Effect of Various Catechins on the IL-12p40 Production by Murine Peritoneal Macrophages and a Macrophage Cell Line, J774.1

Daiju ICHIKAWA, Ayako MATSUI, Miwa IMAI, Yoshiko SONODA, and Tadashi KASAHARA*

Department of Biochemistry, Kyoritsu University of Pharmacy; 1–5–30 Shibakoen, Minato-ku, Tokyo 105–8512, Japan.

Received March 27, 2004; accepted June 18, 2004; published online June 24, 2004

Interleukin-12 (IL-12) is a heterodimeric cytokine comprising p40 and p35 subunits produced mainly by monocytes and macrophages, and plays an essential role in the regulation of the differentiation of Th1 cells. Green tea polyphenols exhibit potent anti-oxidative activities and anti-inflammatory effects by modulating cytokine production. We investigated the effect of catechins on IL-12p40 production in murine macrophages induced by bacterial lipopolysaccharide (LPS). Pretreatment with several catechins at doses of 0.3–30 μM suppressed IL-12 p40 production by murine peritoneal exudate cells (PEC) and J774.1 cells in a dose-dependent manner. Decreases in protein production were primarily due to down-regulation of the transcription of IL-12p40 mRNA. Of the various catechins, (−)-epigallocatechin gallate (EGCG) was the most potent inhibitor, followed by (−)-gallocatechin gallate (GCG) and (−)-epicatechin gallate (ECG). EGCG inhibited LPS-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK), but not Jun N-terminal kinase (JNK), while ECGC augmented LPS-induced phosphorylation of p44/p42 extracellular signal-related kinase (ERK). In addition, both EGCG and GCG inhibited LPS-induced degradation of IκBα with concomitant inhibition of nuclear protein binding to NF-κB site and synthesis of IRF-1. These results suggest that gallate-containing catechins, particularly EGCG, inhibits LPS-induced IL-12p40 production in murine macrophages by inhibiting p38 MAPK while enhancing p44/p42 ERK, leading to the inhibition of IκBα degradation and NF-κB activation.

Key words interleukin-12 (IL-12); catechin; (−)-epigallocatechin gallate (EGCG); NF-κB; p38MAP kinase; extracellular signal-related kinase (ERK)

Tea is one of the most popular beverages in the world and its possible beneficial effects on health have attracted a great deal of attention. The drinking of green tea has been reported to reduce the risk of developing specific cancers including pancreatic and colorectal cancers, while others reported that the risk of gastric cancer is not associated with green-tea consumption. Polyphenols are the most significant group of tea components, especially the catechin group called flavonols. Major tea catechins are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC). These polyphenols are known to have antioxidative activities due to their radical scavenging and metal chelating functions as well as anti-inflammatory activities. EGCG, a principal constituent of green tea, has been particularly well studied and shown to inhibit lipopolysaccharide (LPS)-induced TNFα production, induction of inducible nitric-oxide synthase in monocytes and endothelial cells, and IL-8 production by human keratinocytes or endothelial cells. Several studies have focused on the potential mechanisms of EGCG’s anti-inflammatory and anticancer effects. One mechanism of action is the inhibition of the activation of NF-κB, which regulates the expression of a variety of genes critical for the induction of inflammatory cytokines and immune responses, and/or anti-apoptosis.

Production of IL-12 by macrophages and dendritic cells is critical to host defense against a variety of pathogens. A key function of IL-12 is the induction and maintenance of Th1 responses. As IL-12 is induced in macrophages and dendritic cells by bacteria and bacterial products, it is an important bridge between host innate and adaptive immunity. IL-12 is also involved in the pathogenesis of Th1-mediated chronic inflammatory disorders in mice and humans, such as diabetes mellitus, multiple sclerosis, arthritis, and inflammatory bowel disease. IL-12 is an inducible, heterodimeric, disulfide-linked cytokine composed of 35- and 40-kDa subunits encoded by separate genes. The bioactive IL-12 cytokine is a heterodimeric p70 molecule and both subunits are co-expressed in the same cell to generate the bioactive form. The regulation of IL-12p40 gene expression seems to occur mainly at the level of transcription, in which multiple regulatory elements have been implicated including NF-κB, Ets-2, and members of the IFN regulatory factor (IRF) and CCAAT enhancer binding protein (C/EBP) families.

