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Short communication

Inhibitory effect of cranberry polyphenol on biofilm formation and cysteine proteases of *Porphyromonas gingivalis*

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Background and Objective: The purpose of this study was to investigate the effects of cranberry polyphenol fraction on biofilm formation and activities of Arg-gingipain and Lys-gingipain in *Porphyromonas gingivalis*.

Material and Methods: The polyphenol fraction was prepared by using a glass column packed with Amberlite XAD 7HP and 70% aqueous ethanol as an elution solvent.

Results: Synergistic biofilm formation by *P. gingivalis* and *Fusobacterium nucleatum* was significantly inhibited by the polyphenol fraction at a concentration of 250 μg/mL compared with untreated controls ($p < 0.01$). Arg-gingipain and Lys-gingipain activities in *P. gingivalis* ATCC 33277 and FDC 381 were inhibited significantly at a polyphenol fraction concentration of ≥1 μg/mL ($p < 0.05$).

Conclusion: These findings indicate that the polyphenol fraction inhibits biofilm formation and the Arg-gingipain and Lys-gingipain activities of *P. gingivalis*.

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Dental plaque comprises biofilm communities of mixed species of oral bacteria. *Porphyromonas gingivalis* is a gram-negative bacterium closely associated with chronic periodontitis (1). The pathogenicity of *P. gingivalis* has been attributed to a number of virulence factors, including lipopolysaccharide, fimbriae, hemagglutinin and proteases. Among the latter, Arg-gingipain and Lys-gingipain extracellular cysteine proteases have been isolated from culture supernatants, vesicle membrane fractions and cell extracts of *P. gingivalis* (2,3). These enzymes down-regulate polymorphonuclear neutrophils, degrade extracellular proteins and bio-active peptides such as C5, prekallikrein and kininogen, and modulate host cytokine networks, strongly affecting the virulence of *P. gingivalis* (4,5). The potential contribution of gingipains to the pathophysiology of periodontitis suggests these enzymes as potential therapeutic targets for *P. gingivalis*-associated periodontal disease (6–10).

Cranberry juice and its constituents inhibit the attachment of, and biofilm formation by, oral streptococci, and decrease the levels of *Streptococcus mutans* in saliva (11,12). Furthermore, a high-molecular-mass, nondialyzable material obtained from cranberries prevents the co-aggregation of many oral bacterial pairs (13). Recently, Bodet *et al.* (14–16) reported the effects of high-molecular-weight cranberry fraction on the pathogenicity of *P. gingivalis*. The purpose of the present study was to investigate the effects

of cranberry polyphenol on the biofilm formation and activities of Arg-gingipain and Lys-gingipain in *P. gingivalis*.

Material and methods

Cranberry juice concentrate (40 g, Brix 35°; Nippon Del Monte Corp., Tokyo, Japan) was applied to a glass column packed with Amberlite™ XAD™ 7HP (Rohm and Haas Co., Philadelphia, PA, USA). Water (100 mL) was passed through the column to remove non-phenolic cranberry constituents. To elute cranberry phenolics, aqueous 70% ethanol (100 mL) was passed through the column. The elution was then concentrated under vacuum and lyophilized to give the polyphenol fraction (0.53 g). Total polyphenol was confirmed to be 62% by the Folin & Ciocalteau method (17) (Gallic acid conversion) and total flavanol was confirmed to be 34% by the Vanillin-HCl method (18) (Catechin conversion). The total flavanol value represents the sum of monomeric polyphenols, including catechins, and primary components such as pro-anthocyanidins.

In preliminary experiments, *P. gingivalis* strains did not form a sufficient quantity of biofilm to allow measurement. Therefore, we used a synergistic biofilm model of *P. gingivalis and Fusobacterium nucleatum*. *F. nucleatum* may also play a key role in periodontal disease (19) because of its ability to co-aggregate with various periodontal pathogens that are late colonizers in dental plaque (20). Co-aggregation between *P. gingivalis* and *F. nucleatum* has been studied extensively (21–25). The synergistic pathogenicity of *P. gingivalis and F. nucleatum* has also been reported in subcutaneous abscess models (26,27).

The inhibitory effect of polyphenol fraction on biofilm formation by pairs of these two oral bacterial species on the bottom of cell culture plates (SUMILON Multi Well Plate; Sumitomo Bakelite Co., Ltd, Tokyo, Japan) was then examined.

