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Green tea epigallocatechin-3-gallate alleviates *Porphyromonas gingivalis*-induced periodontitis in mice

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ABSTRACT

Porphyromonas gingivalis causes inflammation, and leads to the periodontitis in gingival tissue damage and bone resorption. Epigallocatechin-3-gallate (EGCG) is a major polyphenol extract from green tea with plenty of pharmacological functions. The aim of this study was to determine whether continuous oral intake of EGCG would alleviate *P. gingivalis*-induced periodontitis. Eight-week BALB/c mice were administered with EGCG (0.02%) or vehicle in drinking water. They were fed normal food and orally infected with *P. gingivalis* every 2 days, up to a total of 20 times, and then sacrificed at 15 weeks of age. The *P. gingivalis*-challenged group markedly increased alveolar bone resorption of the maxillae in BALB/c mice by Micro-CT detection, and administration of EGCG resulted in a significant reduction in bone loss. Inflammation cytokine antibody array and enzyme linked immunosorbent assay revealed that some inflammatory mediators in serum were increased by *P. gingivalis* infection, but were lowered after EGCG treatment. High positive areas of IL-17 and IL-1β in the gingival tissue were observed in the *P. gingivalis*-challenged mice, and were reduced by EGCG treatment. Real-time polymerase chain reaction (PCR) analyses also showed the expressions of IL-1β, IL-6, IL-17, IL-23, TNF-α and other mediators in gingival tissue were higher in *P. gingivalis*-challenged mice, and were down-regulated with EGCG treatment, except IL-23. Our results suggest that EGCG, as a natural healthy substance, probably alleviates *P. gingivalis*-induced periodontitis by anti-inflammatory effect.

1. Introduction

Periodontitis is a common oral disease and public health problem worldwide. It is a chronic disease resulting in the destruction of tooth supporting tissues, including the resorption of alveolar bone and periodontal ligament, eventually leading to tooth loss and relating to some systemic diseases [1]. Many studies have indicated that chronic inflammatory and immune responses, activated by periodontal infectious pathogens cause damage from gingival tissue to alveolar bone, and can play an important role in the development of periodontitis [2,3].

Porphyromonas gingivalis, a Gram-negative, non-motile, obligate anaerobic bacterium, is one of the most important pathogens associated with chronic periodontitis [4]. The periodontal pathogen infection involves the initial of inflammatory response, and then the host response is amplified by related immune cells in periodontal tissue, as well as inducing bone resorption correlating with inflammatory mediators such as cytokines, chemokines and adhesion molecules [5]. The pathogenicity of *P. gingivalis* including various virulence factors, such as lipopolysaccharide (LPS), fimbriae, capsule, hemagglutinins and gingipains can induce inflammatory responses in periodontal tissue and alveolar bone resorption [6]. *P. gingivalis* infection not only causes the inflammation in periodontitis, but also relates to systemic diseases, such as cardiovascular disease [7]. Therefore, the prevention of periodontitis caused by *P. gingivalis* is important in both oral and systemic health. Although there are many clinical treatments for periodontal therapy, novel biocompatible natural substances are of interest for prevention and subservience for periodontal treatment.

Green tea is one of the most popular beverages worldwide. Catechin is a natural substance extracted from green tea, and the most abundant catechin is Epigallocatechin-3-gallate (EGCG), which is considered to have protective effects against diabetes, hypertension, cancer and cardiovascular diseases [8]. EGCG has been shown to possess a variety of pharmacological functions, such as anti-oxidative [9], anti-mutagenic [10], anti-angiogenic [11], anti-inflammatory [12] and anti-bacterial [13] effects. We have demonstrated that EGCG attenuates *P. gingivalis*-induced atherosclerosis, owing to its biocompatible properties for anti-inflammatory and anti-oxidative effects [14]. Previous studies have shown that the ratio of *P. gingivalis* was higher than other oral bacteria in chronic periodontitis, and that it invaded periodontal tissue to increase the expressions of inflammatory mediators [15,16], especially interleukins (IL)-1β, IL-6, IL-17 and tumor necrosis factor (TNF)-α.

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Several studies have also shown that IL-6 and TNF-α are associated with periodontitis in vivo and in vitro, and are down-regulated by EGCG treatment. However, few studies have reported that EGCG mediates IL-17and IL-1β, which are responsible for receptor activator for nuclear factor-κ B ligand (RANKL) expression and host immune response in periodontal disease during *P. gingivalis* infection. Therefore, the purpose of this study was to investigate whether continuous oral intake of EGCG alleviates the development of periodontitis induced by *P. gingivalis* infection in BALB/c mice, and to demonstrate the anti-inflammatory activities of EGCG by inhibiting inflammatory cytokines expression, including IL-17and IL-1β, as a potential therapeutics to prevent periodontal disease.

2. Materials and methods

2.1. Bacterial strain

P. gingivalis strain FDC381 was cultured on anaerobic basal agar plates (Oxoid LTD, England) enriched with 5% sheep blood under anaerobic condition with 80% N_2, 10% CO_2 and 10% H_2 at 37 °C for 3–5 days. Cultures were then inoculated into brain heart infusion broth (Oxoid LTD, England), supplemented with 5 μg/mL hemin and 0.4 μg/mL menadione (Sigma-Aldrich, USA), and grown for 2 days until it reached an optical density at 600 nm (OD_{600}) of 1.0, corresponding to 10^9 CFU/mL. The cultured cells were then centrifuged at 8000 *g* for 20 min at 4 °C and diluted by phosphate-buffered saline (PBS) with 2% carboxymethylcellulose (CMC, Sigma-Aldrich, USA) for oral infection.

