Inhibitory Effect of Tea Catechins on Collagenase Activity

Masaharu Makimura, Masatomo Hirasawa,† Kihei Kobayashi,‡ Jiro Indo,§ Senji Sakanaka,§ Takashi Taguchi,* and Shigeo Otake**

A MAJOR PURPOSE OF THIS STUDY was to examine inhibitory effect of the catechin derivatives from Japanese green tea *Camellia sinensis* on collagenase activity. The crude tea catechins, which contain (+)-catechin (C), (−)-epicatechin (EC), (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and (−)-epigallocatechin gallate (EGCg), were tested for their ability to inhibit the prokaryotic and eukaryotic cell derived collagenase activities. Among the tea catechins tested, ECg and EGCg showed the most potent inhibitory effect on collagenase activity when an optimal concentration of tea catechins (100 μg/ml) was added to reaction mixture containing collagenase and collagen. Preincubation of collagenase with tea catechins reduced the collagenase activity as well. In contrast to ECg and EGCg, the other four tea catechins (C, EC, EGC, and GC) did not show any collagenase inhibitory effect. Our results suggest that the steric structure of 3-galloyl radical is important for the inhibition of collagenase activity. The collagenase activity in the gingival crevicular fluid from highly progressive adult periodontitis was completely inhibited by the addition of tea catechins. These results demonstrated that tea catechins containing galloyl radical possess the ability to inhibit both eukaryotic and prokaryotic cell derived collagenase. *J Periodontol 1993; 64:630–636.*

Key Words: Collagen; collagenase; gingival crevicular fluid; catechin; tea.

The destruction of collagen fiber by a specific enzyme, collagenase, is one of the unique characteristics of periodontal disease (PD). Thus, collagenase has been shown to be an important pathogenic factor for the development of PD.[1-5] Numerous reports demonstrated a positive correlation between the occurrence of high collagenase activity in the exudate from the gingival crevice and the severity of PD.[1,2,6,7] It has been reported that collagenase is mainly produced by inflammatory infiltrative cells and tissue cells such as neutrophil,[8] macrophage,[9] fibroblast, and epithelial cell.[10] Further, PD related bacteria including *Prevotella* and *Porphyromonas*[11,12] species, *Actinobacillus actinomycetemcomitans*,[12] and *Clostridium histolyticum*[13] were also capable of producing collagenase.

A number of investigators have reported that antibiotics such as tetracyclines possess beneficial effects in the treatment of PD.[14-17] The effectiveness of this family of antibiotics is thought to reflect its ability to suppress the growth of subgingival microorganisms.[18] Alternatively, it was shown that PD can be prevented by the inhibition of collagenase which was produced by both bacterial and mammalian cells. Thus, tetracyclines were shown to inhibit gingival collagenolytic enzyme activity and skin collagen resorption in both conventional and germfree rats.[19] Further, this antibiotic also inhibited collagenase activity in vitro.[20] Based on these findings, part of the mechanism of the therapeutic effect exhibited by tetracyclines is explained by the inhibition of collagenase activity.[4,19-23]

In this report, we have studied the effect of individual tea catechins extracted from Japanese green tea on GCF collagenolytic enzyme activity in order to provide possible alternative tools for the treatment of PD. Our results provided new evidence that specific forms of tea catechins (e.g., ECg and EGCg) can inhibit the activity of collagenase from both eukaryotic and prokaryotic cells.

MATERIALS AND METHODS

Tea Catechins

The tea catechins used in this study were originally isolated from the leaf of *Camellia sinensis*, known as Japanese green tea and found to contain six major catechins: (+)-catechin (C), (−)-epicatechin (EC), (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), and

*Nihon University School of Dentistry at Matsudo, Department of Clinical Pathology, Matsudo, Japan.
†Department of Microbiology.
‡Department of Complete Denture Prosthodontics.
§Central Research Laboratories, Taiyo Kagaku Co., Ltd., Yokkaichi, Japan.

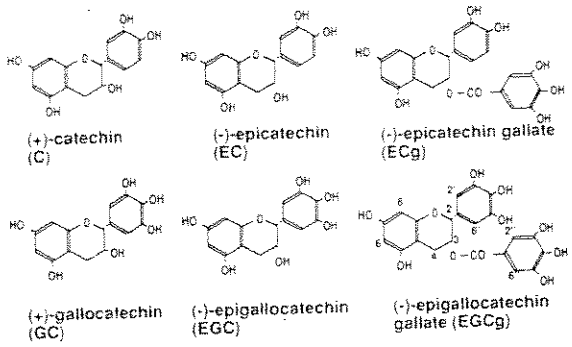

Figure 1. Structures of tea catechins.

