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Green tea catechin inhibits the activity and neutrophil release of Matrix Metalloproteinase-9

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ABSTRACT

Green tea (*Camellia sinensis*; 綠茶 lǜ chá) extracts have been shown to possess anti-oxidant and anti-inflammatory effects in various cell types. Green tea extract (GTX) has been shown to significantly inhibit the activity of collagenase-3 (matrix metalloproteinase-13 (MMP-13)) *in vitro*. MMPs, such as MMP-9, are known to be involved in many inflammatory diseases including periodontal disease. GTX and a major catechin, epigallocatechin-gallate (EGCG), were examined for their ability to inhibit purified MMP-9 activity and its release from stimulated neutrophils.

Methanol extract of Green tea and commercially purchased EGCG (>95 % purity) were tested *in vitro* for their ability to inhibit MMP-9 activity and/or its release from neutrophils using a β-casein cleavage assay and gelatin zymography, respectively. Statistical analysis was performed by Student's t-test.

GTX and EGCG at 0.1% (w/v) completely inhibited the activity of MMP-9. In addition, GTX and EGCG (0.1 %) significantly inhibited ($p < 0.001$) the release of MMP-9 from formyl-Met-Leu-Phe (FMLP)-stimulated human neutrophils by 62.01% ± 6.717 and 79.63% ± 1.308, respectively. The inhibitory effects of GTX and EGCG occurred in unstimulated neutrophils (52.42% ± 3.443 and 62.33% ± 5.809, respectively). When the inhibitory effect of EGCG was further characterized, it significantly inhibited the release of MMP-9 from the FMLP-stimulated human neutrophils in a dose-dependent manner.

The effects of GTX and EGCG on MMPs could be extrapolated to clinical/*in vivo* studies for the development of oral care products to prevent or treat chronic inflammatory diseases including periodontal diseases.

1. Introduction

Bacterial invasion stimulates infiltrated neutrophils to produce reactive oxygen species (ROS). These ROS modulate various enzyme activities including protein kinases, ion channels, membrane receptors and transcriptional factors like nuclear factor kappa B (NFκB). In turn, these stimulate the production of cytokines and host matrix metalloproteinases (MMPs).[1]

MMPs are a family of at least 27 zinc-containing endopeptidases that are classified as collagenases, gelatinases, stromelysins, membrane-type MMPs and others.[2] MMP-9 (gelatinase-B) is believed to be involved in many inflammatory diseases including periodontal diseases, tumor growth and metastasis, arthritis and cardiovascular diseases.[3–6] MMP-9 is expressed in neutrophils constitutively before they are released from the bone marrow to the circulation as part of the innate defense armory.[7] The expression of the MMPs also occurs at the tissue level after neutrophil migration upon infection in an inductive manner. MMP-9 is stored in neutrophils' granules and released upon activation.[7]

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Periodontal diseases involve inflammation of the supporting connective tissues with subsequent loss of alveolar bone.[4,5] Overproduction and/or activation of the host derived MMPs eventually lead to the destruction of the periodontal extracellular matrix.[6] MMP-9 can be activated by hypochlorous acid (HOCl) generated by neutrophil myeloperoxidase (MPO) utilizing H_2O_2 as a substrate.[7] This suggests that ROS production and MMP-mediated periodontal diseases are directly related.[8]

The main polyphenols in Green tea (綠茶 lǜ chá) extract (GTX) are derivatives of catechin (flavanols) that include epigallocatechin gallate (EGCG), epigallocatechin, gallocatechin, gallocatechin gallate, epicatechin and epicatechin gallate. GTX has anti-oxidant activities. GTX and its catechins have been shown to inhibit ROS production including superoxide (O_2^-) and nitric oxide (NO) *in vitro* in a dose-dependent manner.[9] Green tea catechins have also been shown to be effective in the prevention and treatment of periodontal disease and dental caries due to their anti-microbial activity.[10,11] Their inhibition of bacterial amylase activity and the growth of oral microorganisms have been documented.[12,13] GTX has also been shown to have anti-inflammatory and anti-proliferative properties.[14,15]

Since the activation and release of MMP-9 from stimulated neutrophils upon infection play a part in inducing periodontal diseases and other chronic inflammatory diseases, any agent that could inhibit the production of ROS and also inhibit the activity or release of MMP-9 from stimulated neutrophils would be a novel "multi-pronged" mechanism for the prevention and treatment of various inflammatory diseases including periodontal disease. In the present study, the ability of GTX and its major catechin, EGCG, to inhibit the activity of MMP-9 and its release from stimulated neutrophils were examined *in vitro*.