In this study, we attempted to determine how the various catechins affect LPS-induced IL-12p40 production. We also demonstrated that the gallate-containing catechins, (−)-ECG, (−)-EGCG and (−)-GCG, suppressed IL-12 and IL-12p40 mRNA markedly, possibly through the inhibition of the p38 MAPK and NF-κB activation pathways.

MATERIALS AND METHODS

Reagents and Antibodies The LPS (from Escherichia coli, O111: B4) and catechins were purchased from Sigma-Aldrich (Tokyo, Japan). The catechins used were as follows: (+)-catechin (CA), (−)-CA, (+)-epicatechin (EC), (−)-EC, (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (−)-gallocatechin (GC), and (−)-gallocatechin gallate (GCG). These compounds were dissolved in 99.5% ethanol.

Mouse monoclonal antibodies against phospho-p44/p42 ERK and phospho-SAPK/JNK, and rabbit polyclonal antibodies against total-p44/p42 ERK, total-SAPK/JNK, total-p38 and phospho-p38 were purchased from Cell Signaling Technology (Tokyo, Japan). Goat polyclonal antibody against actin and rabbit polyclonal antibodies against IκBα and IRF-1 were purchased from Santa Cruz Biotechnology (Sant
Cruz, CA, U.S.A.). Peroxidase-conjugated anti-rabbit IgG, anti-goat IgG and anti-mouse IgG were obtained from Dako (Dako-Japan, Tokyo, Japan).

Murine Peritoneal Exudate Cells (PEC) and J774.1 Cells Female BALB/c mice were injected ip. with 0.5 ml of a 1:1 mixture of Freund’s complete adjuvant (Sigma-Aldrich) and Freund’s incomplete adjuvant, and PEC were harvested 7 d later. The cells were washed, and incubated in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 1% FBS in the presence or absence of 1 μg/ml of LPS. The macrophage cell line J774.1 was obtained from Japanese Cancer Research Resources (JCRB, Kamiyoga, Tokyo, Japan). It was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Nissui Seiyaku, Tokyo, Japan) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum (Gibco BRL, NY, U.S.A.).

Measurement of IL-12p40, TNFα and NO Concentrations PEC and J774.1 cells were incubated in 48-well plates (Nalge Nunc International, Tokyo, Japan) at 5×10^5 and 1×10^5 cells per well, respectively. Cells were treated with catechins 24 h before stimulation with LPS. Supernatants were harvested 24 h after stimulation. Cytokine concentrations in the supernatants were determined using a specific ELISA kit for IL-12p40 and TNFα (Pepro Tech, London, U.K.). Nitrite concentrations in culture supernatants were determined with the Griess reaction. Cell cytotoxicity was determined by the release of lactate dehydrogenase (LDH) using LDH Cytotoxic Test (Wako Chemicals, Tokyo, Japan).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) RNA was prepared using an RNA purification kit (Quiagen, Tokyo). RT-PCR analysis was performed according to the manufacturer’s directions (Takara RNA Isolation and Its Binding to the NF-κB Consensus Sequence were done as described elsewhere.27) In EMSA, 5 μg nuclear protein was incubated with 32P-labeled NF-κB consensus probe (5′-AGTTGAGGGACCTTCCACGGC-3′) and bound complex was separated on 5% TBE gel by electrophoresis in TBE buffer, dried, and visualized by autoradiography. Western blot and EMSA were performed at least three times, and representative data was shown in each Figure.

Statistical Analysis Statistical differences between control and treatment groups were assessed with Student’s t-test.

