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Regulation of Interleukin-1β–Induced Chemokine Production and Matrix Metalloproteinase 2 Activation by Epigallocatechin-3-Gallate in Rheumatoid Arthritis Synovial Fibroblasts

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Objective. **To evaluate the efficacy of epigallocatechin-3-gallate (EGCG), a potent antiinflammatory molecule, in regulating interleukin-1β (IL-1β)–induced production of the chemokines RANTES (CCL5), monocyte chemoattractant protein 1 (MCP-1/CCL2), epithelial neutrophil–activating peptide 78 (ENA-78/CXCL5), growth-regulated oncogene α (GROα/CXCL1), and matrix metalloproteinase 2 (MMP-2) activity in rheumatoid arthritis (RA) synovial fibroblasts.**

Methods. **Fibroblasts obtained from RA synovium were grown, and conditioned medium was obtained. Cell viability was determined by MTT assay. RANTES, MCP-1, ENA-78, and GROα produced in culture supernatants were measured by enzyme-linked immunosorbent assay. MMP-2 activity was analyzed by gelatin zymography. Western blotting was used to study the phosphorylation of protein kinase C (PKC) isoforms and nuclear translocation of NF-κB.**

Results. **EGCG was nontoxic to RA synovial fibroblasts. Treatment with EGCG at 10 μ*M* or 20 μ*M* significantly inhibited IL-1β–induced ENA-78, RANTES, and GROα, but not MCP-1 production in a concentration-dependent manner. EGCG at 50 μ*M* caused a complete block of IL-1β–induced production of RANTES, ENA-78, and GROα, and reduced production of MCP-1 by 48% ($P < 0.05$). Zymography showed that EGCG blocked constitutive, IL-1β–induced, and chemokine-mediated MMP-2 activity. Evaluation of signaling events revealed that EGCG preferentially blocked the phosphorylation of PKCδ and inhibited the activation and nuclear translocation of NF-κB in IL-1β–treated RA synovial fibroblasts.**

Conclusion. **These results suggest that EGCG may be of potential therapeutic value in inhibiting joint destruction in RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by robust infiltration of leukocytes into the synovium, resulting in hyperplasia of the synovial lining, progressive cartilage destruction, and finally erosion of the underlying bone (1). Synovial fibroblasts mediate joint destruction in RA by producing chemokines that facilitate the expansion and invasion of synovial fibroblasts into the adjacent tissue, and the regulation of these events has been a primary target of therapeutic intervention in RA (2). In response to the proinflammatory cytokines produced by macrophages, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), RA synovial fibroblasts produce chemokines that promote inflammation, neovascularization, and cartilage degradation via activation of matrix-degrading enzymes, such as matrix metalloproteinases (MMPs) (3).

Chemokines are a specialized family of small (8–10-kd), structurally related proteins classified into 4 supergene families, C, CC, CXC, and CX_3C, based on the location of cysteine residues (4). The CC and CXC chemokines are well-established regulators of gene transcription, cell proliferation, and leukocyte trafficking to

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normal and inflamed tissues (5). Chemokines such as epithelial neutrophil–activating peptide 78 (ENA-78/CXCL5), RANTES (CCL5), monocyte chemoattractant protein 1 (MCP-1/CCL2), and growth-regulated oncogene α (GROα/CXCL1) are potent chemotactic agents that have been shown to be constitutively produced by RA synovial fibroblasts and up-regulated upon stimulation with IL-1β (6). These chemokines play a major role in inducing MMP activity and expression in RA synovial fibroblasts (7). IL-1β– or TNFα-mediated up-regulation of chemokines was found to be suppressed by the neutralization of IL-1β, but not TNFα, suggesting a predominant role of IL-1β (7).

Green tea (*Camellia sinensis*) is one of the most commonly consumed beverages in the world, with no significant side effects (8). Extensive studies in several animal models in the past 2 decades have verified the antioxidant, antiinflammatory, and antioncogenic properties of a polyphenolic mixture derived from green tea (9). A majority of pharmacologic properties of green tea are mimicked by its active constituent, epigallocatechin-3-gallate (EGCG) (10). In our earlier studies using human chondrocytes derived from osteoarthritic cartilage, we showed EGCG to be an effective inhibitor of the production of catabolic mediators, such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), by transcriptional and translational regulation (11–13). Recently, we showed that EGCG significantly inhibited the expression and activities of MMP-1 and MMP-13 in human chondrocytes at a physiologically achievable dose (14). Nonetheless, significant gaps remain in our understanding of the mechanism of action of EGCG in RA.

Hence, the present study was undertaken to study the effect of EGCG on IL-1β–induced production of chemokines and MMP-2 activation in RA synovial fibroblasts. EGCG down-regulated IL-1β–induced RANTES, MCP-1, ENA-78, and GROα production and MMP-2 activation in human RA synovial fibroblasts, and it was also effective in blocking chemokine-induced MMP-2 activity in RA synovial fibroblasts. We provide evidence that EGCG specifically inhibits IL-1β–induced protein kinase Cδ (PKCδ) phosphorylation and suppresses chemokine production and MMP-2 activation. Additionally, we show that the protective effects of EGCG are also mediated via NF-κB inhibition.

