Anticytotoxic Effect of Green Tea Catechin on Lipopolysaccharide from Aggregatibacter actinomycetemcomitans

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Abstract
Green tea is an aqueous infusion of the dried unfermented leaves of Camellia sinensis (family Theaceae), for which numerous biological activities have been reported, including antimutagenic, antibacterial, hypcholesterolemic, antioxidant, antitumor and cancer preventive activities. Aggregatibacter actinomycetemcomitans is implicated in the etiology of aggressive periodontitis and chronic periodontitis. We previously reported that LPS from Aggregatibacter actinomycetemcomitans (Aa-LPS) had strong cytotoxic effects against human leukemia cell lines (HL60 cells, THP-1 cells) and human gingival fibroblasts. The purpose of this study was to investigate the anticytotoxic effect of green tea catechin on Aa-LPS. When the cell, Aa-LPS, and the catechin components (EGCg, ECg, C and GC) were incubated, EGCg and Cg showed a strong anticytotoxic effect on Aa-LPS. Furthermore, when the catechin-pretreated cells and Aa-LPS were incubated, EGCg and Cg showed a strong anticytotoxic effect on Aa-LPS. Thus, it was suggested that the gallate moiety included in green tea catechins showed anticytotoxic effects against LPS from Aggregatibacter actinomycetemcomitans.

Introduction
Consumption of catechins has been demonstrated to render a variety of beneficial effects, such as antioxidative, antitumor, antimutagenic, and antihypertensive activities (1, 2, 3, 4, 5, 6). Green tea contains catechins, a class of low molecular weight polyphenols that consist mainly of flavan-3-ol monomers. Catechins are present mainly as catechin (C), catechin gallate (Cg), gallocatechin (GC), gallocatechin gallate (GCg), epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg). Green tea leaves normally contain 10% to 20% of catechins, mainly EGCg (7).

Aggregatibacter actinomycetemcomitans, a Gram-negative oral bacterium, has been implicated as one of the virulent bacteria involved in the immunopathogenesis of chronic inflammatory periodontal disease. Increased frequencies of this periodontopathic bacterium have been observed in patients with chronic inflammatory periodontal disease, particularly those with early onset periodontitis (8, 9). LPS, one of the virulent factors isolated from this periodontopathic bacterium, has been shown to stimulate bone resorption (10), epithelial cell destruction (11) and proinflammatory cytokine production by human cells such as monocytes and fibroblasts (12, 13), indicating that LPS from A. actinomycetemcomitans (Aa-LPS) may play a crucial role in periodontal tissue destruction during the course of chronic inflammatory periodontal disease.

We have reported the cytotoxic effect of Aa-LPS recently (14). In this study, we examined the effect of green tea catechin on the cytotoxic effects of Aa-LPS.

Materials and Methods
Cell culture
HL60 cells, THP-1 cells (Cell Bank, Riken Bioresource Center, Ibaraki, Japan) and HGFs (obtained from Department of Renascent Dentistry, Nihon University School of Dentistry at Matsudo, Chiba, Japan) were maintained in...
RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% of fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) for 48 h at 37°C under a 5% CO2 atmosphere. Before use, the medium was discarded and adherent HGFs were treated with Trypsin-EDTA (0.25 w/v % trypsin, 1 mmol l\(^{-1}\) EDTA-4Na; Wako, Osaka, Japan) for 5 min at 37°C. HL60 cells, THP-1 cells and planktonic HGFs were washed twice in phosphate-buffered saline (PBS; Wako, Japan) to remove the medium and then were resuspended with PBS.

Bacterial cultures and LPS preparation

*A. actinomycetemcomitans* strain Y4 was grown in brain-heart infusion broth supplemented with 1% yeast extract (Becton Dickinson & Co., Sparks, MD) at 37°C under a 5% CO2 atmosphere. LPS was extracted using the hot phenol-water method and partially purified by Sephacryl S-200 HR (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as described by Takada et al. (14).

