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Tea catechins reduce inflammatory reactions via mitogen-activated protein kinase pathways in toll-like receptor 2 ligand-stimulated dental pulp cells

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ABSTRACT

Aims: In this study, we evaluated whether catechins could inhibit the expression of pro-inflammatory mediators induced by dental caries-related bacteria, Streptococci, or pathogen-associated molecular patterns (PAMPs) stimulation in human dental pulp fibroblasts (HDPF). We further determined the mechanisms of the anti-inflammatory activity of catechins.

Main methods: Streptococci or PAMP-stimulated HDPF were treated with catechin, and then the expression and production of pro-inflammatory mediators were determined by RT-PCR and ELISA. Furthermore, the signal transduction pathways activated with toll-like receptor (TLR)2 ligand were assessed by Immunoblot and ELISA using blocking assay with specific inhibitors.

Key findings: Increased expressions of pro-inflammatory mediators are found in inflamed dental pulp, especially in HDPF. We recently reported that dental pulpal innate immune responses may mainly result from the predominantly-expressed TLR2 signaling. Catechins, polyphenolic compounds in green tea, exert protective and healing effects through multiple mechanisms, including antioxidative and anti-inflammatory effects. However, there are no reports concerning the effects of catechins on dental pulp. In this study, we demonstrated that the up-regulated expressions of IL-8 or PGE_2 in Streptococci or PAMP-stimulated HDPF were inhibited by catechins, (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG). In TLR2 ligand-stimulated HDPF, specific inhibitors of extracellular signal regulated kinase (ERK)1/2, p38, c-jun NH_2-terminal kinase (SAP/JNK), NF-κB or catechins markedly reduced the level of pro-inflammatory mediators and the phosphorylation of these signal transduction molecules was suppressed by catechins.

Significance: These findings suggest that catechins might be useful therapeutically as an anti-inflammatory modulator of dental pulpal inflammation.

Introduction

Intradentinal progression of bacteria in the caries process induces inflammatory and immune events that develop to pulpitis (Schein and Schilder 1975; Staquet et al. 2008). The driving force behind the pulpal response seems to lie in the immune system's response to bacteria, which can enhance the production of inflammatory factors (Tsai et al. 2005). Generally, the initial sensing of microbial pathogens is mediated by pattern recognition receptors (PRRs) for pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), and these PAMPs induce the expression of many immune and inflammatory genes via nuclear factor-κB (NF-κB) stimulation. With pulpitis development, increased expressions of pro-inflammatory mediators are found in inflamed pulp, especially in human dental pulp fibroblasts (HDPF) (Huang et al. 1999; Nakanishi et al. 2005). Recently, it has been shown that HDPF respond to Toll-like receptor (TLR)2-, TLR3-, and TLR4-specific agonists (Staquet et al. 2008). We also reported that TLR2 and the nucleotide-binding oligomerization domain (NOD)2 are functionally predominant receptors stimulating the production of pro-inflammatory mediators, such as interleukin (IL)-8, IL-6, monocyte-chemoattractant protein (MCP)-1, and prostaglandin (PG)E_2, suggesting that these receptors play important roles in pulpal immune responses, leading to progressive pulpitis (Hirao et al. 2009).

It has been reported that daily consumption of green tea is associated with many important health benefits, such as a reduced risk of oxidative stress and damage, atherosclerosis, cancer, and cardiovascular diseases (Frei and Higdon 2003; Vita 2003; Crespy and Williamson 2004). The healing properties of green tea are attributable to its abundant polyphenolic compounds, known as catechins. The green tea polyphenols, catechins, include epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), gallocatechin (GC), and catechin. Among these polyphenols,

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EGCG is the major polyphenol component of green tea. Especially, EGCG and ECG showed very strong activity to inhibit cytokine-induced IL-8 production in both nasal fibroblasts and bronchial epithelial cells (Kim et al. 2006). A recent interesting report showed that intraperitoneal administration of EGCG protected mice against lethal endotoxemia, and rescued mice from lethal sepsis (Li et al. 2007), however, there are no reports concerning the effects of catechins on dental pulp tissues.

With regard to dental pulpal inflammation, we focused on catechin modulation of PAMPs and Streptococci-induced inflammatory responses, and selected two catechins, EGCG and ECG, based on their strong anti-inflammatory effects. In this study, we first evaluated whether catechins could inhibit the expression of pro-inflammatory mediators induced by Streptococci or PAMP stimulation in HDPF and further determined the mechanisms of the anti-inflammatory activity of catechins in pulpitis.

