Oral Microbiology Immunology 2004: 19: 118–120
Printed in Denmark. All rights reserved

Oral Microbiology and Immunology

Short communication

Inhibitory effect of green tea catechins on cysteine proteinases in *Porphyromonas gingivalis*

M. Okamoto[1], A. Sugimoto[2], K. -P. Leung[3],K. Nakayama[4], A. Kamaguchi[5], N. Maeda[1]

[1]Department of Oral Bacteriology, Tsurumi University School of Dental Medicine, Kanagawa, Japan, [2]Itoen Ltd, Shizuoka, Japan, [3]US Army Dental Research Detachment, Great Lakes, IL, USA, [4]Department of Microbiology, School of Dentistry, Nagasaki University, Nagasaki, Japan, [5]Department of Oral Microbiology, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan

Okamoto M, Sugimoto A, Leung K-P, Nakayama K, Kamaguchi A, Maeda N. Inhibitory effect of green tea catechins on cysteine proteinases in Porphyromonas gingivalis.
Oral Microbiol Immunol 2004: 19: 118–120. © Blackwell Munksgaard, 2004.

The purpose of this study was to examine the effects of catechins and their derivatives on the activities of Arg-gingipain (Rgp) and Lys-gingipain (Kgp) in *Porphyromonas gingivalis*. Catechin derivatives, which included (−)-epigallocatechin gallate, (−)-epicatechin gallate, (−)-gallocatechin gallate, and (−)-catechin gallate, significantly inhibited the Rgp activity. The 50% inhibitory concentrations (IC50s) of these catechin derivatives for Rgp ranged from 3 to 5 μM. While (−)-epigallocatechin and (−)-gallocatechin moderately inhibited Rgp activity (IC50s, 20 μM), (−) -epicatechin, (+)-catechin, and gallic acid were not effective, with IC50s greater than 300 μM. Further, some of the catechin derivatives tested also inhibited the Kgp activity, though to a lesser extent than inhibition of the Rgp activity. These findings suggest that green tea catechins may have the potential to reduce periodontal breakdown resulting from the potent proteinase activity of *P. gingivalis*.

Key words: catechin; Rgp; Kgp; *Porphyromonas gingivalis*

Masaaki Okamoto, Department of Oral Bacteriology, Tsurumi University School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama, Kanagawa 230-8501, Japan
Tel.: +81 45 581 1001;
fax: +81 45 573 9599;
e-mail: okamoto-m@tsurumi-u.ac.jp
Accepted for publication August 25, 2003

Porphyromonas gingivalis, a gram-negative anaerobe, has been implicated as a major periodontopathic organism, due in part to its frequent recovery from periodontal lesions (22, 25) and the possession of potent virulence factors (6, 16). Among these virulent factors, cysteine proteinases, which include Arg-gingipain (Rgp) and Lys-gingipain (Kgp), are regarded as important virulence determinants, as demonstrated by various *in vitro* assays (1, 2, 12, 15, 27) and testing in murine abscess models (3, 9, 20). Kgp and Rgp cause the development of edema, neutrophil infiltration, and bleeding followed by karikrein/kinin pathway activation, complement pathway activation, and degradation of fibrinogen (9, 27). Further, the enzymes disrupt polymorphonuclear leukocyte functions, as shown by their inhibitory effects on the generation of active oxygen species from the activated cells (11). Therefore, in view of the potential of these enzymes to cause periodontal inflammation, it has been suggested that gingipains could be targets for drug- or vaccine-based treatment strategies. For example, gingipain-specific inhibitors could control adult periodontitis caused by *P. gingivalis* infection (4, 13, 14, 18, 19, 28).

There have been reports showing that the (−)-epigallocatechin gallate (EGCg), which is a predominant component of Japanese green tea catechins, exhibited a number of inhibitory effects on bacteria, tumor cells, and the host's immune systems. The bactericidal effects of tea catechins on pathogenic bacteria, such as *Escherichia coli* O157-H7 (26), *Helicobacter pylori* (17), and methicillin-resistant *Staphylococcus aureus* (29), were demonstrated by their ability i) to damage the bacterial lipid bilayer (8), ii) to inhibit bacterial enzymatic activities, and iii) to bind directly to peptidoglycan, as observed with beta lactams. The tea catechin is also capable of inhibiting collagenase activity associated with *P. gingivalis* (23). Recently, we have reported that EGCg and its derivative inhibit protein–tyrosine phosphatase activity in *Prevotella intermedia*, a putative periodontopathic organism (21). The purpose of this study was to investigate the effects of the EGCg and its derivatives from Japanese green tea on the activities of Rgp and Kgp in *P. gingivalis*.