2.2. Mice and treatments

Eight-week-old female BALB/c Cr Slc (BALB/c) mice (20–25 g), purchased from Vital River Inc. (Beijing, China), were divided randomly into three groups (n = 8 per group) under specific-pathogen-free conditions. The Institutional Animal Care and Use Committee of Peking University Health Science Center approved all the animal protocols (approval number LA2014242). The mice received sterile food and drinking water ad libitum, with (1) and (2) distilled water or (3) 0.02% solution of EGCG from 8 weeks to 15 weeks. EGCG was administered at a dose of 0.02% in drinking water following our previous study [14]. The EGCG derived from green tea (95% purity as determined by HPLC) was purchased from Sigma-Aldrich (Cat. no. E4143, USA) and dissolved in distilled water. Mice were orally inoculated at 2-day intervals, up to a total of 20 times, with (1) 100 μL PBS with 2% CMC or (2) and (3) 10^8 CFU of *P. gingivalis* in 100 μL PBS with 2% CMC, as described previously [17]. At the age of 15 weeks, the mice were sacrificed to collect blood, gingival tissue and maxillae samples.

2.3. Tissue collection and preparation

After the last injection, mice continued to receive food and distilled water with or without EGCG until 15 weeks, and then were euthanized by intraperitoneal pentobarbital sodium (100 μg/μL, Merck, Germany). Blood samples were collected by infraorbital puncture, and serum was isolated by centrifugation at 10,000 rpm for 5 min at 4 °C. The gingival tissue was rapidly removed from mice into a liquid N_2 box and kept at −80 °C until analyzed, or fixed by 10% formalin for immunohistochemical staining. Mice maxillae were harvested and fixed in 4% paraformaldehyde at 4 °C overnight, and then transferred to a 70% ethanol solution. Horizontal bone loss around the maxillary molars was scanned by Micro-CT (Inveon MM CT, Siemens, USA).

2.4. Micro-CT imaging

Micro-CT imaging of mice maxillae was performed using a Siemens Inveon MM CT scanner for the generation of three-dimensional model. Parameters were as follows: 360° rotation, 360 projections, 1500 ms exposure time, 60 kV source voltage, 220 μA beam current, and effective pixel size of 8.82 μm. Acquisitions were reconstructed with a filtered back projection algorithm, matrix size 1024 × 1024 × 448, using Inveon Acquisition Workplace software (Siemens, USA). Images were rotated and adjusted from M1 to M3, and then analyzed by Inveon Research Workplace software (Siemens, USA) to measure the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) for evaluating the bone resorption.

2.5. Mouse inflammation cytokine antibody array

Serum samples were analyzed using the Mouse Inflammation Ab array C1 (RayBiotech, USA) consisting of 40 different inflammation cytokine antibodies, following the manufacturer's recommendation as previously described [18,19]. Signal intensities from the bound cytokines were measured using the equipment of Fusion FX Vilber Lourmat (Marne-la-Vallée cedex, France) and analyzed using mouse inflammation cytokine semi-quantitative software. Six spots of positive control were served by Biotin-conjugated IgG, where it was used to identify the membrane orientation and to normalize the results from different membranes which were being compared. The net optical density level from each spot was determined by subtracting the background optical level from the total raw optical density and the level of each cytokine was represented as a percentage of the positive control.

2.6. Cytokine ELISA

Serum samples were isolated from blood after euthanasia (15 weeks), and cytokine levels were detected using Enzyme-linked immunosorbent assay (ELISA) kits for IL-17 and IL-1β (RayBiotech, USA).

2.7. Immunohistochemical staining

Gingival tissues were fixed by 10% formalin and embedded in paraffin. The sections (5 μm thick) were processed by deparaffinized, rehydrated and heat-induced epitope retrieval. Hydrogen peroxide (3%) was used to block the endogenous peroxidase for 10 min at room temperature. Sections were blocked by goat serum and then incubated with rat anti-mouse IL-17 (Bioss, China) and rat anti-mouse IL-1β (Boster, China) as primary antibodies overnight at 4 °C. After washing with PBS three times, the secondary antibody (IgG) was dropped on at 37 °C for 20 min, and washed by PBS three times. Avidin biotin horseradish peroxidase (S-A/HRP) was then added to the slides for visualization, and they were stained by diaminobenzidine (DAB) for detection. Finally, nuclei were counterstained with Mayer's hematoxylin and mounted with permount (Sinopharm Chemical Reagent, China). For the observation of the positive staining area, images were acquired using an Olympus BX41 microscope (Olympus, Japan) at ×400 magnifications, and analyzed by using Image-Pro Plus software (American Media Cybernetics co, USA).

2.8. Quantitative real-time PCR

Total RNA was purified from gingival tissues using an RNeasy Plus Mini Kit (Qiagen, USA), and then reverse-transcribed using a Primescript RT Master Mix Kit (Takara Bio, Japan) to generate cDNA. Quantitative real-time PCR analysis was performed by using the Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies, USA) in accordance with the manufacturer's protocol. Briefly, the reactions contained 10 μL of 2× SYBR Green (Takara Bio, Japan), each primer at 100 nM and 30 ng of reverse-transcribed RNA. The PCR conditions were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. The dissociation curve analysis was then performed to confirm specificity. Each gene was tested in triplicate, and target RNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Primer sequences details are shown in Table 1.

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