**RESULTS**

Effect of Catechins on IL-12p40 Production by LPS-Stimulated Murine PEC LPS induced significant increases in IL-12p40 production by PEC during incubation for 24 h. To test the effect of the catechins, PEC were pretreated with various catechins at doses of 0.3 to 30 μM for 24 h, then stimulated with LPS (1 μg/ml) for an additional 24 h. While most catechins tended to suppress IL-12p40 production in a dose-dependent fashion, (−)-ECG, (−)-EGCG and (−)-GCG, all containing gallate, had marked inhibitory effects. No significant cytotoxicity, determined by the LDH release assay, was observed at these doses and cell cytotoxicity was rather reduced at 30 μM by (−)-ECG, (−)-EGCG and (−)-GCG (Fig. 1), indicating that the suppression was not ascribable to a direct cytotoxic effect by these catechins.

Effect of Catechins on IL-12p40, TNFα and NO Production in LPS-Stimulated J774.1 Cells We also tested the effect of catechins on LPS-induced IL-12p40 production...
by J774.1 cells in addition to PEC. J774.1 cells were pretreated with various catechins at concentrations ranging from 0.3 to 30 μM for 24 h, then stimulated with LPS (1 μg/ml) for an additional 24 h. (−)-ECG, (−)-EGC, (−)-EGCG, (−)-GC and (−)-GCG decreased IL-12p40 production in a dose-dependent fashion (Fig. 2A). The inhibitory activity of each catechin on IL-12p40 production was calculated as follows: GCG (IC50: 4.8 μM) > EGC (IC50: 7.6 μM) > EGCG (IC50: 19.5 μM) > EGC (IC50: 24.8 μM). Catechins were not cytotoxic at up to 30 μM, the concentration at which marked inhibition was exhibited by (−)-ECG, (−)-EGCG and (−)-GCG (Fig. 2B). Other catechins had little or no effect at the concentrations tested. Since it is reported that EGCG inhibits LPS-induced TNFα production and NO production in mouse macrophages,11,28 we examined effect of catechins on TNFα and NO production induced by LPS. Similar to their effect on IL-12p40 production, (−)-EGC, (−)-EGCG and (−)-GCG decreased TNFα production significantly (Fig. 2C). However, catechins did not modulate LPS-induced NO production significantly at concentrations up to 30 μM (data not shown).

**Effect of Catechins on IL-12p40, TNFα and TLR4 mRNA Expression in LPS-Stimulated J774.1 Cells** To investigate whether catechins also affect the mRNA levels of the cytokines, we examined the effects of catechins on the LPS-induced IL-12p40 mRNA expression in J774.1 cells. IL-12p40 mRNA was detectable at 6 h, and reached maximal at 12 h after LPS treatment (Fig. 3A). Therefore, J774.1 cells, pretreated with catechins at 30 μM for 24 h, were stimulated with LPS for 16 h. As shown in Fig. 3B, (−)-GCG as well as (−)-ECG, (−)-EGC and (−)-EGCG, potently suppressed IL-12p40 mRNA expression. (−)-ECG, (−)-EGCG, (−)-GC and (−)-GCG also decreased TNFα mRNA levels. Other catechins had little or no effect at the concentration tested. The suppression of IL-12p40 and TNFα mRNA expression paralleled the drop in protein levels determined by ELISA. Catechins did not affect the mRNA expression of the LPS receptor, TLR4.

**Effect of Catechins on the LPS-Induced ERK, SAPK/JNK and p38 MAP Kinase Activation in J774.1 Cells** Since LPS activates at least three MAP kinases, i.e., ERK, SAPK/JNK and p38 MAPK in J774.1 cells with a maximal response at 30 min (Fig. 4A), J774.1 cells were pre-treated with catechins at a dose of 30 μM for 24 h, then stimulated with LPS for 30 min. The LPS-induced activation, i.e. phosphorylation, of p38 MAP kinase, was reduced significantly by (−)-ECG, (−)-EGC, (−)-EGCG or (−)-GCG pre-treatment, most potently by (−)-ECG. In contrast, the activation of ERK was little affected or was rather enhanced by (−)-ECG, (−)-EGCG, (−)-GC or (−)-GCG pre-treatment. Similarly, the LPS-induced phosphorylation of SAPK/JNK was unchanged or slightly decreased by (−)-ECG or (−)-EGCG pre-treatment (Fig. 4B).