Catechins

The catechins used in this study were (−)-epigallocatechin gallate (EGCg), (−)-epicatechin gallate (ECg), (+)-catechin (C) and (+)-gallocatechin (GC), purchased from Funakoshi Co. (Tokyo, Japan).

Influence of green tea catechins on cytotoxic effects of Aa-LPS

One hundred microliters of reaction mixture, \(2 \times 10^6\) cells ml\(^{-1}\) each cells, 500 μg ml\(^{-1}\) Aa-LPS and 1 mg ml\(^{-1}\) catechins, were incubated for 60 min at 37°C in an atmosphere of 5% CO2 in the air. After incubation, the reaction mixtures were placed on ice, 100μl trypan blue (0.4%) was added, and surviving cells were counted in a hemocytometer. At least four fields were counted and cell numbers were averaged for each assay. The percent of cell lysis was calculated by dividing the number of dead cells by the total cell number and multiplying by 100. The control reaction was without Aa-LPS.

Influence of green tea catechin-pretreated cells on cytotoxic effect of LPS

Influence of green tea catechin-pretreated cells on cytotoxic effects of LPS was investigated: 1 mg ml\(^{-1}\) catechins and \(2 \times 10^6\) cells ml\(^{-1}\) each cell were incubated for 30 min at 37°C in an atmosphere of 5% CO2 in the air. After incubation, each catechin-pretreated cell was washed twice with PBS. Catechin-pretreated cells were mixed with 500μg ml\(^{-1}\) Aa-LPS and made up to a final volume of 100μl with PBS. Cytotoxicity and calculations were performed as described above.

Statistical Analysis

The data are presented as the mean ± the standard deviation and compared using the two-tailed Student’s *t* test. A *P* value of < 0.05 was considered significant.

Results

**Influence of green tea catechins on cytotoxic effect of Aa-LPS**

The influence of green tea catechins on cytotoxic effect of Aa-LPS was investigated. LPS-untreated HL60 cells as a control showed 4.8% cell lysis after 0 and 60 min of incubation. HL60 cells treated with 500μg ml\(^{-1}\) Aa-LPS showed a 69.0% death rate after 60 min incubation. Aa-LPS-mediated lysis in the presence of catechins was observed to be 69.0% by C, 7.7% by ECg, 66.0% by GC and 12.3% by EGCg (Fig. 1A). The cytotoxic effect of green tea catechins on HL60 cells was not observed (data not shown).

LPS-untreated THP-1 cells as a control showed 7.8% cell lysis after 0 and 60 min of incubation. THP-1 cells treated with 500μg ml\(^{-1}\) Aa-LPS showed a 92.2% death rate after 60 min incubation. Aa-LPS-mediated lysis in the presence of catechins was observed to be 86.0% by C, 21.2% by ECg, 88.3% by GC and 14.0% by EGCg (Fig. 1B). As for the influence of green tea catechins on THP-1 cells, THP-1 cells were slightly lysed by ECg and EGCg; however, C and GC did not influence cell lysis (data not shown).

LPS-untreated HGFs as a control showed 7.8% cell lysis after 0 and 60 min incubation. HGFs treated with 500μg ml\(^{-1}\) Aa-LPS showed a 93.4% death rate after 60 min incubation. Aa-LPS-mediated lysis in the presence of catechins was observed to be 84.5% by C, 8.3% by ECg, 78.3% by GC and 12.0% by EGCg (Fig. 1C). Cytotoxic effects of green tea catechins on HGFs were not observed (data not shown).