The strains used in this study were *P. gingivalis* KDP129, a Kgp deficient mutant, and KDP133, an Rgp-deficient mutant, as previously described (24). Strain KDP129 was used as the source of Rgp and mutant strain KDP133 as the source of crude Kgp. *P. gingivalis* was maintained on Brucella HK agar supplemented with 5% laked sheep blood in an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 at 37 C. For the extraction of the enzymes, bacteria were cultured for 2 days in a brain–heart infusion broth supplemented with 0.5% yeast extract, 0.05% cysteine-HCl, 5 μg/ml of hemin, and 1 μg/ml of menadione under

the anaerobic conditions as described above. The cell-free culture supernatants were obtained by centrifugation of bacterial suspensions at 10,000 × *g* for 20 min at 4°C. Ammonium sulfate was added to culture supernatants until 70% saturation. The precipitated proteins were collected by centrifugation at 10,000 × *g* for 20 min, resuspended in 10 mM Tris buffer, pH 7.6, containing 0.05% Briji 35, and dialysed against the same buffer at 4°C overnight. This was used as the source of enzymes for the following studies.

A colorimetric assay was used for determining the effects of catechins on the activities of Rgp and Kgp. Benzoyl-arginine-*p*-nitroanilide (Sigma Chemical Co., St. Louis, MO) and tosyl-glycine-proline-lysine-*p*-nitroanilide (Sigma Chemical Co.) in 80 μl of 0.1 M Tris-HCl (pH 7.6) containing 1 mM cysteine, were used as the substrate (final concentration, 0.2 mM) for Rgp and Kgp, respectively. The substrate was dispensed into the wells of a 96-well microtiter plate. Crude enzyme extracts (10 μl) and different concentrations of catechins were added to the substrate and incubated at 37°C for 10 min. Final concentrations of catechins in the assay ranged from 0.3 to 300 μM. The absorption at the wavelength of 405 nm was determined by a multiwell microtiter plate reader (Labsystems Multiskan Multisoft, Labsystems, Finland). The inhibition of cysteine proteinase activity of the samples incubated in the presence of catechins was expressed as a percent of the activity exhibited by the enzyme incubated with the substrate alone.

The tea catechins used in this study, which included EGCg, epicatechin gallate (ECg), epigallocatechin (EGC) and epicatechin (EC), (−)-gallocatechin gallate (GCg), catechin gallate (Cg), gallocatechin (GC) and (+)-catechin (C), were purchased from Kurita Industries (Tokyo, Japan). Gallic acid was obtained from Sigma Chemical Co. (Fig. 1).

The effect of catechins on the Rgp activity was shown in Fig. 2. Catechin derivatives, which included EGCg, ECg, GCg, and Cg, significantly inhibited the Rgp activity. The 50% inhibitory concentrations (IC50s) of these catechin derivatives for the Rgp ranged from 3 to 5 μM. While EGC and GC moderately inhibited against the Rgp activity (IC50s, 20 μM), EC, C, and gallic acid were not effective, with the IC50s determined to be greater than 300 μM. Further, the effect of catechins on the Kgp activity was shown in Fig. 3. Some of the catechin derivatives tested inhibited the Kgp activity of