2. Materials and methods

2.1. Reagents

Green tea (*Camellia sinensis*; 綠茶 lǜ chá) was cultivated in the Chonnam province in Korea. The green tea were then stored and processed at the Department of Food and Technology, Chonnam University, Gwangju, Korea. After being air-dried under a fume hood at room temperature, the aerial parts of the plants were crushed using a super mixer (model SM2000, Retch, Germany). The dried materials were extracted in 80% methanol (MeOH) for 24 h and filtered through Whatman No. 2 filter paper. The MeOH extract was then concentrated using a vacuum evaporator (Type N-2N, Eyela, Tokyo, Japan) attached to a cooling aspirator. The concentrated MeOH GTX was then lyophilized (FDU-540, Eyela, Tokyo, Japan). The GTX was then dissolved in 10 mM dimethyl sulphoxide (DMSO) before being diluted in phosphate buffered saline (PBS). The EGCG (95 % purity) was purchased from Calbiochem (La Jolla, CA). EGCG was diluted in PBS prior to conducting the experiments. Human MMP-9 was purified by gelatin sepharose from conditioned media as previously described.[16]

2.2. EGCG analysis

High-performance liquid chromatography (HPLC) equipped with a UV-Vis detector was used to determine the ECGC content using maximal absorption peaks at 280 nm. HPLC analysis was performed on Agilent 1100 system (Palo Alto, CA) using an Eclipse XDB-C18 chromatography column (3.5 μm, 4.6 × 150 mm) with a 20 μL injection volume. A binary mobile phase consisting of solvent systems A and B were used in an isocratic elution with 90:10 A:B. Mobile phase A and B was 2% formic acid (v/v) in ddH_20 and 100% acetonitrile, respectively. The mobile phase flow rate was 1.0 mL/min and the run time was 15 min. The retention time for EGCG was 6.07 min. The amount of ECGC was calculated from a standard curve equation.

2.3. β-Casein cleavage assay

The ability of GTX or EGCG to inhibit the activity of MMP-9 was examined using a β-Casein cleavage assay. Briefly, activated purified samples (3.0 μg/ml) of human MMP-9 were incubated with 1.25 mg/ml β-casein (Mr 21,000) at 37 °C.[17] Samples with or without GTX or EGCG were periodically (0–60 min) removed and the reactions stopped by addition of 1, 10-phenanthroline to a final concentration of 10 mM. The samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie blue and analyzed by densitometry. Serial dilution of GTX was utilized to determine the dose-response.

2.4. Human neutrophils

Buffy coats separated from healthy human donor blood were obtained from the Central Indiana Regional Blood Center (Indianapolis, IN) with Institutional Review Board approval. A double-sucrose gradient, Histopaque-1119 (3 ml) and Histopaque-1077 (3 ml), was used to separate the neutrophils by centrifugation at 20 °C for 35 min. The lower band containing the granulocytes was drawn off. After washing with 10 ml PBS, the cells were centrifuged at 950 rpm for 10 min and the supernatant was discarded. The washing procedure was repeated twice and the cells were resuspended in 10 ml of Roswell Park Memorial Institute (RPMI) 1640 (Sigma Co. St. Louis, MO) media.[18] After counting, the cells were adjusted to 2.2×10^7 cells/ml. Trypan Blue staining was utilized to determine the viability of the harvested cells.