The organisms used were *P. gingivalis* ATCC 33277 and FDC 381, and *F. nucleatum* ATCC 25586, TDC 2 and TDC 20 (ATCC, American Type Culture Collection; FDC, Forsyth Dental Center Collection; TDC, Tokyo Dental College Collection). *P. gingivalis* and *F. nucleatum* were cultured in Tryptic soy broth (Becton Dickinson Co., Sparks, MD, USA) supplemented with 5 μg of hemin and 0.5 μg/mL of menadione, and Tryptic soy broth supplemented with 0.3% yeast extract, 0.25% glucose and L-cysteine-hydrochloride (FN broth), respectively, under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37°C. Biofilm assays were performed according to the protocol of Loo *et al.* (28). Briefly, all bacterial strains were cultured in FN broth supplemented with 5 μg of hemin and 0.5 μg/mL of menadione, and with 250 μg/mL or 500 μg/mL of polyphenol fraction for 1 d under anaerobic conditions. Media and unattached bacterial cells were decanted from the wells, and the remaining planktonic or loosely bound cells were removed by rinsing twice with distilled water. The plates were then blotted on paper towels and air dried, and adherent bacteria were stained with 50 μL of 0.1% crystal violet for 15 min at room temperature. After rinsing twice, each time with 200 μL of distilled water, bound dye was extracted from the stained cells by using 200 μL of 99% ethanol. Biofilm formation was then quantified by measuring the absorbance of the solution at 595 nm with a microtiter plate reader (Model 3550; Bio-Rad Laboratories, Hercules, CA, USA). The Mann–Whitney *U*-test was used for all experiments to identify statistically significant differences.

Results and discussion

The inhibitory effects of polyphenol fraction on synergistic biofilm formation between *P. gingivalis* and *F. nucleatum* are summarized in Table 1. Biofilm formation between all combinations examined was significantly inhibited by the polyphenol fraction at a concentration of either 250 μg/mL or 500 μg/mL, compared with the controls ($p < 0.01$). Labrecque *et al.* (16) showed that cranberry nondialyzable material prevented biofilm formation of *P. gingivalis*. The present study indicated that cranberry polyphenol inhibits not only monobacterial biofilm formation, but also synergistic biofilm formation.

Table 1. Inhibitory effect of cranberry polyphenol on the synergistic biofilm formation of Porphyromonas gingivalis and Fusobacterium nucleatum

Strain	Concentration of cranberry polyphenol (μg/mL)	Biofilm formation (OD_{595})
Porphyromonas gingivalis ATCC 33277 + *Fusobacterium nucleatum* ATCC 25586	0 (control)	0.789 ± 0.410
	250	0.159 ± 0.018*
	500	0.160 ± 0.020*
Porphyromonas gingivalis ATCC 33277 + *Fusobacterium nucleatum* TDC 2	0 (control)	0.821 ± 0.237
	250	0.193 ± 0.016*
	500	0.158 ± 0.016*
Porphyromonas gingivalis ATCC 33277 + *Fusobacterium nucleatum* TDC 20	0 (control)	0.439 ± 0.107
	250	0.189 ± 0.011*
	500	0.164 ± 0.144*
Porphyromonas gingivalis FDC 381 + *Fusobacterium nucleatum* 25586	0 (control)	0.265 ± 0.041
	250	0.162 ± 0.030*
	500	0.161 ± 0.020*
Porphyromonas gingivalis FDC 381 + *Fusobacterium nucleatum* TDC 2	0 (control)	0.454 ± 0.195
	250	0.192 ± 0.040*
	500	0.161 ± 0.020*
Porphyromonas gingivalis FDC 381 + *Fusobacterium nucleatum* TDC 20	0 (control)	0.522 ± 0.153
	250	0.257 ± 0.111*
	500	0.182 ± 0.032*

Cranberry polyphenol was dissolved in Tryptic soy broth supplemented with 0.3% yeast extract, 0.25% glucose and L-cysteine-hydrochloride. Data are the mean values from two fourfold experiments with standard deviations.
*$p < 0.01$ compared with the control.

The inhibitory activity of polyphenol fraction against Arg-gingipain and Lys-gingipain was evaluated by colorimetric assay using synthetic substrates. Bacterial cells of *P. gingivalis* ATCC 33277 and FDC 381 were harvested by centrifugation (10,000 *g* for 20 min), washed and suspended in 50 mM phosphate-buffered saline (pH 7.4) to an optical density at 660 nm (OD_{660}) of 2.0. This cell suspension was used in the colorimetric assay for Arg-gingipain and Lys-gingipain activities. Benzoyl-arginine-*p*-nitroanilide (Sigma Chemical Co., St Louis, MO, USA) and tosyl-glycine-proline-lysine-*p*-nitroanilide (Sigma Chemical. Co.), in 160 μL of 0.1 M Tris-HCl (pH 8.0) containing 1 mM dithiothreitol, were used as substrates (final concentration, 0.5 mM) for Arg-gingipain and Lys-gingipain, respectively. The substrates were dispensed into the wells of a 96-well microtiter plate. Each 20 μL of bacterial cell suspension, and different concentrations of polyphenol, were added to the substrate and incubated at 37°C for 20 min. Adsorption at a wavelength of 405 nm (A_{405}) was determined by the microtiter plate reader. *P. gingivalis* cells exhibited high Arg-gingipain and Lys-gingipain activities [ATCC 33277: Arg-gingipain activity (A_{405})/control (A_{405}) = 1.159/0.055, Lys-gingipain activity (A_{405})/control (A_{405}) = 1.115/0.067; FDC 381: Arg-gingipain activity (A_{405})/control (A_{405}) = 0.910/0.055, Lys-gingipain activity (A_{405})/control (A_{405}) = 0.956/0.067; data represent averages with standard deviations of less than 15%] at the concentrations used in this study. Relative enzymatic activity was determined as follows: [(A_{405} with bacterial cells and polyphenol − A_{405} of control)/(A_{405} with bacterial cells − A_{405} of control)] × 100.