Table 1. Clinical Observations

Patient	Age	Sex	Region	Pocket Depth (mm)	Bone Loss* (%)
A	39	F	10	8	50
B	38	M	6	6	40
C	42	F	7	8	60
D	45	F	10	6	50
E	36	M	10	7	50
F	41	F	10	7	50
G	34	M	6	6	40

* Value of bone loss from the CEJ to the apex of the root was determined from radiographs.

(−)-epigallocatechin gallate (EGCg).[24] Details of the purification and chemical characterization of individual catechins have been described by Sakanaka et al.[24] Briefly, tea catechin was extracted from tea leaves using ethyl acetate. Individual catechins were further fractionated by silica gel column using methanochloroform (10:1) as elution solvent. Each fraction was then purified by recycled high-performance liquid chromatography using a PVA HP-GPC column 'JAIGEL GS-320'.[§] The structural formulas of the individual tea catechins are shown in Figure 1.

Test Collagenase

The commercially available collagenase of *Clostridium histolyticum*# origin which originated from leech## and colagenolytic proteinase from Kamchatka crab‡‡ was used in this study. Further, a supernatant of *Porphyromonas gingivalis* 381 culture was used as collagenolytic proteinase of pathogenic bacteria related to PD.

Test Gingival Crevicular Fluids

Test gingival crevicular fluids (GCF) were collected using a previously described intracrevicular method[3] from the periodontal pocket of 7 patients with highly progressive adult periodontitis (3 male; 4 female, between the ages of 34 and 45) (Table 1).

Collagenase activity was determined using a commercially-available kit.‡‡

Manipulative method. A mixture solution containing 100 μl of 0.1M acetic acid with 0.1% fluorescent collagen and 100 μl of 0.1M-Tris-HCl buffer (containing 0.4M NaCl and 10mM $CaCl_2$) was mixed with 200 μl of test solution (collagenase or GCF sample) and incubated at 35°C for the appropriate period. To terminate the reaction, 10 μl of 80mM o-phenanthroline was added to the mixture. After additional incubation for 60 minutes at 35°C to denature the broken collagen, the mixture was vigorously stirred by adding 400 μl of 0.17M-Tris-HCl buffer (pH 9.5, containing 0.67M-NaCl), with 70% ethanol to precipitate non-denatured collagen. After centrifugation for 10 minutes at 3,000 rpm, the supernatant was measured using a spectrophotofluorimeter§§ at 495 nm of excitation wavelength and 520 nm of fluorescent wavelength. Substrate collagen solution was heated to 80°C for 10 minutes and cooled to room temperature to measure the total amount of collagen.

Detecting the activity of test collagenase in this assay system. To investigate the influence of collagenase concentration and incubation time on the inhibitory effect of catechins, various concentrations of collagenase and incubation time (10, 25, and 50ng/ml and 30, 60, and 120 minutes, respectively) were used. The ratio of decomposed collagen to the total collagen was calculated from the absorbance and indicated by percent digestion. A GCF sample diluted 200 times was used as the test solution and a test was made by setting the reaction time at 120 minutes. The enzyme activity which decomposes collagen in 1 μg per minute was indicated as 1 unit from the absorbance ratio of the decomposed collagen to the total collagen.

Elucidation of the Inhibition of Collagenase Activity by Tea Catechin

Addition of tea catechin to reaction mixture. The same method described above was used to investigate the effect of tea catechins on collagenase activity, except that 100 μl of tea catechin solution (0–100 μg/ml) was added to 100 μl of collagen-collagenase solution (100 ng/ml) and incubation time was 120 minutes.

Pretreatment of collagen with tea catechin. To investigate whether or not the inhibition of collagenase activity by tea catechin is due to the influence on collagen, 2 μl of tea catechin solution (5 mg/ml) was added to 200 μl of collagen solution. After 30 to 60 minutes incubation at 4°C, ethanol was added to the mixture, since ethanol treatment solubilized denatured collagen while native collagen is insoluble. After stirring, the mixture was centrifuged for 10 minutes at 3,000 rpm. The sediment was then dissolved in 0.01M acetic acid prior to use as collagen solution, using the manipulative method. As control, 2 μl of PBS was added to collagenase solution instead of tea catechin.