2.5. Gelatin zymography

To determine if GTX or EGCG could inhibit the release of MMP-9 from the neutrophils, gelatin zymography was utilized. Human neutrophils (10^6/ml) were stimulated with 10^{-6} M *N*-formyl-Met-Leu-Phe (FMLP) for 30 min at 37 °C with or without GTX or EGCG. After incubation, the cells were pelleted by centrifugation at 14,000 rpm for 5 min and the collected supernatant was analyzed for MMP-9 release. The supernatant samples were resolved in 10% SDS-PAGE gels co-polymerized with 1 mg/ml gelatin. After electrophoresis, the gels were washed with solution 1 (50 mM Tris, pH. 7.5, 3 mM NaN_3, 2.5 % Triton X-100), solution 2 (50 mM Tris, pH 7.5, 3 mM NaN_3, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 2.5 % Triton X-100) and solution 3 (50 mM Tris, pH 7.5, 3 mM NaN_3, 5 mM $CaCl_2$, 1 μM $ZnCl_2$) for 20 min each. The gels were then incubated in fresh solution 3 overnight at 37 °C. The gels were later stained with Coomassie blue to visualize the lytic bands. The density of the bands was analyzed by NIH 1.62 Image.

2.6. Statistical analysis

The significance between the control and test groups was determined by the Student's t-test. The data was expressed as mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. EGCG analysis

The amount of ECGC was calculated from the standard curve equation ($r^2 = 0.99917$) and the concentration of ECGC in the 1%

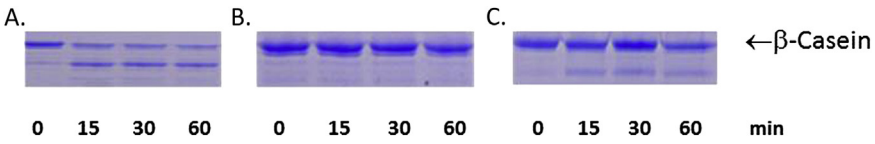

Fig. 1. GTX and EGCG Inhibition of MMP-9 Activity. Activated purified samples (3.0 μg/ml) of human MMP-9 were incubated with 1.25 mg/ml β-casein (Mr 21,000) at 37 °C with or without 0.1% GTX or 0.1% EGCG. Samples were periodically (0–60 min) removed and the reactions stopped by addition of 1, 10-phenanthroline (10 mM). The samples were analyzed by SDS-PAGE and stained with Coomasie blue. (A) control, (B) Green tea extract, and (C) EGCG.

green tea (綠茶 lǜ chá) extract was 0.94 ± 0.01 mg/mL (0.094% ECGC).

3.2. β-casein cleavage assay

Serial dilutions of GTX were used to determine the dose-response. Consequently, 0.1% GTX was chosen for the experiments because it effectively inhibited the cleavage of the β-casein by MMP-9. EGCG at 0.1% (1 mg/ml) also effectively inhibited the activity of MMP-9 compared to the untreated control (Fig. 1).

3.3. Gelatin zymography

Both GTX (0.1 %) and EGCG (0.1%) significantly ($p < 0.001$) inhibited the release of MMP-9 from untreated neutrophils by 51.42 ± 3.443 % and 62.33 ± 5.809 %, respectively. GTX or EGCG at also significantly ($p < 0.001$) inhibited the release of MMP-9 from 10^{-6} M FMLP-stimulated human neutrophils by 62.01% ± 6.717 and 79.63% ± 1.308, respectively (Figs. 2 and 3). When the inhibitory effects of EGCG was further tested in serial dilutions, it significantly ($p < 0.001$) inhibited the release of MMP-9 from the FMLP-stimulated neutrophils in a dose-dependent manner (Fig. 4 and Table 1).

4. Discussion

The anti-oxidant properties of plant extracts have been demonstrated in numerous studies, but little is known about their anti-inflammatory effects related to human neutrophil functions. The current study examined whether extracts from green tea (綠茶 lǜ chá) and its major catechin, EGCG, could modulate MMP-9 activity or release from neutrophils. MMP-9 has been suggested to play a role in many inflammatory diseases including periodontal diseases, cancer cell migration, arthritis and degeneration of central nervous systems. Therefore, testing any compound, which inhibits production of ROS followed by inhibition of MMP-9 and/or its release from stimulated neutrophils would be a novel "multi-pronged" approach to prevent and possibly treat inflammatory conditions.