**Effect of Catechins on LPS-Induced Degradation of IκBα and NF-κB Binding Assay, EMSA** Since the degradation of IκBα and subsequent activation of NF-κB are critical for the expression of the IL-12p40 gene in response to LPS,20 we examined the effect of catechins on the LPS-induced degradation of IκBα. As shown in Fig. 5A, treatment with LPS for 30 min induced marked degradation of IκBα. Various catechins were tested and both (−)-EGCG and (−)-GCG at 30 μM markedly suppressed the degradation. Modera-
ate inhibition was also shown by (−)-ECG, (−)-EGC and (−)-GC (Fig. 5B). In addition, EMSA, an assay for the nuclear NF-κB activation, indicated that (−)-EGCG, (−)-GCG as well as (−)-ECG, markedly suppressed the binding of nuclear extracts to NF-κB consensus oligonucleotide (Figs. 6A, B). Other catechins including (+)-, (−)-CA, (+)-, (−)-EC did not show any inhibition in this EMSA (data not shown). Thus, the marked inhibition of IL-12p40 production by catechins paralleled inhibition of IκBα degradation and subsequent NF-κB activation by these compounds.

**Effect of Various Catechins on LPS-Induced Synthesis of IRF-1**

Finally, as IFN regulatory factor-1 (IRF-1) binds to the IL-12p40 gene promoter and up-regulates the gene expression, we examined the effect of catechins on LPS-induced IRF-1 synthesis. Various catechins at 30 μM were tested for effects on LPS-unstimulated and LPS-stimulated synthesis of IRF-1 in J774.1 cells (Figs. 7A, B). As shown in Fig. 7B, (−)-ECG, and (−)-EGCG pretreatment inhibited most of the LPS-induced synthesis of IRF-1.
DISCUSSION

In the present study, we investigated the effects of various catechins on the production of IL-12 in macrophages activated by LPS. This study demonstrated that ECG, EGCG and GCG inhibited the production of IL-12p40 in murine PEC and a macrophage cell line, J774.1 cells. The most potent inhibition was exhibited by EGCG, followed by GCG and ECG, all of which are gallate-containing compounds. Other catechins had little or no effect on IL-12p40 production. This observation was consistent with the findings by Lin et al. and Yang et al., that EGCG usually has the greatest antioxidant activity or inhibitory activity toward NF-κB.

Although we did not assay IL-12p35 mRNA levels in J774.1 cells, the expression of which was under the limit of detection, we examined IL-12p40 at the protein and mRNA levels. Inhibitory effects of these catechins on the production of IL-12p40 were confirmed at the level of mRNA expression, the level of mRNA expression correlating well with the observed cytokine production. Since IL-12p40 transcription and NF-κB activation were inhibited by NO in murine macrophage and dendritic cells, we also examined the effect of catechins on LPS-induced NO production. However, we found no significant enhancement or suppression, suggesting that NO production contributed little, if any, to the observed suppression of LPS-induced IL-12p40 production by catechins. Lin et al. indicated that various tea polyphenols, particularly teaflavin-3,3′-gallate as well as EGCG, inhibits NO production and iNOS expression in activated murine macrophages, i.e., RAW264.7, by downregulating NF-κB activation. Since RAW264.7 cells produce larger amount of NO in response to LPS than J774.1 cells (data not shown), effects by catechins are presumed to be more prominent in the RAW264.7 cells.

IL-12 is an inducible, heterodimeric disulfide-linked cytokine composed of p35 and p40 subunits. Expression of the p35 subunit is constitutive and ubiquitous. Therefore, the biological activity of IL-12 is regulated mainly by the induction of p40, which was reported to be regulated primarily at the level of transcription. It should be noted that since IL-12 is a key cytokine in Th1-mediated autoimmune responses, downregulation of IL-12 production by catechins may ameliorate the autoimmune diseases. While three MAP kinases, ERK, SAPK/JNK and p38 MAPK, are reported to be involved in the LPS-induced production of NO as well as IL-12p40 by macrophages, ERK appeared to negatively regulate the LPS-induced mRNA expression of IL-12p40. Actually, our results demonstrated that (-)-EGCG and (-)-EGCG and (-)-GCG inhibited the phosphorylation of p38 MAP kinase markedly with a concomitant downregulation of IL-12p40 production. In contrast, activation of ERK MAP kinase was inhibited little or rather enhanced by (-)-EGCG, (-)-G, and (-)-GCG. The apparent opposing effects of catechins are yet to be explored, but the ERK MAP kinase activation pathway possibly mediates suppression of transcription factors including IRF-1 and IkB kinases as was substantiated by our previous study.