[§]Japan Analytical Industry Co., Tokyo, Japan
#Amano, Taiyo Kagaku Co., Ltd., Yokkaichi, Japan
##Collagenase [illegible] U.K.

‡‡Collagen Giken, Tokyo, Japan.
§§650-10S, Hitachi, Tokyo, Japan.

Pretreatment of collagenase with tea catechin. To directly demonstrate the inhibition of collagenase activity by tea catechin, collagenase solution was preincubated with tea catechin (50 μg/ml) for 60 minutes at 4°C prior to the manipulative method. The enzyme activity was compared with control sample which was pretreated with an equal volume of buffer without tea catechin.

Inhibition of Collagenase Activity by Refined Polyphenol Compounds

An experiment was performed by a similar manipulative method described above; 50 μg/ml of the refined individual tea catechins were subjected to collagen-collagenase mixture.

Inhibitory Effect of Collagenase Activity in GCF by Tea Catechin

A mixture containing 100 μl of tea catechin solution (100 μl/ml) and 100 μl of the GCF sample diluted 100× obtained from 7 patients was used as sample solution for manipulative method. The sample solution not containing tea catechin was used as a control.

RESULTS

Determination of Test Collagenase Activity

To determine an optimal condition for the collagenase activity in this manipulative assay system, different concentrations of *C. histolyticum* derived collagenase (10 to 50 ng/ml) were added to reaction mixture containing a known amount of collagen (500 μg/ml). These reaction mixtures were then incubated for different time periods (30 to 120 minutes). The activity of collagenase was elevated according to the increased amount of collagenase (Fig. 2). Further, it was shown that the collagenase activity increased with the extension of incubation time. Thus, the reaction mixture containing 50 ng/ml of collagenase demonstrated approximately 85 to 90% of collagen digestion after 2 hours of incubation. Based on this finding, collagenase concentration at 50 ng/ml and incubation time of 120 minutes were used in the manipulative method to determine the inhibitory effect of tea catechin and the individual tea catechins on bacterial collagenase activity.

Inhibition of Collagenase Activity by Tea Catechin

Addition of tea catechin to reaction mixture. To investigate the effect of tea catechins on collagenase activity, various concentrations of tea catechin (0, 25, 50, and 100 μg/ml) were added to the *C. histolyticum* collagenase assay solution and to the 100 μl of double concentrated collagen-collagenase solution in the manipulative assay. As shown in Figure 3, addition of increased concentration of tea catechin resulted in decreased collagenase activity. Approximately 50% of collagenase activity was reduced at concentration of 50 μg/ml of tea catechin. Further, total inhibition of the collagenase activity was achieved by 100 μg/ml of tea catechin. The values are indicated in the percentage of the sample activity against control (Figure 3).

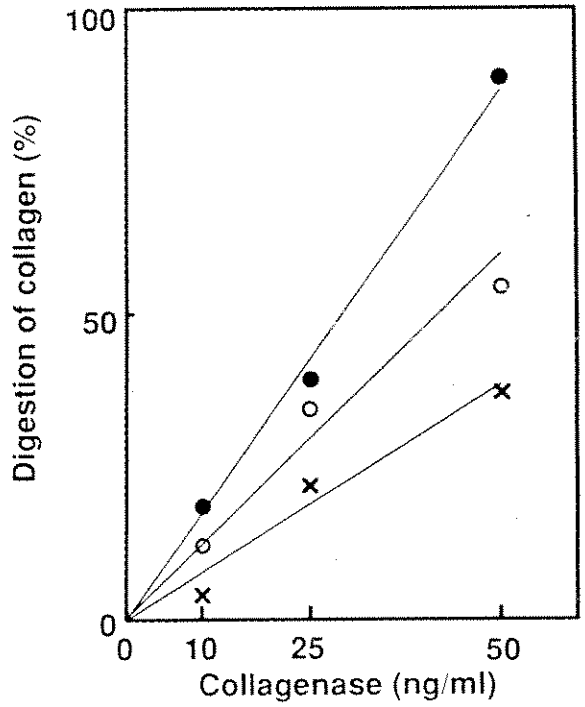

Figure 2. Digestion of collagen by varying concentration of collagenase in different incubation time: × = 30 minutes; ○ = 60 minutes, and ● = 120 minutes.