**Influence of cytotoxic effects of LPS on green tea catechin-pretreated cells**

LPS-untreated HL60 cells as a control showed 7.0% cell lysis after 0 and 60 min of incubation. HL60 cells treated with 500μg ml\(^{-1}\) Aa-LPS showed a 93.4% death rate after 60 min of incubation. Aa-LPS-mediated lysis in the presence of catechins was observed to be 84.5% by C, 8.3% by ECg, 78.3% by GC and 12.0% by EGCg (Fig. 2A).
Fig. 1 Anticytotoxic effect of green tea catechins on Aa-LPS. (A), HL60 cells; (B), THP-1 cells; (C), HGFs. The results are expressed as the mean ± standard deviation of four independent experiments. Asterisks indicate $P < 0.05$ vs. each cell without catechin and incubated with catechins. 1, cell alone; 2, cell incubated with Aa-LPS; 3, cell incubated with Aa-LPS and C; 4, cell incubated with Aa-LPS and ECg; 5, cell incubated with Aa-LPS and GC; 6, cell incubated with Aa-LPS and EGCg.

Fig. 2 Cytotoxic effects of Aa-LPS on catechin-pretreated cells. (A), HL60 cells; (B), THP-1 cells; (C), HGFs. The results are expressed as the mean ± standard deviation of four independent experiments. Asterisks indicate $P < 0.05$ vs. each cell without catechin and incubated with catechins. 1, cell alone; 2, cell incubated with Aa-LPS; 3, C-pretreated cells incubated with Aa-LPS and; 4, ECg-pretreated cells incubated with Aa-LPS; 5, GC-pretreated cells incubated with Aa-LPS; 6, EGCg-pretreated cells incubated with Aa-LPS.
LPS-untreated THP-1 cells as a control showed 9.3% cell lysis after 0 and 60 min of incubation. THP-1 cells treated with 500μg ml⁻¹ Aa-LPS accounted for 90.0%. Aa-LPS-mediated lysis of THP-1 cells pretreated with catechins accounted for 90.3% by C, 21.3% by ECg, 90.3% by GC and 20.0% by EGCg (Fig. 2B).

LPS-untreated HGFs as a control showed 14.7% cell lysis after 0 and 60 min of incubation. THP-1 cells treated with 500μg ml⁻¹ Aa-LPS accounted for 90.0%. Aa-LPS-mediated lysis of HGFs pretreated with catechins accounted for 83.3% by C, 12.7% by ECg, 84.7% by GC and 14.7% by EGCg (Fig. 2C).

**Discussion**

Catechin components of green tea are responsible for the observed antibacterial activity, antioxidative, antitumor, antimutagenic, and antihypertensive activities. We have previously reported the cytotoxic effect of Aa-LPS on human leukemia cell and HGFs(14); therefore, we examined the influence of green tea catechin on the cytotoxic effects of Aa-LPS.

Aa-LPS showed strong cytotoxic effects on each cell. When Aa-LPS, catechin components and each cell were incubated, ECg and EGCg strongly inhibited the cytotoxic effects of Aa-LPS on each cell. It was considered that ECg and EGCg prevent cell lysis by binding to each cell or Aa-LPS. Furthermore, we investigated the cytotoxic effects of Aa-LPS on catechin-pretreated cells. Aa-LPS was not able to lyse on ECg- and EGCg-pretreated cells. These findings suggested that the gallate moiety of green tea catechins may inhibit the cytotoxic effect of Aa-LPS by covering the LPS binding site on the cell-surface. The formation of catechin/protein aggregates has been reported(16). It has been reported that EGCg is able to bind CD antigen and laminin receptor on the cell surface(17, 18). Otake et al. (19) and Hattori et al. (20) have demonstrated that EGCg and ECg inhibit streptococcal glucosyltransferase. Also, Makimura et al. have demonstrated the inhibitory effects of EGCg and ECg on collagenase activity (21). It was suggested that gallate forms of catechins were bound to the proteinaceous LPS binding sites on the cell surface such as TLR4 and CD14 and inhibited the interaction between each cell and Aa-LPS.

A cup of tea (about 150ml) contains 250 to 300mg of catechins. Taken together with the bactericidal activity of tea(22, 23), our results further suggest that tea or components of tea may be candidates for the prevention of periodontal disease.

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**References**

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