We demonstrated that catechins, particularly (-)-EGCG and (-)-GCG, prevented IkBα degradation, thus resulting in a suppression of NF-κB activation. Actually, we indicated that (-)-EGCG and (-)-GCG, as well as (-)-EGC, clearly suppressed nuclear factor binding to the NF-κB consensus sequence, suggesting that these catechins downregulate binding of transcription factor to the NF-κB sequence. Alternatively, this observation may suggest that catechins act directly or indirectly on IkBα degradation possibly through inhibition of the activation of IkBα kinases, IKKα or IKKβ, as has been demonstrated in an intestinal epithelial cell line. It was also demonstrated that EGCG inhibited the phosphorylation of IkBα-GST by acting on IKK activity at rather high doses. While EGCG induces apoptosis in several cancer cells including human epidermal carcinoma A4321 cells, albeit at higher the doses (at 40—80 μM) than those used in our study, EGCG inhibits constitutive and TNFα or LPS-mediated activation of NF-κB in these cells. Several anti-inflammatory agents including aspirin and sodium salicylate, as well as a number of antioxidants, such as pyrrolidine dithiocarbamate (PDTC), N-acetylcysteine (NAC) and vitamin E, can suppress NF-κB activity. The suppression by these antioxidants may not necessarily be unilateral, but the IKK complex composed of IKKα, IKKβ, and IKKγ is a potential target for developing anti-inflammatory drugs, since the activation of many immune and inflammatory genes including cytokines depends on NF-κB activation.

Structure-activity relationship on the catechins has not extensively been studied, but it is apparent that gallate group is essential for the inhibition of NF-κB activation and subsequent IL-12 production, since catechins lacking gallate group are less potent in inhibiting IL-12 production as shown in this study as well as in other papers. While EGCG induces apoptosis in several cancer cells, particularly teaflavin-3,3′-gallate as well as EGCG, inhibits NO production and iNOS expression in activated murine macrophages, i.e., RAW264.7, by downregulating NF-κB activation. Since RAW264.7 cells produce larger amount of NO in response to LPS than J774.1 cells (data not shown), effects by catechins are presumed to be more prominent in the RAW264.7 cells. Recently, Ahn et al. have reported that EGCG suppresses LPS-induced IL-12 production through inhibition of MAPK and NF-κB activation by dendritic cells, proposing that EGCG is a potent inhibitor of dendritic cell maturation. Other important regulatory elements affecting mouse IL-12p40 promoter activity are IRF-1 and interferon consensus sequence binding protein (ICSBP/IRF8), both IRF family members, since IL-12p40 expression is impaired in ICSBP-deficient mice and transfection of ICSBP together with IRF-1 restored the expression. We did not examine the role of the IRF family in this study, but (-)-ECG and (-)-EGCG in particular inhibited IRF-1 synthesis. It should be noted that these catechins downregulated the synthesis of IRF-1 family members, which finally resulted in the downregulation of IL-12p40 transcription. Further study is needed to clarify the mechanism by which catechins act on the IRF family of transcription factors.

In conclusion, we demonstrated that gallate-containing catechins, EGCG as well as GCG and ECG, are the potent inhibitors on IL-12 production. These catechins inhibited LPS-induced NF-κB activation by inducing rapid degradation of IkBα. While these observations are consistent to the previous findings reported by other groups, we stress that these catechins inhibited p38 MAPK activation, while rather augmented p44/p42 ERK, suggesting differential role of catechins regulating MAPK and ERK activation. In addition,
how gallate-containing catechins, particularly EGCG and GCG interact with IKK and IRF families, has not been studied extensively, but should be explored further to elucidate the anti-inflammatory action of these catechins.

REFERENCES