



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

Kouji Hirao, Hiromichi Yumoto*, Tadashi Nakanishi, Kayo Mukai, Kanako Takahashi, Daisuke Takegawa, Takashi Matsuo

Department of Conservative Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, Japan

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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 PGE₂ 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 NH₂-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.

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Introduction

Intradental 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)E₂, 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), galliccatechin (GC), and catechin. Among these polyphenols,

* Corresponding author. Department of Conservative Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima, 770-8504, Japan. Tel./fax: +81 88 633 7340.

E-mail address: yumoto@dent.tokushima-u.ac.jp (H. Yumoto).

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% CO₂. 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). Muramyl dipeptide (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 PGE₂ (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 κ B α (I κ B) α antibody (Sigma-Aldrich), phospho-I κ B α antibody, mitogen-activated protein kinase (MAPK), phospho-MAPK family antibody, NF- κ B p65 antibody or phospho-NF- κ B 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, I κ B α and NF- κ B 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

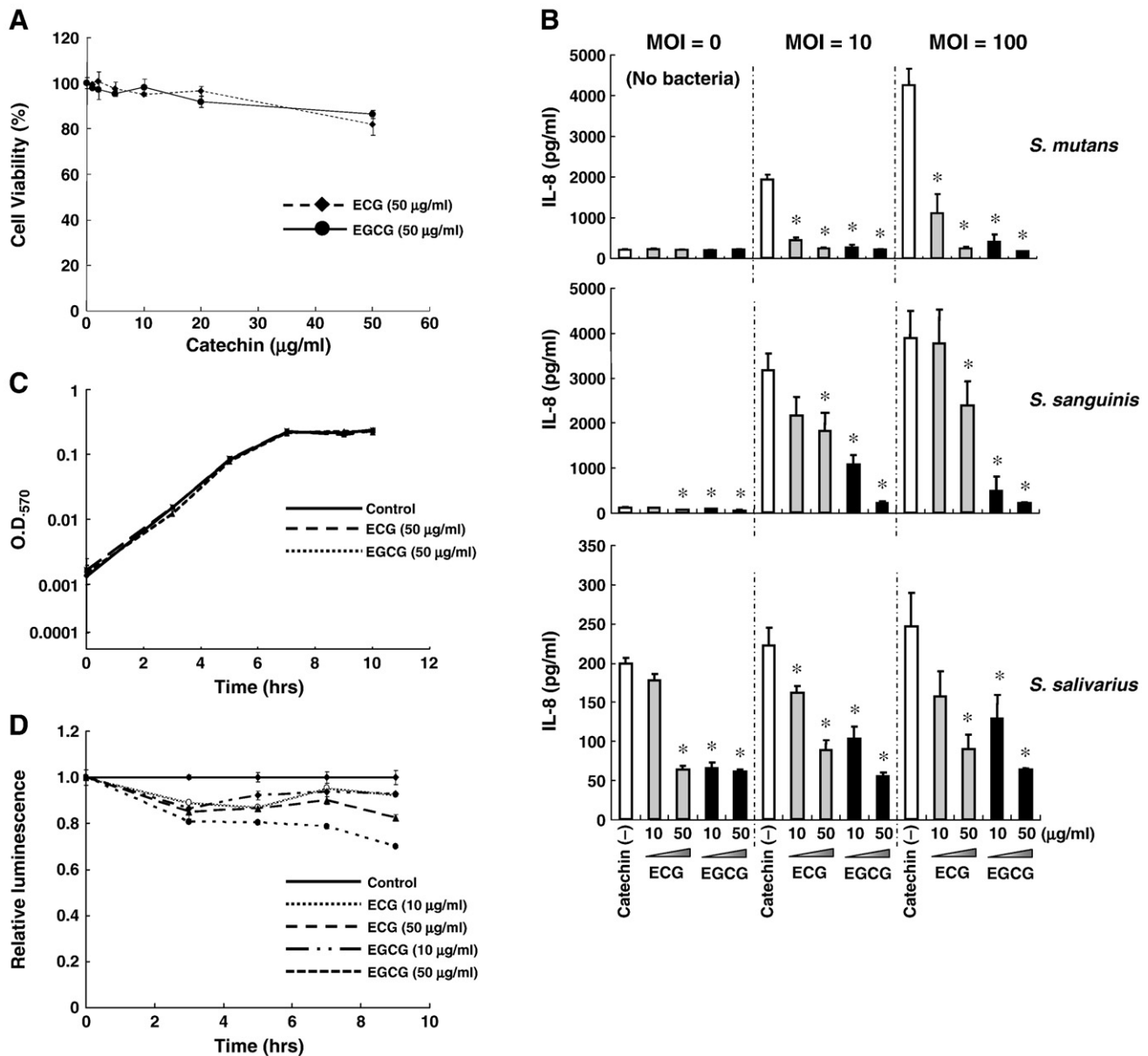


Fig. 1. The effects of catechins on cell viability, IL-8 production in streptococcal-stimulated HDPF, and antimicrobial activity of *S. mutans*. (A) HDPF were cultured with ECG or EGCG (up to 50 µg/ml) for 24 h. Percent cell viability was expressed as the percentage of viability derived from cells without catechin treatment. (B) HDPF were cultured with ECG or EGCG (10 or 50 µg/ml) and/or *S. mutans*, *S. sanguinis* or *S. salivarius* (multiplicity of infection; MOI = 0, 10 or 100) for 48 h. IL-8 level in