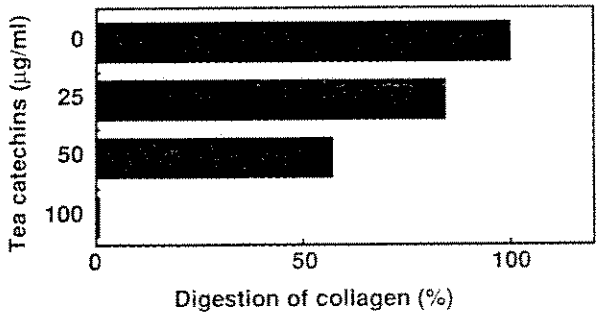

Figure 3. Effect of different concentration of tea catechins on collagenase activity. Various concentrations of tea catechins (0 to 100 μg/ml) were added to the reaction mixture containing 500 μg/ml of collagen and 50 ng/ml of collagenase and incubated for 120 minutes at 35°C.

Effect of pretreatment of collagen with tea catechin. Since optimal concentration of tea catechin (50 to 100 μg/ml) inhibited the collagenase activity in the reaction mixture containing collagenase and collagen, it was important to clarify the mechanism for the inhibitory activity of tea catechin. Therefore, in the next series of experiments, collagen was pretreated with tea catechin prior to the assay to determine whether tea catechin affected collagen or collagenase. Figure 4 shows the value of collagenase activ-

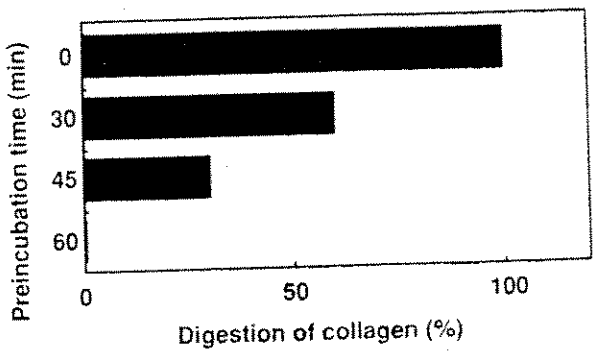

Figure 4. Effect for the preincubation of collagen with tea catechins in different incubation periods (0 to 60 minutes). Collagen (500 μg/ml) was preincubated with 100 μg/ml of tea catechins for 0 to 60 minutes prior to the reaction with 50 ng/ml of collagenase.

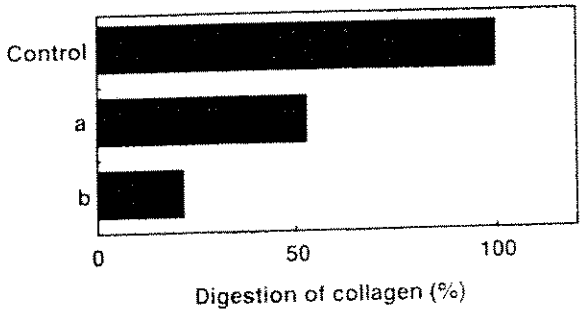

Figure 5. Influence for the preincubation of collagenase with tea catechins. As positive control, 500 μg/ml of collagen was mixed with 50 ng/ml of collagenase. In experiment a 500 μg/ml of collagen was mixed with 50 μg/ml of tea catechins and 50 ng/ml of collagenase without any preincubation. In experiment b, 50 ng/ml of collagenase was preincubated with 50 μg/ml of tea catechins prior to the reaction with 500 μg/ml of collagen.

ity when collagen was pretreated with 100 μg/ml tea catechin for 30, 45, or 60 minutes. Values are presented as a percentage of the activity at the time when collagen was pretreated with the control solution for 60 minutes (100%). A decrease of collagenase activity was observed with extension of the pretreatment time with tea catechin and was inhibited completely by treatment of more than 60 minutes. Collagenase activity was decreased 5 to 10% by pre-treatment with ethanol.