Materials and methods

Cell culture

Clinically healthy pulp tissue samples were obtained from non-carious teeth extracted for orthodontic reasons under informed consent at Tokushima University Hospital. This study was performed with approval from and compliance with Tokushima University Ethics Committee. HDPF were established from explant cultures of pulp tissues as described previously (Adachi et al. 2007), and cultured in Dulbecco's Modified Eagle's Medium (Gibco, NY) supplemented with 10% fetal bovine serum (JRH Biosciences, KS), 1 mM sodium pyruvate (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37 °C in a humidified atmosphere of 5% CO_2. Confluent monolayers were stimulated at passages 5 to 10.

Bacteria

Streptococcus mutans MT8148 (kindly provided by Dr. T. Ooshima, Osaka University, Osaka, Japan), *S. sanguinis* 90-1 and *S. salivarius* 184-2 (clinically isolated at Tokushima University Hospital) grown in Brain–Heart-Infusion broth (Difco, MI) were harvested in the stationary phase. Bacterial numbers were determined spectrophotometrically with a standard curve and adjusted with antibiotics-free medium.

Reagents

Pam3CSK4 (TLR2 ligand) and ultra pure *Escherichia coli* LPS (TLR4 ligand) were purchased from InvivoGen (San Diego, CA). Muramyldipeptide (MDP) was purchased from Sigma-Aldrich (Walkersville, MD). ECG and EGCG were purchased from Sigma-Aldrich. PD98059 and SP600125 were purchased from Merck Biosciences Ltd. (Darmstadt, Germany). SB203580 and SN50 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BIOMOL Research Laboratories (Plymouth Meeting, PA), respectively.

Cell proliferation assay

TetraColor ONE Cell Proliferation Assay System (Seikagaku Corporation, Tokyo, Japan) was used to evaluate cell proliferation activity by monitoring the amount of formazan, which is in proportion to the number of live cells, using a microplate reader according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were used to quantify IL-8, IL-6 (R&D Systems, MN), MCP-1 (Pepro Tech, London, UK), and PGE_2 (Cayman Chemical, MI) in cell culture supernatants.

Determination of total adenosine triphosphate (ATP)

BacTiter-Glo Microbial Cell Viability Assay (Promega Corporation, WI) was used to evaluate the antimicrobial activity of catechins on *S. mutans* by monitoring the luminescence signal using a microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's instructions.

RT-PCR

Total RNA from HDPF was isolated with NucleoSpin RNA II (MACHEREY-NAGEL, Düren, Germany), and 100 ng RNA was utilized for each RT-PCR. RT-PCR was performed in two steps as follows. cDNA synthesis was performed with an RNA PCR Kit (TaKaRa, Shiga, Japan) and specific gene transcripts were amplified with ReddyMix PCR Mix (ABgene, Surrey, UK). The primers and PCR conditions for amplification of IL-8, COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were described previously (Liu et al. 2008; Hirao et al. 2009). GAPDH was used as an internal control. PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

PAMP-stimulated HDPF were collected with RIPA lysis buffer (Santa Cruz Biotechnology). Protein concentrations in lysates were quantified with a bicinchoninic acid protein assay kit (Sigma-Aldrich). An equal amount of protein was then loaded onto a 5–20% SDS-PAGE gel, followed by electrotransfer to a polyvinylidene difluoride (PVDF) membrane. The membrane was first incubated with Inhibitor κBα (IκB)α antibody (Sigma-Aldrich), phospho-IκBα antibody, mitogen-activated protein kinase (MAPK), phospho-MAPK family antibody, NF-κB p65 antibody or phospho-NF-κB p65 (Ser536) (Cell Signaling Technology, MA). After washing, the membrane was reacted with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich). Protein bands were finally visualized on X-ray film using the ECL system (GE Healthcare, Buckinghamshire, UK). In addition to total MAPK family, IκBα and NF-κB antibodies, an equal loading of gels was confirmed by immunoblot with anti-actin antibody (Sigma-Aldrich) as an internal control.

Statistical analysis

All statistical analyses were performed using the unpaired Student's *t* test. Differences were considered significant when the probability value was less than 1% ($P<0.01$).

Results

Effects of catechins on cell viability, IL-8 production in streptococcal-stimulated HDPF, and antimicrobial activity for S. mutans

We first assessed the cytotoxicity of catechins to the HDPF by cell proliferation assay (Fig. 1A). HDPF viability was not inhibited in the presence of catechins (up to 50 μg/ml) after 24 h culture, therefore, the concentrations of 10 and 50 μg/ml catechins were used in the following experiments. We previously reported that live, not heat-killed, *S. mutans* stimulation significantly increased the levels of various pro-inflammatory mediators, such as IL-8, -6 and MCP-1 (Hirao et al. 2009). We then examined the effect of catechins on IL-8 production in Streptococci-stimulated HDPF. Treatment with the indicated concentrations of catechins significantly reduced IL-8 production levels from all three streptococcal-stimulated HDPF (Fig. 1B). We also assessed the antimicrobial activity of catechins for *S. mutans* by determining the bacterial growth curve and the quantity of total ATP. Catechins had no