the culture supernatants was determined by ELISA. * $P < 0.01$ versus control (catechin -). (C) *S. mutans* were incubated with ECG or EGCG (50 µg/ml). Bacterial growth was monitored for 11 h by measuring optical density at 570 nm. (D) *S. mutans* were incubated with ECG or EGCG (10 or 50 µg/ml). Total ATP in *S. mutans* was monitored for 9 h using the Bacter-Glo Microbial Cell Viability Assay. All values are expressed as the means \pm SD of triplicate cultures from 1 representative of 3 independent experiments with similar results.

effect on bacterial growth of *S. mutans*, but had a slight inhibitory effect on the total ATP amount in *S. mutans* (Fig. 1C and D).

Inhibitory effects of catechins on IL-8 and PGE₂ production in TLR-2, TLR-4 or NOD2 ligand-stimulated HDPF

Recently, we showed that TLR2, TLR4, and NOD2 expressed in HDPF function to up-regulate the expressions of various pro-inflammatory mediators including IL-8, PGE₂, and cyclooxygenase (COX)-2, a key enzyme for PGE₂ formation (Hirao et al. 2009). Next, we examined whether catechins could inhibit PAMP-induced IL-8 and PGE₂ production in HDPF (Fig. 2A). ECG effectively inhibited IL-8 and PGE₂ production in PAMP-stimulated HDPF. Interestingly, both concentrations of EGCG inhibited IL-8 production, but a high

concentration (50 µg/ml) of EGCG failed to inhibit PGE₂ production in PAMP-stimulated HDPF. We then determined whether RNA values of IL-8 and COX-2 correlate with the values determined by ELISA. These results demonstrated that both mRNA expression patterns correlate with the results derived from ELISA (Fig. 2B).

Cell signaling pathways in TLR2 ligand-stimulated HDPF

Recent reports have suggested that pulpal innate immune responses, such as the up-regulation of pro-inflammatory mediators may mainly result from predominantly-expressed TLR2 signaling (Mutoh et al. 2007; Hirao et al. 2009); however, the cell signaling pathways through TLR2 in HDPF remain unclear. We investigated cell signaling pathways in Pam3CSK4-stimulated HDPF using several specific inhibitors. In