Influence of pretreatment of collagenase with tea catechin. To test the possibility that tea catechin directly inhibits collagenase activity, collagenase was pretreated with tea catechin prior to the assay. Since it was shown that 100 μg/ml of tea catechin totally inhibited the collagenase activity in this assay system (Figure 3), even if the pretreatment did have some effect on enzyme activity, it would not be detected. Thus in this experiment, we used 50 μg/ml of tea catechin concentration, as the concentration which would give one-half [illegible] effect. "Control" in Figure 5 shows [illegible] collagenase activity obtained by the [illegible] method, and "a" indicates values when 50 μg/ml of tea catechin was added to the test collagenase solution concurrently. The value in "b" represents the collagenase activity of pretreated collagenase with 50 μg/ml tea catechin for 60 minutes before the assay. Values are indicated in percentage to the control. Inhibition of collagenase activity was observed in about 40% by concurrent addition of tea catechin, while further increase of the inhibitory effect was observed with pretreatment of collagenase with tea catechin.

Inhibition of Collagenase Activity by Individual Refined Tea Catechins

Since it was shown that tea catechin contained six individual polyphenols (Fig. 1), we investigated involvement of these refined tea catechins for the inhibitory effect on collagenase activity. When individual components of tea catechins were added to the reaction mixture containing collagenase and collagen, ECg and EGCg (50 μg/ml) completely inhibited collagenase activity (Fig. 6). In addition, pretreatment of collagenase with ECg and EGCg resulted in the reduction of enzyme activity (data not shown). On the other hand, other tea catechins including C, EC, GC, and EGC did not suppress collagenase activity (Fig. 6). Further, usage of excess amounts of C, EC, GC, or EGC did not show any inhibitory effect (data not shown). These findings suggested that EC and EGC with galloyl radical are essential components for the inhibitory effect of tea catechin on collagenase.

Inhibitory Effect of Tea Catechin on Other Collagenase or Collagenolytic Proteinase

According to the results of our study, tea catechin seems to have an inhibitory effect on *C. histolyticum* collagenase. We then determined whether tea catechin can act on other collagenase or collagenolytic proteinase. In this regard, collagenase from leech and collagenolytic proteinase from *Paralithodes camtshatica* (Kamchatka crab) were used as examples of eukaryotic-cell derived collagenase. Further, supernatant of *Porphyromonas gingivalis* 381 was employed as an example of collagenolytic proteinase from prokaryotic cells. As expected, all these enzyme activities were reduced in the presence of tea catechin, and 100 μg/ml of tea catechin completely inhibited these enzyme activities (Fig. 7).

Inhibition of Collagenase Activity in GCF by Tea Catechin

Since our results showed that tea catechin can inhibit purified collagenase, we next examined whether this tea catechin can affect collagenase activity in GCF. When GCF was obtained from 7 patients and examined for collagenase activity, an average of 82.3 ± 24.0 units of collagenase activity was noted (Table 2). Addition of tea catechin (100 μg/ml) resulted in the complete inhibition of collagenase activity in these samples. These findings suggest that tea

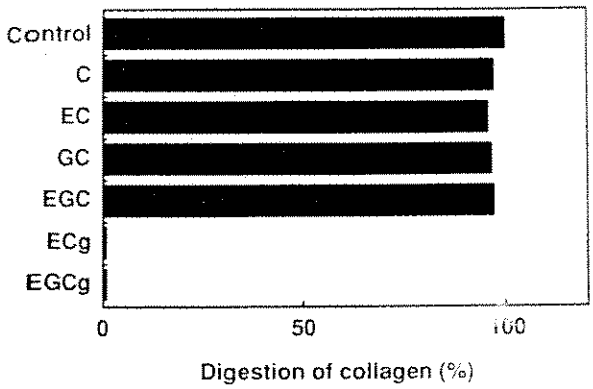

Figure 6. Elucidation of tea catechins which provide inhibition of collagenase activity. 50 μg/ml of each polyphenol compound such as C = (+)-catechin; GC = (+)-gallocatechin; EC = (−)-epicatechin; EGC = (−)-epigallocatechin; ECg = (−)-epicatechin gallate; and EGCg = (−)-epigallocatechin gallate was added to the reaction mixture containing 500 μg/ml of collagen and 50 ng/ml of collagenase.