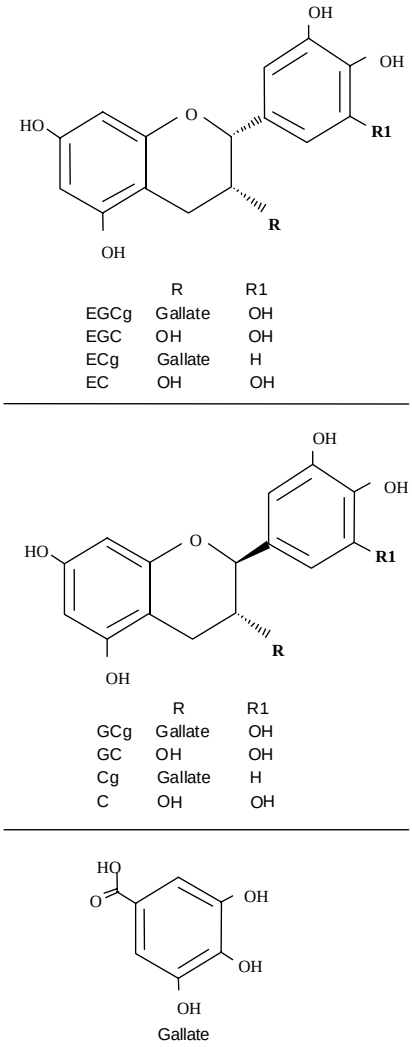

Fig. 1. Chemical structures of Japanese green tea catechins.

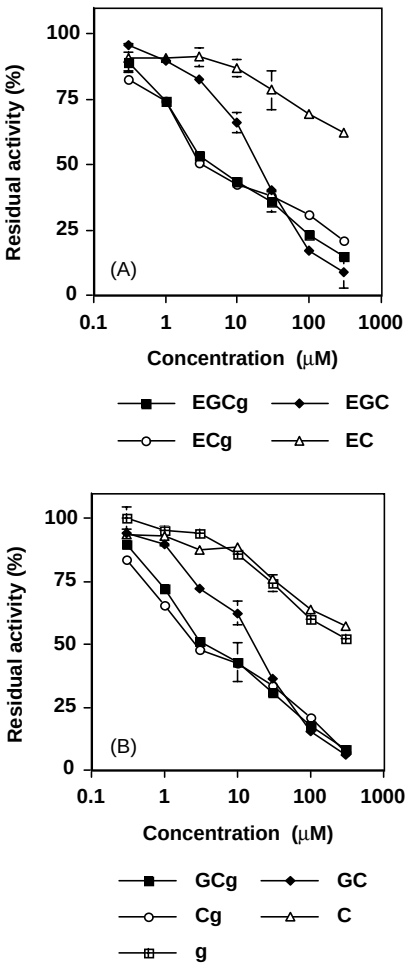

Fig. 2. Effects of EGCg, EGC, ECg, EC (Figure 2A) GCg, GC, Cg, C and gallic acid (Figure 2B) on the Rgp activity. Eighty μl of benzoyl-arginine-*p*-nitroanilide *p*-nitrophenyl phosphate (final concentration 0.2 mM) in 0.1 M Tris-HCl (pH 7.6) containing 1 mM cysteine, and 10 μl of various concentration of catechins were mixed with 10 μl enzyme in the 96-well microtiter plate, and then incubated at 37°C for 10 min. Values are expressed as mean residual activities (%) ± sd to that of the control (without the addition of catechins) from three experiments consisting of trplicate samples.

P. gingivalis, though to lesser extents as compared to their inhibition of the Rgp activity.

The results collectively suggest that some tea catechin derivatives exhibited inhibitory activity against Rgp and to lesser extents against Kgp of *P. gingivalis*. The observation that only tea catechin derivatives containing the galloyl moiety inhibited Rgp and Kgp activities suggests that the inhibitory effect observed is attributable to the presence of the galloyl moiety, which is linked to the 3-OH of the catechin or epicatechin moiety (Fig. 1).

Tetracycline, doxycycline, and chlorhexidine strongly inhibit the collagenase activity (5) of *P. gingivalis,* resulting in the reduction of the severity and progression of periodontal disease in animal models and humans. Our data also suggest that tea catechins were effective in inhibiting the enzymatic activities of *P. gingivalis* in a manner similar to that of chlorhexidine, doxycycline, and non-antimicrobial chemically modified tetracycle derivatives. The effective inhibitory concentrations exhibited by tea catechins were consistent with the observation that tetracycline, at 100 μM, totally inhibits the amidolytic activity of arginine-specific gingipains (RgpA and RgpB) (7, 10). Similarly, both tea catechins and tetracycline inhibited Kgp less efficiently, and higher concentrations of these agents are needed to achieve the similar inhibitory effect as seen against Rgp. Our findings suggest that green tea catechins might be useful in reducing periodontal tissue destruction by interfering

