFU were analyzed with a parametric test (Tukey test) using commercial software (SPSS 15.0 J for Windows; SPSS, Inc., Chicago, IL, USA), and significance was accepted at $p < 0.05$.

Results

Characterization of indocyanine green-loaded nanospheres for antimicrobial photodynamic therapy

The diode laser with a wavelength of 805 nm used in this study is suitable for PDT using ICG (maximum absorption wavelength; 805 nm). We developed an original photosensitizer, ICG-Nano/c, for aPDT. To check the effect of the chitosan coating on the ability to adhere to periodontopathic microorganisms by fluorescent microscopy, we first prepared nanospheres that were loaded with coumarin instead of ICG but had the same particle size (approximately 500 nm) as ICG-Nano/c. Nanospheres without a chitosan coating, which are negatively charged, could not adhere efficiently to

P. gingivalis (Fig. 1B and 1D). In contrast, nanospheres with a chitosan coating, which are positively charged, could adhere to *P. gingivalis* (Fig. 1C and 1E). As a negative control study, we also observed *P. gingivalis* alone (without coumarin-loaded nanospheres) by fluorescent microscopy, and confirmed that no signal was detected. Interestingly, the procedure for chitosan coating made the nanospheres agglutinate *P. gingivalis* (Fig. 1C). However, with non-coated nanospheres (Fig. 1B), *P. gingivalis* was scattered extensively. This agglutinative effect may be an advantage for aPDT. To examine whether this newly developed ICG-Nano/c is suitable for aPDT, we next tried to confirm the ability of ICG-Nano/c to adhere to *P. gingivalis*. As shown in Fig. 1F, ICG-Nano could not adhere to *P. gingivalis*. In contrast, ICG-Nano/c (approximately 500 nm) adhered to *P. gingivalis* (diameter: 1 μm), and an agglutinative effect was observed (Fig. 1G).

We also examined changes in temperature during aPDT using ICG-Nano/c. We examined irradiation in CW mode and with three different pulse durations (RPT 3 ms, RPT 10 ms and RPT 100 ms) mode. Typical results of thermographic measurement in CW mode at 0.5 W for 1 and 2 min are shown in Fig. 2A and 2B, respectively. The highest temperature was observed at 5 mm from the surface of the solution. In the ICG-alone group (Fig. 2C), the results regarding the change in temperature (initial temperature, 26°C) in each group indicate that the increase in temperature in all of the groups is irradiation duration-dependent. As shown in Fig. 2D, we found a similar tendency for diode laser irradiation in the ICG-Nano/c group. The RPT 100 ms group had the lowest rise in temperature (i.e. an increase of 4.23 ± 0.85°C at 1 min). As a negative control, we also tested a distilled water group without any photosensitizer, and the temperature in this group only increased a few degrees (Fig. 2E). Thus, ICG and ICG-Nano/c might absorb diode laser light, which would result in an increase in temperature, as shown in Fig. 2C and 2D.