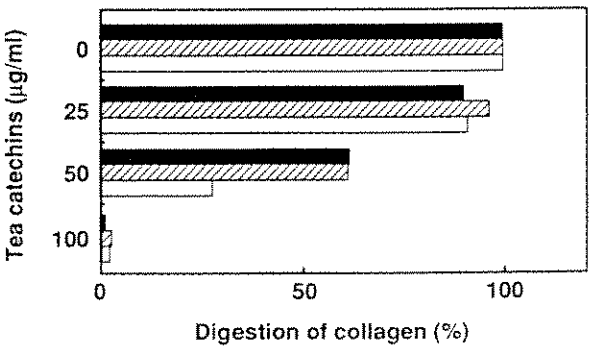

Figure 7. Tea catechin inhibits various collagenolytic proteinase. Different collagenase or collagenolytic proteinase from leech (■), Paralithodes camtshatica (Kamchatka crab), (▨) and Porphyromonas gingivalis *38J (□) were tested. An optimal concentration of tea catechins (100 μg/ml) was added to the reaction mixture containing 50 ng/ml of purified collagenase and 500 μg/ml of collagen. In the case of* P. gingivalis*-derived collagenase, culture supernatants was used as a source of prokaryotic cell derived collagenase.*

catechin can down regulate collagenase activity in GCF isolated from patients with adult periodontitis.

DISCUSSION

It has been shown that collagenase plays an important role in the disruption of the collagen component in the gingival tissues of patients with periodontal disease.[1-5] Collagenase is produced by both prokaryotic and eukaryotic cells; however, bacterium and mammalian cell-derived collagenases possess biochemically distinct characteristics. The collagenase of bacterial origin degrades collagen molecules into small peptides, whereas the collagenase of mammalian origin catalyzes collagen molecule to 3/4 and 1/4 fragments by cleaving it at a certain point.[1] It has been demonstrated that

Table 2. Effect of Tea Catechin on Collagenase Activity in Gingival Crevicular Fluid

Patient	Non-Treated	Treated*
A	82.3†	—‡
B	48.6	—
C	60.2	—
D	96.5	—
E	103.2	—
F	70.5	—
G	114.8	—

*Concentration of 100 μg/ml.
†Unit/ml.
‡Not detected.

the most collagenase activity seen in GCF is of the latter origin.[1,5,6,8,10,25,26] However, it is also presumed that the collagenase generated by the major periodontal pathogenic bacteria such as *Porphyromonas gingivalis*, *Prevotella* species, or *Actinobacillus actinomycetemcomitans* may be involved in the destruction of gingival connective tissues.[27]

Several reports have suggested that the improvement in periodontal conditions treated by tetracyclines or doxycyclines results from their ability to inhibit collagenase activity in addition to the antibacterial function.[19-23] These studies have provided a possibility that prevention of periodontal disease can be achieved by inhibition of collagenase activity. In this regard, we have made an attempt to test the possible influence of tea catechin on the eukaryotic and prokaryotic cell-generated collagenase activities. Our experimental results demonstrated that tea catechin possesses inhibitory effects on *C. histolyticum* collagenase activity (Fig. 3).

Among various refined tea catechins, remarkable inhibitory activity was observed by ECg and EGCg, but not with other polyphenols such as C, EC, EGC, or GC (Fig. 6). A common characteristic of ECg and EGCg is the presence of a galloyl radical (Fig. 1). These findings suggest that presence of 3-galloyl in the tea catechin structure can be important in this inhibitory effect for collagenase activity (Fig. 6). In a previous report,[28] tea catechins containing galloyl radical possessed inhibitory effect on glucosyltransferase isolated from *Streptococcus mutans*. Further, other studies[29,30] reported that gallotannins also inhibited glucosyltransferase activity. The results we obtained support this concept, since only tea catechins containing galloyl radical (ECg and EGCg) exhibited collagenase inhibitory activity.

In the present study, pretreatment of collagenase with an optimal concentration of tea catechin resulted in the reduction of its activity (Fig. 4). Our separate study results indicated that tea catechins did not denature collagenase molecule, since SDS-PAGE analysis and precipitation tests of tea catechin containing ECg and EGCg with collagenase were weaker when compared with tannic acid. However a distinct sediment was observed with the tea catechin treatment. This finding suggests that tea catechin may aggregate, rather than denature collagenase molecules, which results in the inhibition of enzyme activity (unpublished

data). It was also indicated that the inhibition occurs by the pretreatment of collagen with tea catechin (Fig. 5). Taken together, it is possible to postulate that the inhibitory mechanisms of tea catechin on collagenase activity could be a result of binding of ECg and EGCg to collagenase, which may lead to the aggregation of the enzyme and the blockage of enzymatic activity. This possibility is also supported by the previous studies which described the inhibitory effect of tea catechin and refined tea catechins on the adsorption of *Streptococcus mutans* to saliva-coated hydroxyapatite disk.[28,31]

