Green Tea Catechins Quench the Fluorescence of Bacteria-Conjugated Alexa Fluor Dyes

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Abstract: Accumulating evidence suggests that Green tea polyphenolic catechins, especially the (-)-epigallocatechin gallate (EGCG), can be cross-linked to many proteins, and confer a wide range of anti-bacterial activities possibly by damaging microbial cytoplasmic lipids and proteins. At the doses that conferred protection against lethal polymicrobial infection (induced by cecal ligation and puncture), EGCG significantly reduced bacterial loads particularly in the liver and lung. To elucidate its bactericidal mechanisms, we determined whether EGCG affected the fluorescence intensities of bacteria-conjugated Alexa Fluor 488 or 594 dyes. When mixed with unconjugated Alexa Fluor 488 or 594 dyes, EGCG or analogs did not affect the fluorescence intensity of these dyes. In a sharp contrast, EGCG and some analogs (e.g., Catechin Gallate, CG), markedly reduced the fluorescence intensity of Gram-positive \textit{Staphylococcus aureus}-conjugated Alexa 594 and Gram-negative \textit{Escherichia coli}-conjugated Alexa 488. Interestingly, co-treatment with ethanol impaired the EGCG-mediated fluorescence quenching of the G\textsuperscript{+} \textit{S. aureus}, but not of the G\textsuperscript{-} \textit{E. coli}-conjugated Alexa Fluor dyes. In light of the notion that Alexa Fluor dyes can be quenched by aromatic amino acids, it is plausible that EGCG exerts antimicrobial activities possibly by altering microbial protein conformations and functions. This possibility can now be explored by screening other fluorescence-quenching agents for possible antimicrobial activities.

Keywords: Alexa Fluor 488, Alexa Fluor 594, antimicrobial, EGCG, fluorescence intensity, macrophage, sepsis, tissue bacterial load.

INTRODUCTION

As sequelae of systemic bacterial infections, sepsis and septic shock remain the major cause of increased mortality in the intensive care units, accounting for high health care costs every year [1]. The emergence of antibiotic resistance (AR) has further compromised the successful application of antibiotic drugs in the clinical management of bacterial infections [2-4]. It is thus essential to develop high-efficiency and low-resistance antimicrobial agents to fight against lethal microbial infections.

Brewed from the leaves of \textit{Camellia sinensis}, Green tea has been associated with multiple health benefits against oxidative stress [5], atherosclerosis [5], cancer [6], cardiovascular diseases [7], as well as lethal bacterial infections [8, 9]. These healing properties are attributable to its abundant polyphenolic catechins, such as the epigallocatechin-3-gallate, EGCG, which accounts for up to 50 mg in a single cup of tea [10]. During the last two decades, extensive evidence has emerged to support a wide spectrum of anti-bacterial [11-14], anti-viral [15-17], or anti-fungal properties for EGCG and other polyphenolic catechins [18]. For instance, it is shown that EGCG can render bacteria more sensitive to various anti-bacterial agents [13, 19-21], thereby impairing their resistance to antibiotics. EGCG exerts these anti-bacterial activities partly by damaging bacterial cytoplasmic lipids [22-25], membrane proteins [26], or cytoplasmic enzymes (e.g., gelatinase, protein tyrosine phosphatase, dihydrofolate reductase, or DNA gyrase) [27-29]. Indeed, scanning electron microscopic analysis revealed that EGCG treatment caused alterations in both bacterial cell morphology [30] and outer membrane (e.g., wrinkling with perforations) [31]. As a possible consequence, EGCG impaired the cytoplasmic membrane efflux pump activities, and consequently elevated intracellular antibiotics concentrations, supporting the possible use of EGCG as an adjuvant in antibacterial therapy [19].