The current study demonstrated that GTX (0.1%) and EGCG (0.1%) significantly inhibited the activity of MMP-9 and its release from stimulated human neutrophils in a dose-dependent manner. EGCG has been shown to exert the greatest anti-oxidant activity among the catechins (flavanols) as the most hydroxylated catechin among the polyphenols from GTX. It is esterified to gallic acid (3, 4, 5-trihydroxy benzoic acid) at the 3-OH group in the C ring. Although there is no electron delocalization between the A and B rings (to be stabilized after donating electrons) due to the saturation of the heterocyclic C ring, it can be stable by resonance between the A and B aromatic ring structures.[19] Green tea catechins, including EGCG, chelate transient metal ions (iron or copper) involved in Fenton-reaction via the dihydroxy phenolic structure of the B ring.[19] This could explain the inhibitory effects of EGCG on MMPs. This also prevents further oxidative stress-mediated cell damage (Fig. 5). The amount of ECGC in 0.1% GTX was 0.094 mg/ml and 1 mg/ml in the 0.1% ECGC. The level of MMP-9 inhibition by 0.1% ECGC (1 mg/ml) was not significantly different from the MMP-9 inhibition by 0.1% GTX (0.094% ECGC). This clearly indicates that other components in the GTX had MMP-9 inhibitory activities.

The soybean isoflavone (genistein) and pine needle extract (contains a flavonol, quercetin) have been shown to inhibit the neutrophil respiratory burst activation either alone or together in a synergistic manner.[20] Compared to the flavanols such as EGCG, these flavonols have structural advantages with 4- oxo and 2, 3-unsaturated double bonds in C ring enabling them delocalize electrons more efficiently. However, EGCG has been shown to have the same degree of anti-oxidant capacity due to its highly hydroxylated aromatic ring structures.[19] In addition, genistein, quercetin and EGCG, are all known inhibitors of protein tyrosine kinases owing to the similarity of their B ring structure to tyrosine

Fig. 2. 0.1 % GTX and EGCG Inhibit MMP-9 Release. Human neutrophils (10^6 cells/ml) were stimulated with and without 10^{-6} M *N*-formyl-Met-Leu-Phe (FMLP) for 30 min at 37 °C with or without GTX and EGCG. Samples were analyzed by zymography (1 mg/ml gelatin). After incubation overnight, the gels were stained with Coomassie blue to visualize the lytic bands. Lane 1: Untreated (Control), Lane 2: 0.1% GTX, Lane 3: 0.1% EGCG, Lane 4: 10^{-6} M FMLP, Lane 5: 10^{-6} M FMLP/0.1 %GTX, Lane 6: 10^{-6} M FMLP/0.1% EGCG.

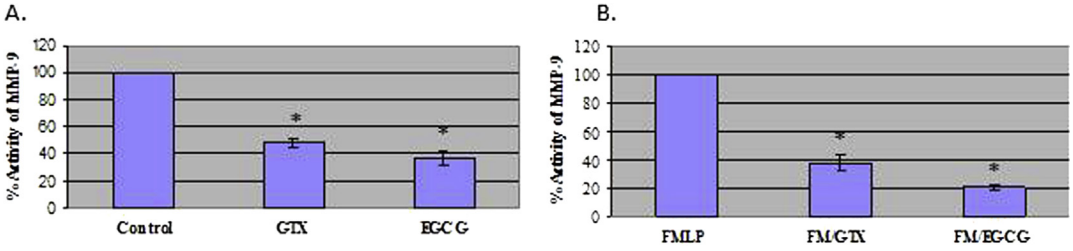

Fig. 3. Inhibition of MMP-9 release. MMP-9 released from the untreated or treated neutrophils calculated from the zymogram utilizing NIH 1.62 Image. 0.1% (w/v) GTX and EGCG significantly ($p < 0.05$) inhibited MMP-9 release from untreated neutrophils (Panel A) or FMLP-stimulated neutrophils (Panel B). Data were expressed as mean of 5 determinants ± SEM. The zymograams were standardized to each other utilizing the untreated control and FMLP-treated control within each group. * Statistically significant at $p < 0.001$.