Finally, tea catechin inhibited the collagenolytic activity in GCF obtained from patients with adult periodontitis. It has been shown that most of the collagenase activity in GCF originates from the prokaryotic cells.[1,5,6,8,10,25,26] However, it is also suggested that the collagenase generated by the major periodontal pathogenic bacteria may be involved in the destruction of gingival connective tissues.[27] In this regard, collagenase and collagenolytic proteinase from eukaryotic cell origin and culture supernatants from *Porphyromonas gingivalis* were employed in this study in order to examine the inhibitory effect of tea catechin. A remarkable inhibitory effect of both eukaryotic and prokaryotic cell derived collagenase activity was observed by tea catechin treatment (Figs. 6 and 7). These results suggest a possibility that tea catechins containing ECg and EGCg can prevent the digestion of collagen by collagenase in periodontal disease. It should be noted that the concentration of tea catechin and its refined catechins, which possess inhibitory effect on collagenase activity, might be physiologically relevant, since 100 ml of green tea contains approximately 50 to 100 mg of tea catechin and the daily consumption of green tea per person in Japan is 300 to 400 ml. Although a high amount of tea catechins are consumed on a daily basis, it is not known whether effective concentration of tea catechins is present in GCF or circulating blood. However, as presented in this study, tea catechin has the ability to directly reduce collagenase activity. Continuous application of tea catechins on a daily basis can be considered as a useful and practical method for the prevention of periodontal diseases.

