Inhibitory Effect of Tea Catechins on Collagenase Activity

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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 sterical 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.6–9 It has been reported that collagenase is mainly produced by inflammatory infiltrative cells and tissue cells such as neutrophil, macrophage, fibroblast, and epithelial cell.10 Further, PD related bacteria including *Prevotella* and *Porphyromonas* species, *Actinobacillus actinomycetemcomitans,* and *Clethriondium histolyticum* 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.4–23

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 EGC) 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
Collagenase activity was determined using a commercially-available kit.21

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 CaCl2) 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 0.57M-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-collagen solution (100 ng/ml) and incubation time was 120 minutes.

Pre-treatment 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 ng/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.
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 \( \mu \text{g/ml} \)) 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 \( \mu \text{g/ml} \) 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 \( \mu \text{l} \) of tea catechin solution (100 \( \mu \text{l/ml} \)) and 100 \( \mu \text{l} \) of the GCF sample diluted 100 \( \times \) 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 \( C. \) \textit{histolyticum} derived collagenase (10 to 50 ng/ml) were added to reaction mixture containing a known amount of collagen (500 \( \mu \text{g/ml} \)). 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 \( \mu \text{g/ml} \)) were added to the \( C. \) \textit{histolyticum} collagenase assay solution at the 100 \( \mu \text{l} \) 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 \( \mu \text{g/ml} \) of tea catechin. Further, total inhibition of the collagenase activity was achieved by 100 \( \mu \text{g/ml} \) of tea catechin. The values are indicated in the percentage of the sample activity against control (Figure 3).

Effect of pretreatment of collagen with tea catechin. Since optimal concentration of tea catechin (50 to 100 \( \mu \text{g/ml} \)) inhibited the collagenase activity in the reaction mixture containing collagen and collagenase, 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-
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 Parasilphodes canstia (Kamchatka crab) were used as examples of cukaryotic-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...
CATECHIN INHIBITION OF COLLAGENASES

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 were added to the reaction mixture containing 300 μg/ml of collagen and 50 μg/ml of collagenase.

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

<table>
<thead>
<tr>
<th>Patient</th>
<th>Non-Treated</th>
<th>Treated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>82.3%</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>48.6%</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>60.2%</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>96.5%</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>103.2%</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>70.5%</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>114.8%</td>
<td>-</td>
</tr>
</tbody>
</table>

*Concentration of 100 μg/ml.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Unit/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>μg/ml</td>
</tr>
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</table>

| Not detected. |

Digestion of collagen (%)

Figure 7. Tea catechin inhibit various collagenolytic proteinase. Different collagenase or collagenolytic proteinase from leech (Ⅰ), Parabacteroides distasonis (Camellia sinensis), (Ⅱ) and Porphyromonas gingivalis (Ⅲ) (Ⅳ) were tested. An optimal concentration of tea catechin (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 the most collagenase activity seen in GCF is of the latter origin.1,5,8,10,15,20 However, it is also presumed that the collagenase generated by the major periodontal pathogenic bacteria such as Porphyromonas gingivalis, Prevotella species, or Actinobacillus actinomyctetemcomitans may be involved in the destruction of gingival connective tissues.21

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 periodental 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 H. 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, E, EC, 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 studies29,30 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 cation 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 ECg to collagenase, which may lead to the aggregation of the enzyme and the blockade 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.16-31

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 proteolytic cells.1,5,8,10,12,26 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 proteins 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 200 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


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Accepted for publication January 11, 1993.