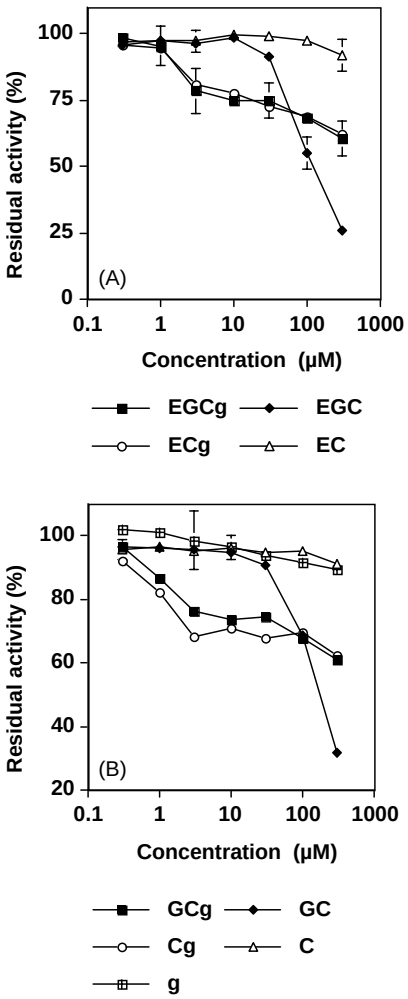

Fig. 3. Effects of EGCg, EGC, ECg, EC (Figure 3A) GCg, GC, Cg, C and gallic acid (Figure 3B) on the Kgp activity. Materials and methods were the same as Fig. 2, except for the use of tosyl-glycine-proline-lysine-*p*-nitroanilide as the substrate.

with the potent protease activity of *P. gingivalis*.

Acknowledgments

This investigation was supported in part by a Grant-in Aid for Scientific Research (C)No. 14571756 from the Ministry of Education, Science and Culture of Japan.