Parallel evidence has also indicated that EGCG can be cross-linked to various proteins [9, 16, 32-37], and consequently induces protein conformational alterations [35], suggesting EGCG as a potent protein remodeling agent. For instance, EGCG binds covalently to sulfhydril groups of membrane proteins, leading to inter-molecular protein cross-linking and accompanying conformational changes [38]. Notably, these protein conformational changes could be monitored by employing novel fluorescence quenching
techniques [39]. For instance, the Alexa Fluor dyes have been popularly used to fluorescently label various macromolecules because of their relative insensitivity to many chemicals. However, their fluorescence can be quenched by aromatic amino acids (e.g., tyrosine, tryptophan and histidine) in solution-based titrations [40], or intramolecularly when these aromatic residues physically interact with the coupled fluorescence dyes [40]. In the present study, we sought to test the possibility that EGCG may alter the fluorescence of bacteria-conjugated Alexa Fluor dyes, providing a basis for future screening of fluorescence-quenching agents for potential anti-bacterial activities.

MATERIALS AND METHODS

Animal Model of Polymicrobial Infection (Sepsis)

To evaluate the bactericidal activities of EGCG, we determined its effects on bacterial elimination in an animal model of polymicrobial infection induced by cecal ligation and puncture (CLP) as previously described [9, 41-43]. Briefly, the cecum of BALB/c mice (7-8 weeks, 20-25 g) was ligated at 5.0 mm from the cecal tip, and then punctured once with a 22-gauge needle to allow leakage of bacteria into the peritoneal cavity. EGCG stock solution was prepared in isotonic saline solution at a concentration of 1.0 mg/ml. At 24 and 48 h post CLP, EGCG saline solution (0.1 ml, equivalent to 4.0 mg/kg) was intraperitoneally administered into mice using small (26-gauge) syringe needles. At 52 h post CLP, peritoneal lavage fluid and animal organs were harvested using sterile techniques to quantitate tissue bacterial loads. Briefly, peritoneal lavage was performed with 5 ml of sterile saline using an 18-gauge syringe needle. The liver, spleen, kidneys, and lungs were harvested using sterile techniques, and homogenized at 4°C in 4 volumes of sterile saline. Immediately, the homogenates were serially diluted, and aliquots of several dilutions (50 μl) were plated onto Luria broth agar plates in triplicates (Fisher Scientific, Pittsburgh, PA). Following incubation at 37°C for 24 hours under aerobic conditions, the bacterial Colony-forming units (CFU) were counted manually under transmitted light. The tissue bacterial loads were then calculated, and expressed as CFU per mg of wet tissue. This study was approved and performed in accordance with the guidelines for the care and use of laboratory animals at the Feinstein Institute for Medical Research, Manhasset, New York, USA.

Cell Culture

Murine macrophage-like RAW 264.7 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin (Gibco), at 37°C in a humidified 5% CO2 atmosphere. Alexa Fluor 594-conjugated Staphylococcus aureus or Alexa Fluor 488-labeled Escherichia coli (Invitrogen, Carlsbad, CA, USA) were dissolved in 1 x PBS, and added to macrophage cultures (at 1.0 μg/ml). Following incubation for 2 h, (-)-Epigallocatechin Gallate (EGCG, 20 μM) was added and incubated for 30 min. Following intensive washings, the intensity of fluorescence was estimated by fluorescence microscopy or flow cytometry, respectively. For flow cytometry, the fluorescence of phagocyted bacteria was analyzed with a FACSCalibur instrument (Becton Dickinson) equipped with CellQuest software. For each sample, at least 1 x 10⁶ cells were collected and analyzed.

Effects of EGCG on the Fluorescence of Alexa Fluor Dyes

Alexa Fluor 594 and Alexa Fluor 488 carboxylic acids (Invitrogen, Carlsbad, CA, USA) were dissolved in 1 x PBS at the concentration of 250 ng/ml. Bacteria-conjugated Alexa Fluor dyes were re-suspended into 1 x PBS to generate bacterial suspension at the concentration of 40 μg/ml. To determine whether EGCG affects the intensity of Alexa Fluor dyes, the unconjugated or bacteria-conjugated fluorescence dyes were exposed to EGCG (20 μM) or analogs for 30 min. The intensity of the fluorescence was measured using a fluorescence microscope (Carl Zeiss Microimaging) or a Fluorescence Spectrophotometer (F-7000, Hitachi High Technologies America, Inc.).