Degradation obtained in the absence of the polyphenol fraction was given a value of 100%. The effects of polyphenol on Arg-gingipain and Lys-gingipain activities are shown in Fig. 1. The polyphenol fraction was found to inhibit Arg-gingipain and Lys-gingipain activities in a dose-dependent manner. The activities of both *P. gingivalis* ATCC 33277 and FDC 381 were significantly inhibited by polyphenol fraction at ≥1 μg/mL ($p < 0.05$). Polyphenol fraction at 100 μg/mL showed an 87–91% inhibitory effect on gingipain activities in both strains.

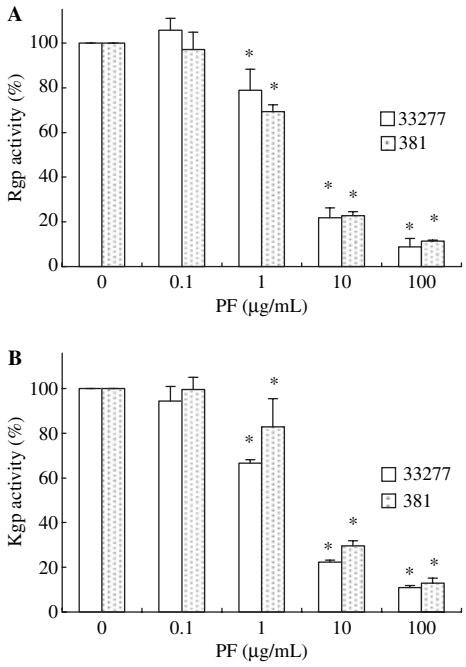

Fig. 1. Effects of polyphenol fraction on Arg-gingipain (A) and Lys-gingipain (B) activities in *Porphyromonas gingivalis* ATCC 33277 and FDC 381. Degradation in the absence of the polyphenol fraction was given a value of 100%. $^{*}p < 0.05$ between the polyphenol fraction at various concentrations and controls without the polyphenol fraction. Kgp, Lys-gingipain; PF, polyphenol fraction; Rgp, Arg-gingipain.

Kadowaki *et al.* (29) reported that gingipain inhibitors reduced the pathogenicity of *P. gingivalis*. Our results showed that cranberry polyphenol efficiently inhibited Arg-gingipain and Lys-gingipain activities in *P. gingivalis*. Bodet *et al.* (15) reported that a high-molecular-weight nondialyzable material fraction of cranberry significantly reduced both Arg-gingipain and Lys-gingipain activities at a concentration of 50 μg/mL and 25 μg/mL, respectively. Our cranberry polyphenol preparation significantly reduced these activities at a low concentration of 1 μg/mL compared with nondialyzable material, suggesting that small polyphenol molecules also effectively inhibit both proteases. It has been reported that Japanese green tea-purified catechins, especially (-)-epigallocatechin gallate, inhibit pathogenic factors of *Escherichia coli* 0157:H7 (30), *Helicobacter pylori* (31) and methicillin-resistant *Staphylococcus aureus* (32). (-)-Epigallocatechin gallate inhibited the growth and adherence of *P. gingivalis* to buccal epithelial cells (33), and Okamoto *et al.* (34) demonstrated that green tea catechins inhibited the Arg-gingipain and Lys-gingipain activities of *P. gingivalis*. We have compared the inhibitory activities of (-)-epigallocatechin gallate and cranberry polyphenol, and found that polyphenol fraction inhibited at the same level as (-)-epigallocatechin gallate (data not shown).

Our results showed that cranberry polyphenol is highly efficient at inhibiting proteases of *P. gingivalis* and its synergistic biofilm formation with *F. nucleatum*. This suggests that cranberry polyphenol has the potential to inhibit the development of dental plaque, and to prevent, or reduce the severity of, *P. gingivalis*-associated periodontal disease.

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