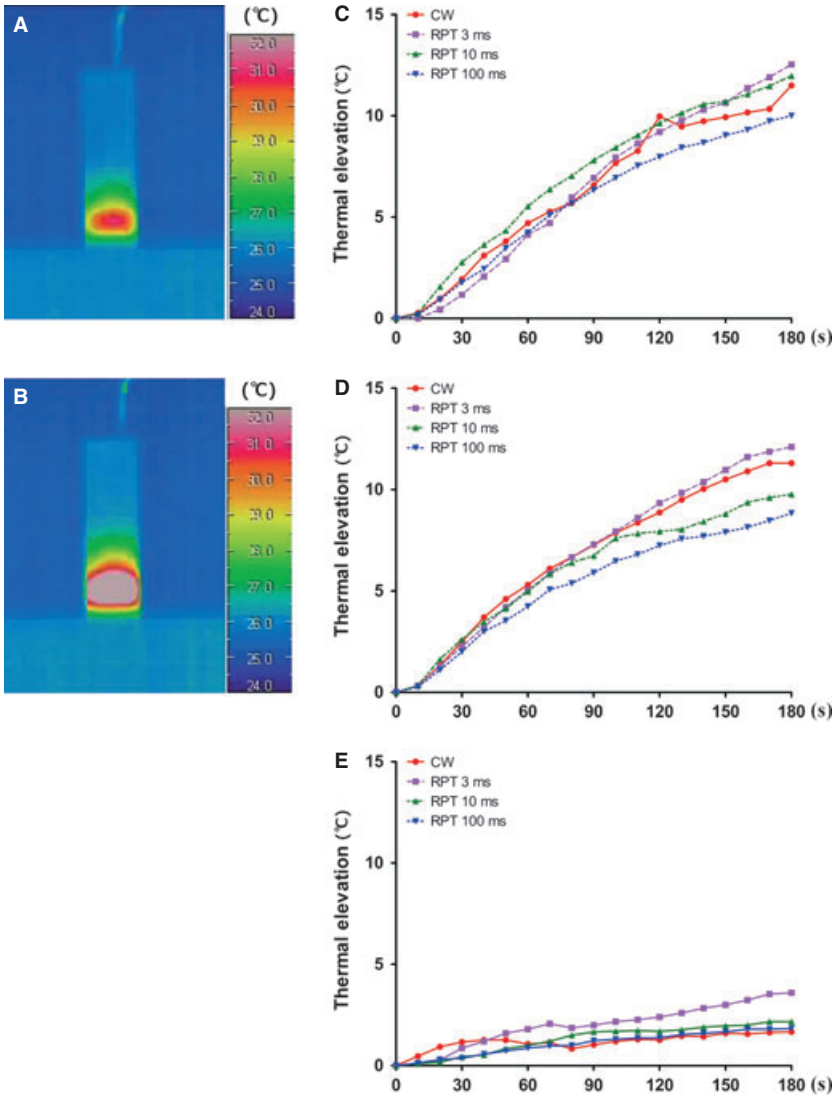

Fig. 2. Effect of laser irradiation with the newly developed photosensitizer on changes in temperature. (A,B), The ICG-Nano/c solution was irradiated with the diode laser (CW, 0.5 W) for 1 (A) or 2 (B) min. Thermography shows that the highest temperature was recognized 5 mm from the surface of the solution. The increase in temperature at 2 min was spread peripherally compared to that at 1 min. Typical results of three independent experiments are shown. (C–E) ICG alone (C) or ICG-Nano/c (D) solution as well as distilled water (E) was irradiated with the diode laser at various pulse ranges. In all of the groups, the temperature increased (initial temperature; 26°C) in an irradiation duration-dependent manner. Typical results from two (C,E) or three (D) independent experiments are shown. CW, continuous wave mode; ICG-Nano/c, ICG-loaded nanospheres coated with chitosan; RPT, repeated pulse mode.

Bactericidal effect of diode laser irradiation with indocyanine green-loaded nanospheres with a chitosan coating

To test the bactericidal effect of various settings of aPDT on a periodontal pathogen, *P. gingivalis* (10^8 CFU/mL) mixed with ICG-Nano/c (10 mg/mL) were irradiated by a diode laser for 1 min. The viability of *P. gingivalis* was significantly decreased ($p < 0.001$) in all of the laser irradiation groups (CW, RPT 3 ms, RPT 10 ms and RPT 100 ms) compared to the non-treatment control group (Fig. 3A). The peak bactericidal effect was found in the RPT 100 ms group. We next