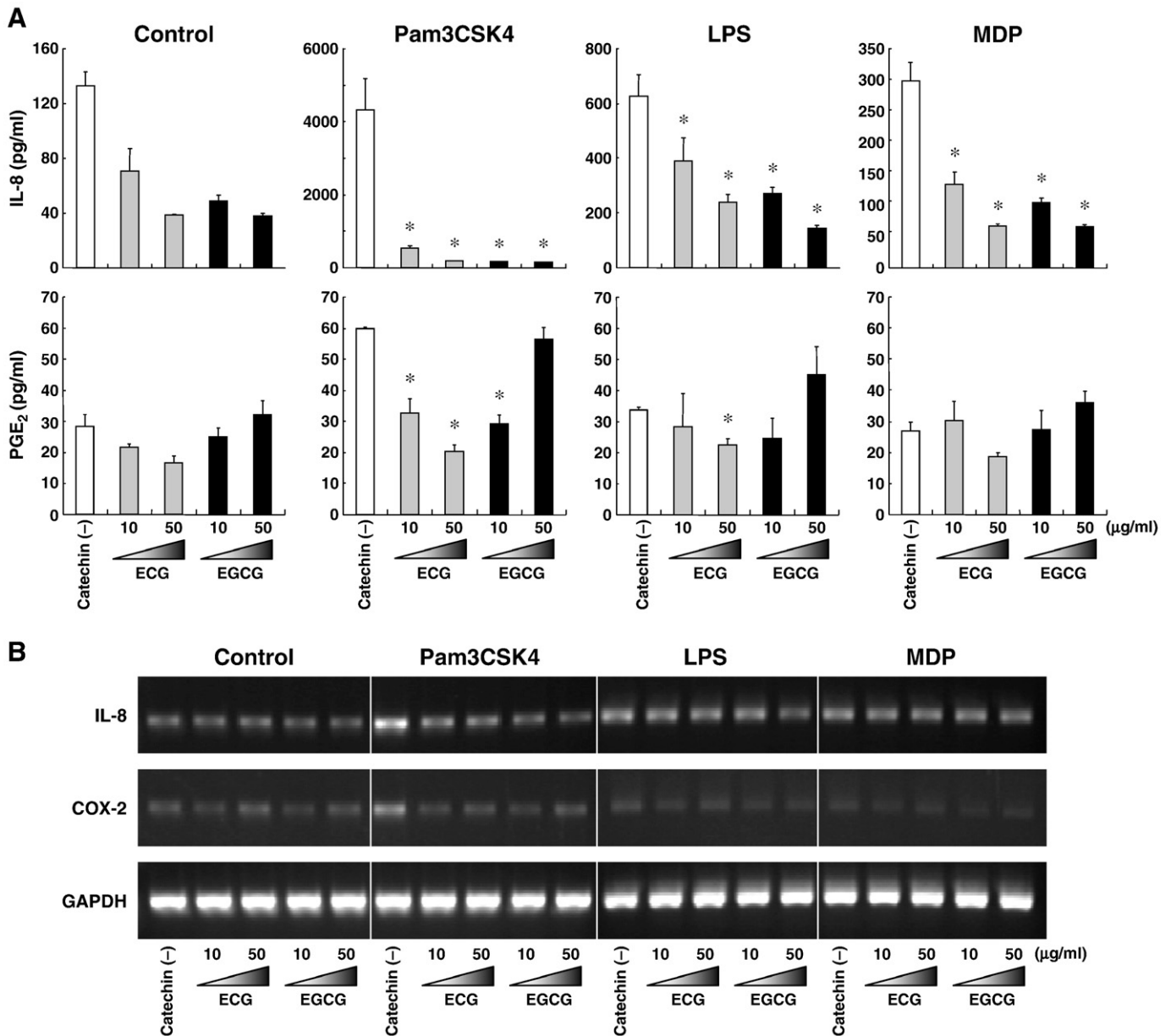


Fig. 2. The effects of catechins on IL-8 and PGE₂ production in TLR2, TLR4 or NOD2 ligand-stimulated HDPF. HDPF were cultured with ECG or EGCG (10 or 50 $\mu\text{g/ml}$) and/or Pam3CSK4 (0.01 $\mu\text{g/ml}$), LPS (0.01 $\mu\text{g/ml}$), MDP (10 $\mu\text{g/ml}$) for 24 h. (A) IL-8 and PGE₂ levels in culture supernatants were determined by ELISA and expressed as the mean \pm SD of triplicate cultures from 1 representative of 3 independent experiments with similar results. * $P < 0.01$ versus control (catechin -). (B) After incubation with catechins and/or PAMPs, total RNA was extracted, and mRNA expressions of IL-8 and COX-2 were analyzed by RT-PCR.