REFERENCES

1. Larivée J, Sodek J, Ferrier JM. Collagenase and collagenase inhibitor activities in crevicular fluid of patients receiving treatment for localized juvenile periodontitis. *J Periodont Res* 1986; 21:702–715.
2. Fullmer HM. Metabolic hydrolysis of collagen. In: Boyer PD, ed. *The Enzymes, Hydrolysis: Peptide Bonds*. 3rd ed. New York: Academic Press Inc; 1971: 301–343.
3. Robertson PB, Simpson J. Collagenase: Current concepts and relevance to periodontal disease. *J Periodontol* 1976; 47:29–33.
4. Golub LM, Wolff M, Lee HM, et al. Further evidence that tetracyclines inhibit collagenase activity in human crevicular fluid and from other mammalian sources. *J Periodont Res* 1985; 20:12–23.
5. Uitto V-J, Raeste A-M. Activation of latent collagenase of human leukocytes and gingival fluid by bacterial plaque. *J Dent Res* 1978; 57:844–851.
6. Golub LM, Ste[illegible] NS, Mandel ID. Some characteristics [illegible] and its relationship to [illegible] diseases in humans. *J Dent Res* [illegible]
7. Golub LM, Kaplan R, Mulvihill JE, Ramamurthy NS. Collagenolytic activity of crevicular fluid and of adjacent gingival tissue. *J Dent Res* 1979; 58:2132–2136.
8. Lazarus GS, Daniels JR, Brown RS, Bladen HA, Fullmer, HM. Degradation of collagen by a human granulocyte collagenolytic system. *J Clin Invest* 1968; 47:2622–2629.
9. Wahl LM, Wahl SM, Mergenhagen SE, Martin GR. Collagenase production by lymphokine-activated macrophages. *Science* 1975; 187:261–263.
10. Fullmer HM, Gibson WA, Lazarus GS, Bladen HA, Whedon KA. The origin of collagenase in periodontal tissues of man. *J Dent Res* 1969; 48:646–651.
11. Gibbons RJ, MacDonald JB. Degradation of collagenous substrates by *Bacteroides melaninogenicus*. *J Bacteriol* 1961; 81:614–621.
12. Robertson PB, Lantz M, Marucha PT, Kornman KS, Trummel CL, Holt SC. Collagenolytic activity associated with *Bacteroides* species and *Actinobacillus actinomycetemcomitans*. *J Periodont Res* 1982; 17:275–283.
13. Alfano MC, Morhart RE, Metcalf G, Drummond JF. Presence of collagenase from *Clostridium histolyticum* in gingival sulcal debris of a primitive population. *J Dent Res* 1974; 53:142.
14. Slots J, Mashimo P, Levine MJ, Genco, RJ. Periodontal therapy in humans. I. Microbiological and clinical effects of a single course of periodontal scaling and root planing and of adjunctive tetracycline therapy. *J Periodontol* 1979; 50:495–509.
15. Genco RJ. Antibiotics in the treatment of human periodontal diseases. *J Periodontol* 1981; 52:545–558.
16. Kornman KS, Karl EH. The effect of long-term low dose tetracycline therapy on the subgingival microflora in refractory adult periodontitis. *J Periodontol* 1982; 53:604–610.
17. Williams RC, Leone CW, Jeffcoat MK, Scott ER, Goldhaber P. Tetracycline treatment of periodontal disease in the beagle dog. I. Clinical and radiographic course over 12 month-maximal effect on rate of alveolar bone loss. *J Periodont Res* 1981; 16:659–665.
18. Walker CB, Gordon JM, McQuidkin SJ, Nielbloom TA, Socransky SS. Tetracycline: Levels achievable in gingival crevice fluid and *in vitro* effect on subgingival organisms. II. Susceptibilities of periodontal bacteria. *J Periodontol* 1981; 52:613–616.
19. Golub LM, Lee HM, Lehrer G, et al. Minocycline reduces gingival collagenolytic activity during diabetes. Preliminary observations and a proposed new mechanism of action. *J Periodont Res* 1983; 18:516–526.
20. Golub LM, Ramamurthy N, McNamara TF, et al. Tetracyclines inhibit tissue collagenase activity. A new mechanism in the treatment of periodontal disease. *J Periodont Res* 1984; 19:651–655.
21. Golub LM, Goodson JM, Lee HM, Vidal AM, McNamara TF, Ramamurthy NS. Tetracyclines inhibit tissue collagenases. Effects of ingested low-dose and local delivery systems. *J Periodontol* (Suppl.) 1985; 56:93–97.
22. McCulloch CAG, Birek P, Overall C, Aitken S, Lee W, Kulkarni G. Randomized controlled trial of doxycycline in prevention of recurrent periodontitis in high risk patients: antimicrobial activity and collagenase inhibition. *J Clin Periodontol* 1990; 17:616–622.
23. Goulb LM, Wolff M, Lee HM, et al. Evidence that tetracyclines inhibit collagenase activity in human crevicular fluid and from other mammalian sources. *J Periodont Res* 1985; 20:12–23.
24. Sakanaka S, Kim M, Taniguchi M, Yamamoto T. Antibacterial substances in Japanese green tea extract against *Streptococcus mutans*, a cariogenic bacterium. *Agric Biol Chem* 1989; 53(9):2307–2311.
25. Kowashi Y, Jaccard F, Cimasoni G. Increase of free collagenase and neutral protease activities in the gingival crevice during experimental gingivitis in man. *Arch Oral Biol* 1979; 24:645–650.
26. Sodek J, Hurum S, Feng J. A highly sensitive and specific assay for vertebrate collagenase. *J Periodont Res* 1981; 16:425–433.
27. Slots J, Genco RJ. Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: Virulence factors in colonization, survival, and tissue destruction. *J Dent Res* 1984; 63:412–421.

28. Otake S, Makimura M, Kuroki T, Nishihara Y, Hirasawa M. Anticaries effects of polyphenolic compounds from Japanese green tea. *Caries Res* 1991; 25:438–443.
29. Kakiuchi N, Hattori M, Nishizawa M, Yamagishi T, Okuda T, Namba T. Studies on dental caries prevention by traditional medicines. VIII. Inhibitory effect of various tannins on glucan synthesis by glucosyltransferase from *Streptococcus mutans*. *Chem Pharm Bull* 1986; 34:720–725.
30. Wu-Yuan CD, Chen CY, Wu RT. Galltannins inhibit growth, water-insoluble glucan synthesis, and aggregation of mutans streptococci. *J Dent Res* 1988; 67:51–55.
31. Sakanaka S, Sato T, Kim M, Yamamoto T. Inhibitory effects of green tea polyphenols on glucan synthesis and cellular adherence of cariogenic streptococci. *Agric Biol Chem* 1990; 54:2925–2929.
32. Maeda S, Nakagawa M. General chemical and physical analyses on various kinds of green tea (in Japanese). *Chagyo kennkyuhoukoku* 1977; 45:85–92.

Send reprint requests to: Dr. Masaharu Makimura, Department of Clinical Pathology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-Nishi, Matsudo, Chiba 271, Japan.

Accepted for publication January 11, 1993.