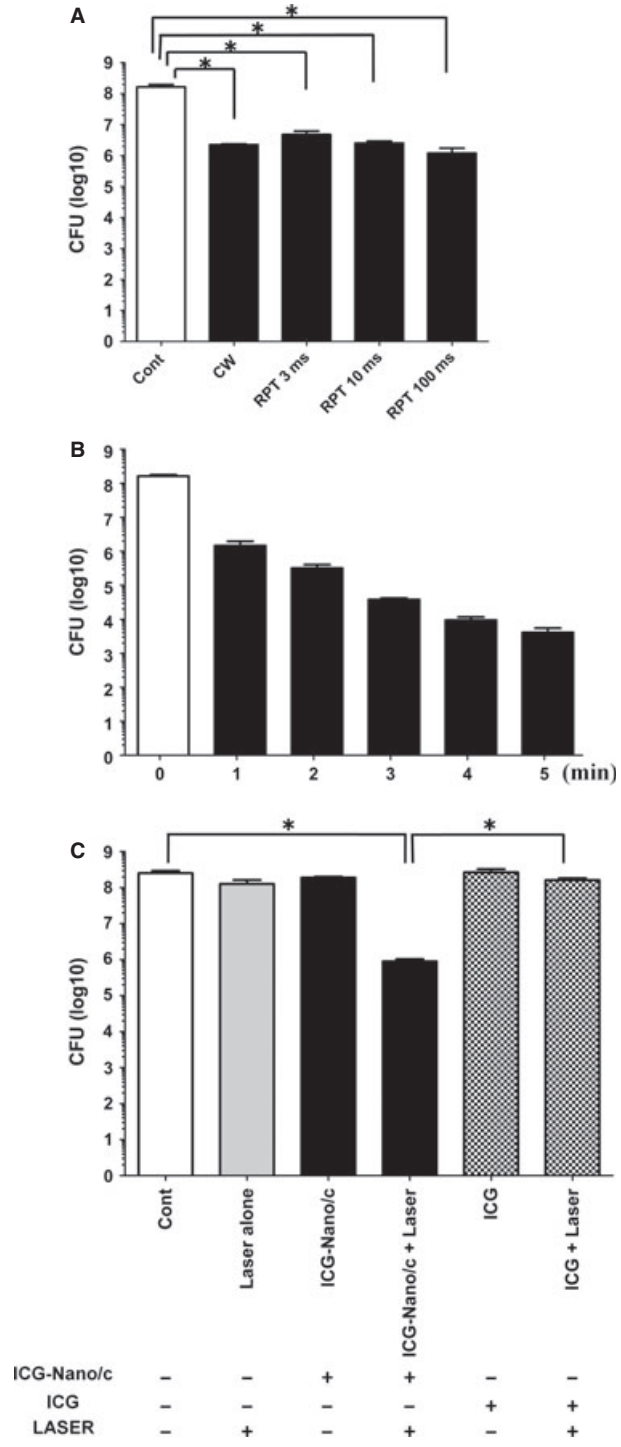

Fig. 3. Effect of antimicrobial photodynamic therapy using ICG-Nano/c. (A) *P. gingivalis* (10^8 CFU/mL) mixed with ICG-Nano/c were irradiated with the diode laser at various pulse ranges for 1 min. All of the laser irradiation groups showed a significant reduction of CFU. (B) *P. gingivalis* (10^8 CFU/mL) mixed with ICG-Nano/c were irradiated with the diode laser (average 0.5 W). Laser irradiation was performed from 1 to 5 min with RPT 100 ms, at a duty cycle of 10%. The bactericidal effect increased in an irradiation duration-dependent manner. (C) *P. gingivalis* (10^8 CFU/mL) mixed with photosensitizer were irradiated by the diode laser for 1 min. The power setting of the laser was 0.5 W (average), at a duty cycle of 10%, in RPT 100 ms mode. The viability of *P. gingivalis* was significantly decreased under laser irradiation with ICG-Nano/c compared to that in the non-treatment control group. Laser irradiation with ICG (ICG + laser group) or without the photosensitizer (laser alone group) had no effect on the number of bacteria. The photosensitizer (ICG-Nano/c or ICG) alone also had no effect on the viability of *P. gingivalis*. Values are the mean ± standard deviation of triplicate assays. *$p < 0.001$. CW, continuous wave mode; ICG-Nano/c, ICG-loaded nanospheres coated with chitosan; RPT, repeated pulse mode.

examined the effect of the duration of irradiation on the viability of *P. gingivalis* in the RPT 100 ms group. As shown in Fig. 3B, the number of bacteria was decreased in a time-dependent manner. However, irradiation for a prolonged duration might produce a hyperthermic effect in addition to an aPDT effect.

It is important to exclude the hyperthermic effect of treatment to assess accurately the effect of aPDT. As hyperthermia begins to have an effect above 44°C, a temperature rise of < 5°C (from body temperature) would be better for assessing the actual effect of aPDT. In this study, we could only monitor the increase in temperature from 26°C. However, at least from the results in Fig. 2D, diode laser irradiation with ICG-Nano/c for 1 min was thought to be suitable for verifying the actual effect of aPDT. *P. gingivalis* (10^8 CFU/mL) mixed with or without ICG-Nano/c (10 mg/mL) was irradiated by a diode laser for 1 min. We also performed tests with ICG to estimate the value of the newly developed ICG-Nano/c. We confirmed that the number of bacteria was significantly reduced by laser irradiation with ICG-Nano/c (ICG-Nano/c + laser group) ($p < 0.001$) (Fig. 3C). Interestingly, laser irradiation both with ICG (ICG + laser group) and without photosensitizer (laser-alone group) had no effect on the number of bacteria in this setting. The photosensitizer (ICG-Nano/c or ICG) alone also had no effect on the viability of *P. gingivalis*.

Discussion

PDT using ICG and a diode laser has been reported as a method for the treatment of cancer (14,35), but there have been no reports of its bactericidal effect for the treatment of infectious diseases. In this study, we investigated aPDT using a nanoparticle formulation of ICG against *P. gingivalis*, which is detected at a high frequency in periodontal pockets of patients with advanced periodontitis.