Pam3CSK4-stimulated HDPF, IL-8 and IL-6 productions were reduced by p38 inhibitor SB203580 and NF- κ B inhibitor SN50, and MCP-1 production was inhibited by c-jun NH₂-terminal kinase (SAP/JNK) inhibitor SP600125 as well as p38 and NF- κ B inhibitors (Fig. 3A). These inhibitory effects with specific inhibitors were dose-dependent (Fig. 3B), however, MAPK/extracellular signal regulated kinase (ERK) kinase 1 inhibitor PD98059 did not inhibit the production of these pro-inflammatory mediators in Pam3CSK4-stimulated HDPF (Fig. 3A).

Effects of catechins on the phosphorylation of MAPKs in TLR2 ligand-stimulated HDPF

To determine whether the inhibitory effect of catechins on IL-8 and PGE₂ productions in Pam3CSK4-stimulated HDPF is involved in MAPKs phosphorylation, the phosphorylation of MAPKs was analyzed

by immunoblot analysis. Both ECG and EGCG inhibited the phosphorylation of p38 MAPK, SAP/JNK, and ERK1/2 (Fig. 4). Furthermore, the effect of EGCG was greater than that of ECG for all tested signal molecules.

Effects of catechins on the phosphorylation of I κ B α and p65 subunit of NF- κ B in TLR2 ligand-stimulated HDPF

It has been reported that the regulation of NF- κ B activation following stimulation involves at least 2 independent signal transduction pathways. The best-characterized mechanism for the activation of NF- κ B involves the phosphorylation of I κ B α . An alternative I κ B-independent mechanism for the activation of NF- κ B is I κ B α -independent and involves direct phosphorylation of the most transcriptionally active p65 subunit of NF- κ B at multiple Ser residues by several kinases, such as