Statistical Analysis

Tissue bacterial CFU were presented as scatter dot plots (with means). Differences between groups were compared by the Mann-Whitney U test. A P value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Accumulating evidences indicated that Green tea possessed a wide spectrum of anti-microbial [11-14] and antiviral [16, 17] activities, but the anti-microbial mechanisms remained poorly understood. In the present study, we demonstrated that intraperitoneal administration of EGCG did not significantly reduce bacterial loads in the peritoneal cavity and the blood (data not shown). However, it significantly reduced bacterial CFU in the liver and lung tissue (Fig. 1), suggesting that EGCG facilitated bacterial elimination in selective organs during systemic polymicrobial infection. The underlying antibacterial mechanisms may be attributable to the possibilities that EGCG facilitates bacterial elimination either directly by interacting and inhibiting their growth, or indirectly by modulating hydrogen peroxide production [44] or macrophage-associated innate immune responses. For patients with severe lung infections (e.g., pneumonia) due to antibiotics-resistant bacteria, other effective therapies are urgently needed. It is possible that EGCG serves as one such potential adjuvant therapy due to its immunomodulatory [45, 9] and antimicrobial activities [11-14, 46].

Indeed, EGCG can be cross-linked to various proteins [9, 16, 32-34], and consequently change their tertiary structures and biological functions [35], suggesting EGCG as a potent protein remodeling agent. For instance, EGCG binds to HSV-1 virus envelope glycoproteins [16] or influenza haemagglutinin [17], and consequently induces the formation of viral protein complexes. To test the possibility that EGCG facilitated bacterial elimination by conjugating to bacterial proteins, we determined whether EGCG quenched fluorescence of the Alexa Fluor dyes chemically conjugated to various bacteria (e.g., S. aureus and E. coli) via amine-
Fig. (1). EGCG facilitated bacterial elimination during polymicrobial sepsis. BALB/c mice were subjected to polymicrobial sepsis by cecal ligation and puncture (CLP), and intraperitoneally administered with EGCG at 24 and 48 h post CLP. At 52 h post CLP, animal organs were harvested via sterile techniques, and the number of bacterial colony formation units (CFU) was counted. Each dot represents output (the average CFU of triplicate agar plates) from each animal, and the horizontal lines represent the means of five animals in each experimental group. *, $P < 0.05$ for comparison between EGCG and saline groups.

reactive groups. Indeed, EGCG and an analog, CG, markedly reduced the fluorescence intensity of bacteria-conjugated Alexa Flour 488 or 594 dyes (Fig. 2). In a sharp contrast, other analogs (e.g., C or G) or antibiotics (e.g., Amphotericin B and Erythromycin, data not shown) did not affect the fluorescence intensity of bacteria-conjugated Alexa Flour dyes (Fig. 2). Furthermore, these fluorescence quenching properties appeared to attribute to the presence of the galloyl moiety, which was ester-linked with the 3-OH of the Green tea catechins (Fig. 2).

It is known that the relative concentrations of EGCG (> 800 µg/ml) needed to inhibit the growth of G⁻ bacteria (e.g., E. coli, K. pneumoniae, S. typhi, P. mirabilis, P. aeruginosa, and S. marcescens) are relatively higher than the doses (50-100 µg/ml) of EGCG needed to inhibit G⁺ bacteria (S. aureus, S. epidermidis, S. hominis, and S. haemolyticus). This variation may be attributable to the difference in the structure of the G⁻ and G⁺ bacterial cell walls, or the affinities of EGCG to various cell wall components [47]. Interestingly, we found that co-treatment with the absolute ethanol rendered G⁺ (but not G⁻) bacteria resistant to EGCG-mediated quenching of the bacteria-conjugated Alexa Fluor dyes (Fig. 3). It was possible that the ethanol-mediated dehydration/condensation of G⁺ peptidoglycan hindered the access of EGCG to the peptidoglycan or the inner cytoplasmic membrane proteins, two classes of macromolecules implicated in EGCG binding [48]. Consistently, alternative strategies to block the access of EGCG to targeted bacterial macromolecules (such as by adding exogenous proteins) uniformly impaired EGCG’s antibacterial activities [47].