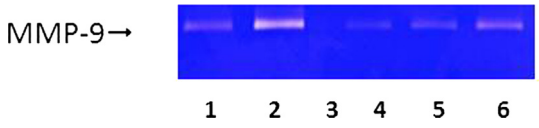

Fig. 4. Dose-dependant inhibition of MMP-9 Release by EGCG. Human neutrophils (10^6 cells/ml) were stimulated with 10^{-6} M FMLP for 30 min at 37 °C with and without EGCG. The samples were analyzed by zymography (1 mg/ml gelatin). After incubation overnight, the gels were stained with Coomassie blue to visualize the lytic bands. Lane 1: Control, Lane 2: 10^{-6} M FMLP, Lane 3: 10^{-6} M FMLP/0.2 % EGCG, Lane 4: 10^{-6} M FMLP/0.1 % EGCG, Lane 5: 10^{-6} M FMLP/0.05 % EGCG, Lane 6: 10^{-6} M FMLP/0.025 % EGCG.

Table 1
Dose-dependent Inhibition of FMLP-stimulated Neutrophil MMP-9 Release by EGCG.

Treatments	% Inhibition
FMLP-stimulated	0.00
FMLP/0.2 % EGCG	87.86 ± 1.65[a]
FMLP/0.1 % EGCG	82.56 ± 2.36[a]
FMLP/0.05 % EGCG	77.20 ± 3.37[a]
FMLP/0.0025 % EGCG	60.27 ± 5.61[a]

The inhibition of the release of MMP-9 from FMLP-stimulated neutrophils was calculated from the gelatin zymographs using NIH image 1.62. Data are expressed as mean ± SEM.
[a] Statistically significant at $p < 0.001$.

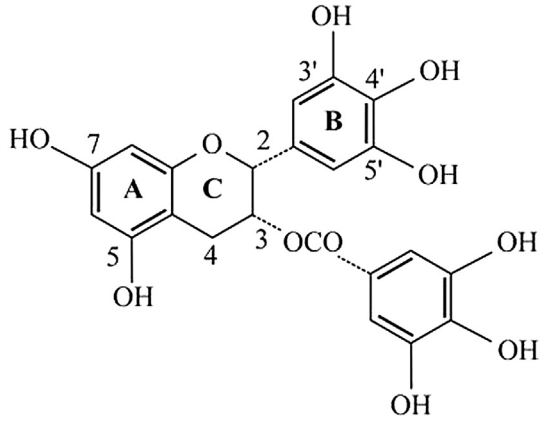

Fig. 5. Structure of epi-gallocatechin-gallate (EGCG).

residue.[19] The ability to inhibit protein tyrosine kinase results in the inhibition of NFκB followed by inhibition of pro-inflammatory gene expressions such as the MMPs and COX-2.[21] Previous studies have demonstrated that both genistein and pine needle extract completely inhibited the release of MMP-9 from phorbol myristate acetate (PMA)-activated neutrophils.[22] The current data with EGCG is consistent with these findings. Others also have shown that the kinase-mediated expression of pro-inflammatory genes (i.e., MMPs and cytokines) is inhibited by genistein.[23]

5. Conclusion

Plant polyphenols modify free radical generation and signaling pathways to regulate pro-inflammatory gene expressions.[21] Furthermore, the bioavailability of plant polyphenols has been assessed in humans[24,25] and EGCG is considered as to be GRAS (generally recognized as safe) by the Food and Drug Administration (FDA). The inhibitory ability of the GTX on the activity or release of MMP-9 could be, in part, caused by EGCG. Therefore, the findings from this study with GTX and EGCG could be extrapolated to clinical/*in vivo* studies for the development of oral care products to prevent or treat chronic inflammatory diseases including periodontal diseases.

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