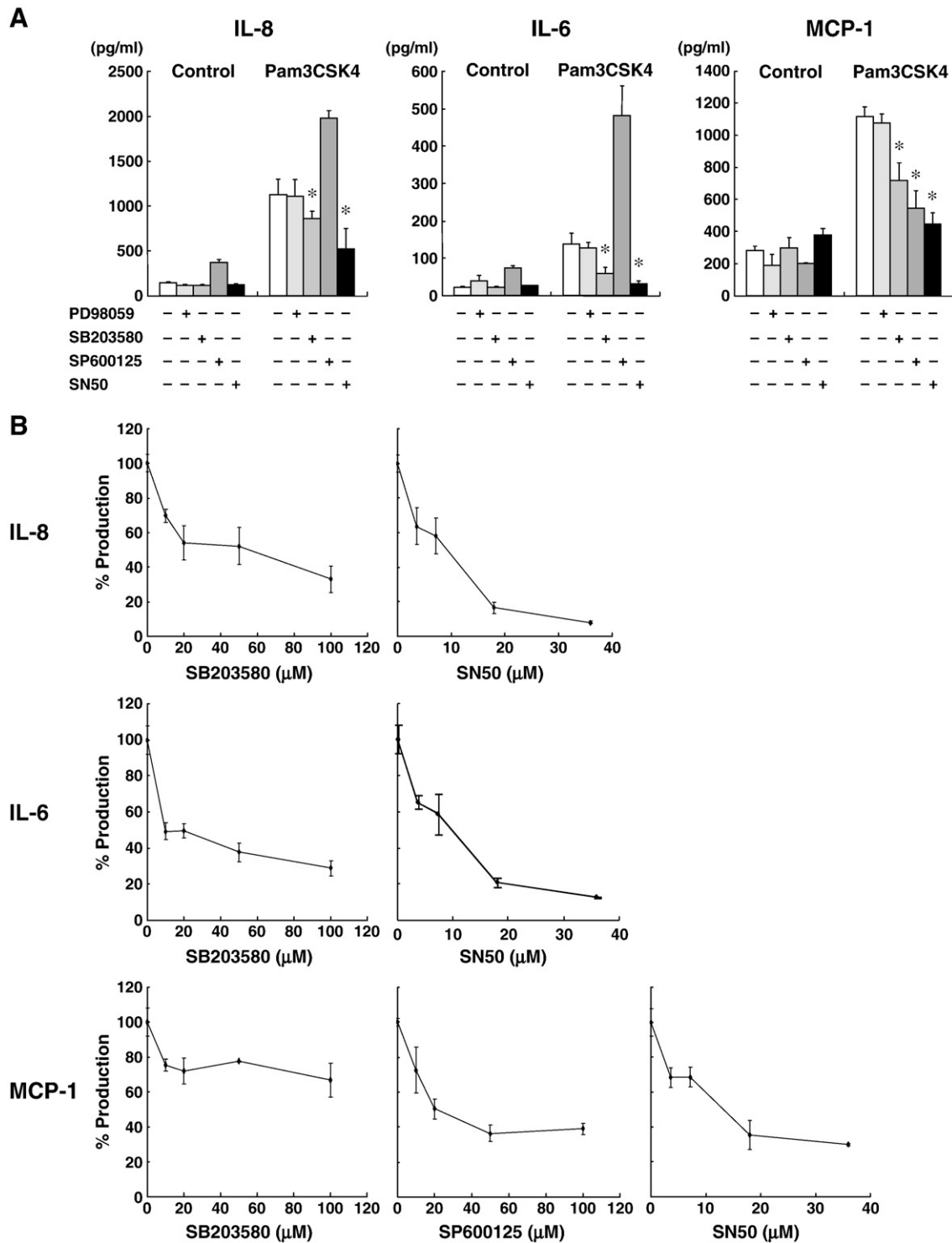


Fig. 3. The effect of MAPKs and NF- κ B inhibitors on cytokine and chemokine productions in TLR2 ligand-stimulated HDPF. (A) HDPF were treated with PD98059 (25 μ M), SB203580 (10 μ M), SP600125 (10 μ M) or SN50 (9 μ M) for 1 h followed by stimulation with Pam3CSK4 (0.01 μ g/ml) for 12 h. IL-8, IL-6 and MCP-1 levels in the culture supernatants were determined by ELISA, and expressed as the mean \pm SD of triplicate cultures from 1 representative of 3 independent experiments with similar results. * P <0.01 versus with Pam3CSK4 stimulated group values without inhibitor treatment. (B) HDPF were treated with SB203580, SP600125 or SN50 at the indicated concentrations for 1 h followed by stimulation with Pam3CSK4 (0.01 μ g/ml) for 12 h. Percent production of IL-8, IL-6 or MCP-1, shown on the y-axis, is relative to each production from the DMSO-treated control (control 100%). Values represent the means of 4 determinations from a representative experiment. Error bars indicate SDs.

inhibition of I κ B kinase (IKK) (Sakurai et al. 1999; Schmitz et al. 2001; Wheeler et al. 2004). Recently, it has been also shown that peptidoglycan, TLR2 ligand, induces the phosphorylation of p65 subunit of NF- κ B (Asehnoune et al. 2005). We next evaluated the effects of catechins on NF- κ B activities using anti-phospho-I κ B α and anti-phospho-NF- κ B p65 antibodies (Fig. 5). Pam3CSK4-induced phosphorylations of I κ B α and

p65 subunit of NF- κ B were increased compared to control or catechin only. However, co-treatment with Pam3CSK4 and catechin resulted in decreased phosphorylations of I κ B α and p65 subunit of NF- κ B after 1 h of catechin treatment. Catechin alone had no significant effect on both phosphorylations. These data demonstrate that catechins suppress Pam3CSK4-induced phosphorylations of I κ B α and NF- κ B in HDPF.