To test the possibility that EGCG directly interacts with the Alexa Fluor dyes to quench the fluorescence, Alexa Fluor 594/488 dyes were separately mixed with EGCG, and

![Diagram of Alexa Fluor 594 and 488 dyes](image)

Fig. (2). EGCG reduced the fluorescence intensity of bacteria-conjugated Alexa Fluor dyes. Bacteria-conjugated Alexa Fluor dyes (Alexa 594 – S. aureus, Alexa Fluor 488 – E. coli) were re-suspended in 1×PBS (1 µg/ml), and seeded onto 24-well culture plates. After incubation for 4 h, EGCG or various analogs (e.g., CG, C, or G) were added to fluorescence-labeled bacteria. Approximately 30 min later, images were acquired with a fluorescence microscope with a digital camera controlled by Meta-Morph software (Universal Imaging; Downingtown, PA).
the fluorescence intensities of the mixtures were measured by fluorescence spectrophotometer. Surprisingly, both EGCG and CG failed to reduce the fluorescence intensity of the unconjugated Alexa Fluor 594/488 dyes even after a prolonged incubation (Fig. 4A). In a sharp contrast, EGCG and CG obviously reduced the fluorescence intensity of bacteria-conjugated Alexa 488 and 594 (Fig. 4B), confirming that EGCG and CG indeed reduced the fluorescence intensity of bacteria-conjugated Alexa Fluor dyes. In light of the notion that the fluorescence quenching of Alexa Fluor dyes could serve as a sensitive indicator of protein conformational changes [39], we proposed that EGCG and CG induced the fluorescence quenching possibly through cross-linking to bacterial proteins to induce changes in both conformations and biological functions.

Previous studies suggested that EGCG could be internalized into macrophage cytoplasmic vesicles [9],...
thereby inhibiting intracellular survival of various microbes (e.g., *Listeria monocytogenes* or *Mycobacterium tuberculosis*) in macrophage cultures [49, 50]. It was thus important to determine whether EGCG could similarly reduce the fluorescence intensity of ingested bacteria-Alexa Flour dyes in macrophage cultures. As predicted, the fluorescence-labeled bacteria were rapidly phagocytosed by macrophages within 2 hours (Fig. 5A). Interestingly, EGCG treatment similarly reduced the fluorescence intensity of bacteria-conjugated Alexa Flour 594 within macrophages (Fig. 5A). This reduction of the fluorescence intensity was confirmed by flow cytometry analysis of macrophage cultures: EGCG similarly reduced the mean fluorescence intensity from 51.6 in control to 22.4 in EGCG-treated macrophages (Fig. 5B).

In conclusion, here we demonstrated that EGCG facilitated bacterial elimination in selective organs (e.g., the liver and lung) in an animal model of lethal polymicrobial infection. Furthermore, we found that EGCG effectively quenched the fluorescence of bacteria-conjugated, but not the unconjugated, Alexa Flour dyes. It is plausible that EGCG may be cross-linked to bacterial proteins to induce their conformational and functional changes. These conformational alterations may allow the physical proximity and chemical interaction between the Alexa Fluor dyes with aromatic amino acid residues (e.g., tyrosine, tryptophan, or histidine) of bacterial proteins, leading to quenching of the fluorescence signals (Fig. 6). Thus, our experimental data suggest that Green tea catechins exert anti-microbial activities possibly by altering microbial protein conformations and functions. Given the extensive evidence in supporting the antibacterial activities of EGCG, it is important to screen other fluorescence-quenching agents for possible antimicrobial activities in future studies. Further

Fig. (6). Proposed model for EGCG-mediated anti-microbial activities. EGCG may be cross-linked to various microbial proteins to induce possible conformational changes, which allows physical proximity and chemical interaction between the Alexa Fluor dyes with aromatic amino acid residues, leading to quenching of the fluorescence signals. It is now possible that Green tea catechins exert anti-microbial activities partly by altering microbial protein conformations and functions.
investigation in this area may prompt the development of novel antibiotics to conquer lethal microbial infections.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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