The photosensitizer used in PDT absorbs the laser light and induces the production of a reactive oxygen species, singlet oxygen, from oxygen dissolved in the tissue. Nevertheless, the effects of singlet oxygen have a very narrow range and persist for a very short time. To overcome these disadvantages, we developed a new photosensitizer, ICG-Nano/c, the surface of which was given a positive charge by a coating of chitosan to promote adherence to bacteria. In the present study, we showed that these positively charged nanospheres could adhere to *P. gingivalis*. Moreover, we confirmed that ICG-Nano/c could adhere to other periodontal pathogens, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* (data not shown).

When a target is irradiated by a laser with an appropriate wavelength, the target will absorb the energy and produce heat. In this study, the temperature increased in an irradiation duration-dependent manner when ICG-Nano/c was irradiated with the diode laser at the lowest output of the device (average output 0.5 W) under each condition (CW and RPT modes). In addition, with an increase in the pulse width, the rate of increase in temperature decreased among groups with the same total energy level. When biological tissue is irradiated with a laser, the tissue temperature rises corresponding to tissue properties and laser parameters (wavelength, power density and duration of irradiation), and tissue degeneration occurs with an increase in temperature. Previous reports have suggested that short-term temperature changes of ±5°C do not influence tissue (36). For periodontal treatment, careful consideration is necessary because the laser will irradiate the inner regions of the periodontal pockets located close to the dental pulp and alveolar bone. While dental pulp is not influenced by a temperature increase of up to 5°C (37), the increase in temperature should be inhibited as much as possible. Thus, inhibition of the increase in temperature may be necessary for periodontal treatment. Our results suggest that low-level diode laser irradiation (average output 0.5 W) with ICG-Nano/c in RPT 100 ms (duty cycle 10%) mode for 1 min (temperature increase of 4.23 ± 0.85°C) may be suitable for clinical application.

aPDT is a new unique approach for the less-invasive treatment of periodontitis. aPDT has recently been attracting attention, and the development of photosensitizers for PDT has been progressing rapidly. Thus far, studies on PDT using toluidine blue or methylene blue with an absorption wavelength of about 600 nm have been performed (38–44). Kömerik *et al.* (38) reported that the number of *P. gingivalis* markedly decreased using laser irradiation at 600 nm with toluidine blue (absorption wavelength, about 630 nm), and Zanin *et al.* (45) reported that treatment with light and toluidine blue reduced an artificial biofilm, with a maximum reduction of 99.9%. We also explored a new photosensitizer specific for aPDT to broaden options for the use of aPDT in periodontal treatment, and eventually focused on ICG, which can be used for PDT in cancer treatment. In this newly developed ICG-Nano/c, the surface of ICG-Nano was given a positive charge by a chitosan coating to improve its ability to adhere to bacteria. In addition, chitosan has been reported to have an antibacterial effect against some bacteria (46–48). In the present study, the bactericidal effect of irradiation on *P. gingivalis* was significantly greater with ICG-Nano/c than when ICG alone was used, and we speculate that the adhesiveness of the particles compensated for the narrow range of action of singlet oxygen. However, a few limitations of our study must be considered while interpreting the present results. First, we did not confirm the generation of singlet oxygen in our experiments. Second, this *in vitro* study only used a single bacterial strain in the planktonic phase, not biofilms, to determine the bactericidal effect. Fontana *et al.* (41) have reported that the effect of aPDT with methylene blue for biofilms was half of that for bacteria in the planktonic phase. Third, the present study did not cover the effects of ICG-Nano/c on host cells. Because ICG has been used in PDT for cancer, we consider that ICG-Nano/c

alone or ICG-Nano/c with low-level diode laser irradiation might have some undesirable effects on host cells (e.g., pocket epithelial cells). Further studies are needed to confirm the concentration of singlet oxygen and the effect on the bacterial biofilm or on host cells by aPDT with ICG-Nano/c with an 805 nm low-level diode laser irradiation.

Conclusions

Within the limits of this study, ICG-Nano/c with low-level diode laser irradiation (0.5 W, 805 nm) showed an aPDT-like effect, and it might be a potential new photosensitizer for aPDT to achieve clearance of periodontal pathogens including *P. gingivalis*. The maximum absorption wavelength of ICG (ICG-Nano/c) is approximately 800 nm, a tissue-penetrating wavelength. We propose that an 805 nm wavelength diode laser with ICG-Nano/c might enable a bactericidal effect by external irradiation (from the oral epithelial side), which is quite unique and interesting in terms of a new approach to a periodontal pocket or furcation. Further investigations, including clinical trials, are needed to confirm the potential use of ICG-Nano/c in the periodontal treatment of non-reachable sites (i.e., a furcation area) or medically compromised patients.

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