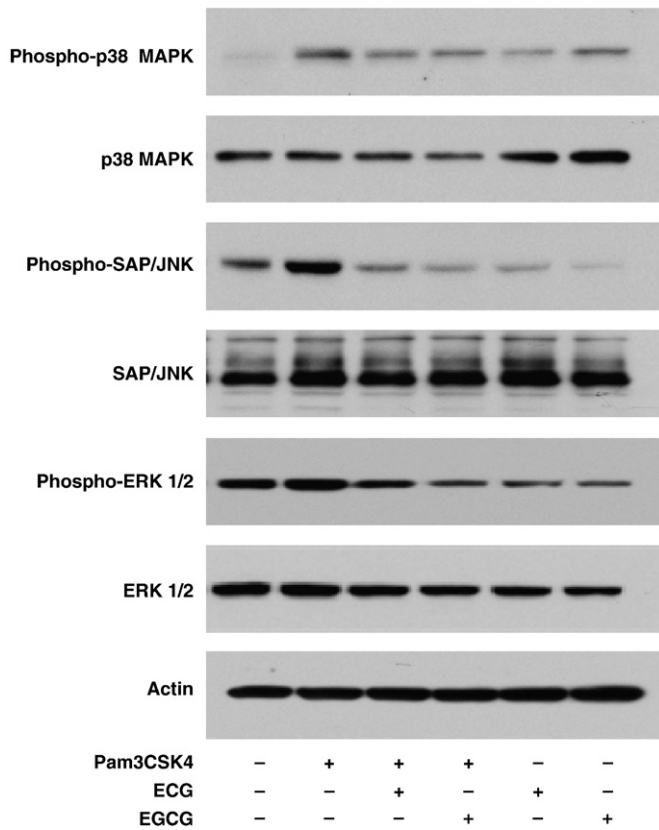


Fig. 4. The effects of catechins on the phosphorylation of MAPKs in TLR2 ligand-stimulated HDPF. HDPF were treated with ECG or EGCG (50 µg/ml) and/or Pam3CSK4 (0.01 µg/ml) for 1 h. The phosphorylation of MAPKs was determined by immunoblot analysis. Equal loading of gels was confirmed by immunoblot with anti-actin antibody. These results are representative of three independent experiments and a representative blot image with anti-actin antibody is shown.

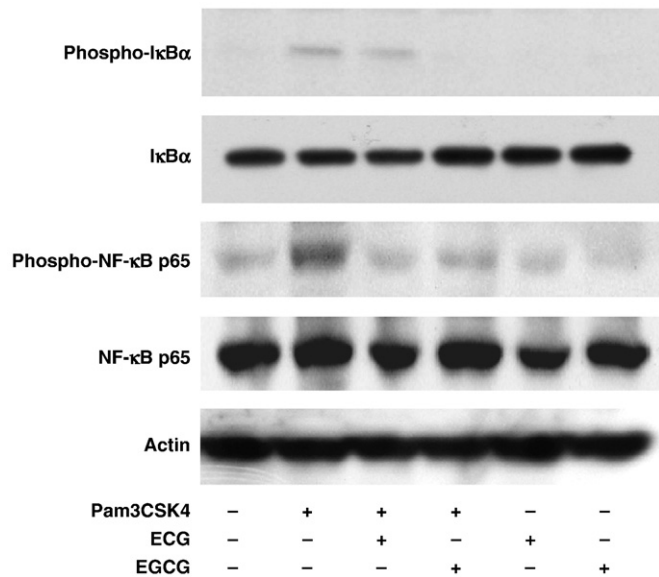


Fig. 5. The effects of catechins on the phosphorylation of IκBα and p65 subunit of NF-κB in TLR2 ligand-stimulated HDPF. HDPF were treated with ECG or EGCG (50 µg/ml) and/or Pam3CSK4 (0.01 µg/ml) for 1 h. The phosphorylation of IκBα and p65 subunit of NF-κB was determined by immunoblot analysis. Equal loading of gels was confirmed by immunoblot with anti-actin antibody. These results are representative of three independent experiments and a representative blot image with anti-actin antibody is shown.

Discussion

When dentin is destroyed by caries, pulp cells are challenged with caries-related bacteria, such as *S. mutans*, or bacterial products, and pulpitis is finally developed (Hahn and Liewehr 2007). Previous reports have shown that various pro-inflammatory mediators are expressed in pulp tissue in response to several inflammatory stimuli and PAMPs, and play important roles in the enhancement of pulpal inflammation (Nakanishi et al. 1995, 2005; Nagaoka et al. 1996; Tokuda et al. 2001). Recently, we reported that TLR2 and NOD2 are functionally predominant receptors stimulating the production of pro-inflammatory mediators, such as IL-8, IL-6, MCP-1, and PGE₂ in HDPF (Hirao et al. 2009). Here, we demonstrated that both ECG and EGCG can inhibit IL-8 production in HDPF stimulated with caries-related bacteria *Streptococci*. We also confirmed that catechins (up to 50 µg/ml) have no cytotoxicity for HDPF by cell proliferation assay and no antimicrobial activity for *S. mutans* by the determination of bacterial growth. In addition, the present results showed that both ECG and EGCG can inhibit IL-8 production in HDPF stimulated with PAMPs, such as Pam3CSK4, LPS and MDP. These findings suggest that catechins, EGCG and ECG, have an anti-inflammatory effect on HDPF in caries-related bacteria-induced pulpal inflammation without antimicrobial and cytotoxic effects, and may have therapeutic potential for the treatment of pulpal inflammation.