References

1. Abe N, Kadowaki T, Okamoto K, et al. Biochemical and functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of *Porphyromonas gingivalis* in periodontal disease. J Biochem (Tokyo) 1998: **123**: 305–312.
2. Curtis MA, Aduse-Opoku J, Rangarajan M. Cysteine proteases of *Porphyromonas gingivalis*. Crit Rev Oral Biol Med 2001: **12**: 192–216.
3. Genco CA, Potempa J, Mikolajczyk-Pawlinska J, Travis J. Role of gingipains R in the pathogenesis of *Porphyromonas gingivalis*-mediated periodontal disease. Clin Infect Dis 1999: **28**: 456–465.
4. Gibson FCr, Genco CA. Prevention of *Porphyromonas gingivalis*-induced oral bone loss following immunization with gingipain R1. Infect Immun 2001: **69**: 7959–7963.
5. Grenier D, Plamondon P, Sorsa T, et al. Inhibition of proteolytic, serpinolytic, and progelatinase-b activation activities of periodontopathogens by doxycycline and the non-antimicrobial chemically modified tetracycline derivatives. J Periodontol 2002: **73**: 79–85.
6. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000 1999: **20**: 168–238.
7. Houle MA, Grenier D, Plamondon P, Nakayama K. The collagenase activity of *Porphyromonas gingivalis* is due to Arg-gingipain. FEMS Microbiol Lett 2003: **221**: 181–185.
8. Ikigai H, Nakae T, Hara Y, Shimamura T. Bactericidal catechins damage the lipid bilayer. Biochim. Biophys. Acta 1993: **1147**: 132–136.
9. Imamura T. The role of gingipains in the pathogenesis of periodontal disease. J Periodontol 2003: **74**: 111–118.
10. Imamura T, Matsushita K, Travis J, Potempa J. Inhibition of trypsin-like cysteine proteinases (gingipains) from *Porphyromonas gingivalis* by tetracycline and its analogues. Antimicrob Agents Chemother 2001: **45**: 2871–2876.
11. Kadowaki T, Yoneda M, Okamoto K, Maeda K, Yamamoto K. Purification and characterization of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from the culture supernatant of *Porphyromonas gingivalis*. J Biol Chem 1994: **269**: 21371–21378.
12. Kadowaki T, Nakayama K, Okamoto K, et al. *Porphyromonas gingivalis* proteinases as virulence determinants in progression of periodontal diseases. J Biochem (Tokyo) 2000: **128**: 153–159.
13. Kitano S, Irimura K, Sasaki T, et al. Suppression of gingival inflammation induced by *Porphyromonas gingivalis* in rats by leupeptin. Jpn J Pharmacol 2001: **85**: 84–91.
14. Kuboniwa M, Amano A, Shizukuishi S, Nakagawa I, Hamada S. Specific antibodies to *Porphyromonas gingivalis* Lys-gingipain by DNA vaccination inhibit bacterial binding to hemoglobin and protect mice from infection. Infect Immun 2001: **69**: 2972–2979.
15. Kuramitsu HK. Proteases of *Porphyromonas gingivalis*: what don't they do? Oral Microbiol Immunol 1998: **13**: 263-270.
16. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev 1998: **62**: 1244–1263.
17. Mabe K, Yamada M, Oguni I, Takahashi T. In vitro and in vivo activities of tea catechins against *Helicobacter pylori*. Antimicrob Agents Chemother 1999: **43**: 1788–1791.
18. Moritz AJ, Cappelli D, Lantz MS, Holt SC, Ebersole JL. Immunization with *Porphyromonas gingivalis* cysteine protease: effects on experimental gingivitis and ligature-induced periodontitis in *Macaca fascicularis*. J Periodontol 1998: **69**: 686–697.
19. Nakagawa T, Sims T, Fan Q, et al. Functional characteristics of antibodies induced by Arg-gingipain (HRgpA) and Lys-gingipain (Kgp) from *Porphyromonas gingivalis*. Oral Microbiol Immunol 2001: **16**: 202–211.
20. O'Brien-Simpson NM, Paolini RA, Hoffmann B, et al. Role of RgpA, RgpB, and Kg*p* proteinases in virulence of *Porphyromonas gingivalis* W50 in a murine lesion model. Infect Immun 2001: **69**: 7527–7534.
21. Okamoto M, Takano K, Maeda N. Distribution of the tetracycline resistance determinant *tetQ* gene in oral isolates of black-pigmented anaerobes in Japan. Oral Microbiol Immunol 2001: **16**: 224–228.
22. Okamoto M, Leung KP, Ansai T, Sugimoto A, Maeda N. Inhibitory effects of green tea catechins on protein tyrosine phosphatase in *Prevotella intermedia*. Oral Microbiol Immunol 2003: **18**: 192–195.
23. Sakanaka S, Aizawa M, Kim M, Yamamoto T. Inhibitory effects of green tea polyphenols on growth and cellular adherence of an oral bacterium, *Porphyromonas gingivalis*. Biosci Biotechnol Biochem 1996: **60**: 745–749.
24. Shi Y, Ratnayake DB, Okamoto K, et al. Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgpA*, *rgpB*, *kgp*, and *hagA*. J Biol Chem 1999: **274**: 17955-17960.
25. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998: **25**: 134–144.
26. Sugita-Konishi Y, Hara-Kudo Y, Amano F, et al. Epigallocatechin gallate and gallocatechin gallate in green tea catechins inhibit extracellular release of Vero toxin from enterohemorrhagic *Escherichia coli* O157:H7. Biochim Biophys Acta 1999: **1472**: 42–50.
27. Travis J, Pike R, Imamura T, Potempa J. *Porphyromonas gingivalis* proteinases as virulence factors in the development of periodontitis. J Periodontal Res 1997: **32**: 120–125.
28. Yonezawa H, Ishihara K, Okuda K. Arg-gingipain a DNA vaccine induces protective immunity against infection by *Porphyromonas gingivalis* in a murine model. Infect Immun 2001: **69**: 2858–2864.
29. Zhao WH, Hu ZQ, Okubo S, Hara Y, Shimamura T. Mechanism of synergy between epigallocatechin gallate and beta-lactams against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 2001: **45**: 1737–1742.