It was also observed that ECG could reduce PGE₂ production in these stimulated HDPF, but a high concentration of EGCG failed to reduce this production. Consistent with this observation, a previous report has shown that EGCG up-regulates COX-2 expression and PGE₂ production in a Raw264.7 macrophage cell line (Park et al. 2001). On the other hand, other studies found that COX-2 was inhibited by EGCG-treated colorectal cancer cell lines and human chondrocytes (Ahmed et al. 2002; Peng et al. 2006); therefore, our results and previous studies suggest that EGCG regulates COX-2-mediated inflammation through different mechanisms in different cell systems and EGCG-treated regulation of the inflammatory pathway might be cell-type specific (Kim et al. 2007).

MAPKs have been implicated in many physiologic processes, including cell proliferation, differentiation, and death. The three major types of MAPKs in mammalian cells are ERK, the p38 MAPKs, and JNK. NF-κB is an oxidative-sensitive transcription factor that plays a critical role in the regulation of various genes, which are important in cellular responses, including inflammation, innate immunity, growth, and cell death. In this study, we investigated that Pam3CSK4-induced signaling cascades lead to increased production of cytokines and chemokines in HDPF. Our results showed that Pam3CSK4-induced activation of p38 increased IL-8, IL-6, and MCP-1 production, and SAP/JNK activation by Pam3CSK4 stimulation up-regulated MCP-1 expression. These findings also propose a signaling cascade between MAPK pathways and TLR2 ligand-induced pro-inflammatory mediator expression. Here, we also demonstrated that Pam3CSK4 induces both IκBα-dependent and -independent signal transduction pathways for the activation of NF-κB. Recently, catechins have been shown to inhibit the MAPK pathway and NF-κB activity (Dong et al. 1997; Shimizu et al. 2005; Chae et al. 2007; Kim et al. 2007; Hayashi et al. 2008). Consistent with these reports, our findings suggest that the inhibition of MAPK activation and NF-κB activity might be a good therapeutic strategy for PAMP-induced pulpal inflammation, and propose that the inhibitory effect of catechins on pro-inflammatory mediator expression with PAMPs or caries-related bacteria stimulation is mediated by blocking the MAPK pathway with subsequent inhibition of NF-κB activity in HDPF.

Recent reports have shown that biological activities of EGCG are mediated through binding to the cell-surface 67-kDa laminin receptor (67LR) (Tachibana et al. 2004; Fujimura et al. 2008). Moreover, the same group showed that 67LR is highly associated with lipid rafts, which regulate membrane function in eukaryotic cells to mediate various

cellular functions of EGCG (Fujimura et al. 2005). Lipid rafts contain specific kinases, which are well-known enzymes enabling the generation of second messengers within the cell by catalyzing the phosphorylation of specific substances (Fujimura et al. 2005), and are signaling platforms for immunomodulative signal transduction and membrane traffic pathways (Simons and Ikonen 1997). Therefore, the association of 67LR with a lipid raft may explain the findings that EGCG has marked inhibitory effects on kinase activity and subsequent selective phosphorylation of downstream proteins, as reported in previous studies (Malinoff and Wicha 1983; Tachibana et al. 2004). Thus, it will be interesting to determine whether disruption of receptor clustering can decrease pulpal inflammation; however, this issue remains unclear and currently under investigation.

Conclusion

We elucidated the anti-inflammatory effect of catechins occurring by inhibiting the expression of cytokines and chemokines in HDPF treated with caries-related bacteria and PAMPs. In addition, catechins inhibited MAPK phosphorylation and subsequently suppressed both $\text{I}\kappa\text{B}\alpha$ -dependent and -independent signal transduction pathways for the activation of NF- κB , leading to the expression of cytokines and chemokines induced by caries-related bacteria and TLR2 ligand. These findings suggest that understanding the mechanisms underlying PAMP-induced pro-inflammatory reactions in HDPF is important for the development of new therapeutic strategies and treatments for pulpitis.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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