MATERIALS AND METHODS

Antibodies and reagents. EGCG was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal anti–phospho-PKCδ, anti–phospho-PKCα/βII, anti–phospho-PKCε, and anti-rabbit horseradish peroxidase–linked secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti–β-actin and anti–NF-κB p65 were purchased from Sigma-Aldrich and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. All signaling inhibitors used in this study were purchased from Calbiochem (La Jolla, CA). Enzyme-linked immunosorbent assay kits for MCP-1, ENA-78, GROα, and RANTES were purchased from R&D Systems.

Culture of human RA synovial fibroblasts. Fibroblasts were isolated from synovium obtained from RA patients who had undergone total joint replacement surgery or synovectomy, according to a protocol approved by the institutional review board of the University of Michigan Medical School. Fresh synovial tissues were minced and digested in a solution of Dispase, collagenase, and DNase. Cells were used at passage 5 or older, at which time they were a homogeneous population. RA synovial fibroblasts were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2. Upon confluence, cells were passaged by brief trypsinization, as previously described (15). All treatments were performed in serum-free medium.

Preparation of EGCG solution. A stock solution of EGCG (10 m*M*) was prepared in water, sterile filtered with 0.2-μm syringe filters, and stored at -20°C in aliquots. Fresh EGCG solution was used in each experiment and directly added to the culture medium.

Treatment of RA synovial fibroblasts with IL-1β and EGCG. To study the effect of EGCG on cell viability, RA synovial fibroblasts (2×10^4/well) were plated in 96-well, flat-bottomed tissue culture plates (Corning, Corning, NY) and cultured in RPMI 1640 plus 10% FBS for 6 hours. This was then replaced with fresh medium and culture continued for 24 hours. EGCG (10–60 μM) was added to RA synovial fibroblasts in serum-free medium and the culture was incubated at 37°C for another 24 hours. Two hours prior to termination, 20 μl of MTT dye (5 mg/ml in sterile phosphate buffered saline [PBS]; Invitrogen, Carlsbad, CA) was added to each well and further incubated at 37°C. At the end of incubation, cells were washed twice with PBS, solubilized in 100 μl of DMSO at 37°C for 5 minutes, and read at an optical density of 570 nm.

To study the effect of EGCG on chemokine production, RA synovial fibroblasts were incubated with or without EGCG (10–50 μM) in serum-free medium for 12 hours, followed by stimulation with IL-1β (10 ng/ml) for 24 hours. After 24 hours, culture supernatant was collected and centrifuged at 10,000*g* for 5 minutes at 4°C to remove particulate matter, and stored at -80°C in fresh tubes. Using commercially available kits (R&D Systems), culture supernatants were used to determine the quantities of RANTES, MCP-1, ENA-78, and GROα. To study the signaling mechanism of chemokine production by IL-1β, RA synovial fibroblasts were incubated with PKC inhibitors (Rottlerin and Gö 6976; 10 μM) and NF-κB inhibitor (pyrrolidine dithiocarbamate [PDTC]; 200 μM) for 2 hours, followed by stimulation with IL-1β for 24 hours, and processed as above.

Western immunoblotting and analysis. To study the effect of EGCG on signaling events, RA synovial fibroblasts were incubated with or without EGCG (1–20 μM) in serum-free RPMI 1640 for 12 hours, followed by stimulation with

IL-1β for 20 minutes. Cells were lysed in cell lysis buffer containing 100 m*M* Tris (pH 7.4), 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* NaF, 20 m*M* NaP_2O_4, 2 m*M* Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 1 m*M* phenylmethylsulfonyl fluoride [PMSF], and protease inhibitors (1 tablet/10 ml; Roche, Indianapolis, IN). Protein was measured using a BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were loaded and separated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA). Blots were probed using rabbit polyclonal antibodies specific for phosphorylated PKCα/βII, PKCδ, and PKCε isoforms, and rabbit polyclonal anti–β-actin. The immunoreactive protein bands were visualized by enhanced chemiluminescence. Densitometric analysis of the bands was performed using UN-SCAN-IT software, version 5.1 (Silk Scientific, Orem, UT), and the data were analyzed using Prism software (GraphPad Software, San Diego, CA).

Gelatin zymography. MMP-2 activity in conditioned medium was measured as previously described (16). Briefly, 15 μl of conditioned medium was added to 15 μl of 2× nonreducing sample buffer, resolved under nonreducing conditions on 7.5% SDS–polyacrylamide gels polymerized with 1 mg/ml gelatin (type B from bovine skin; Sigma) as a substrate, and electrophoresed at 125V. Following electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 minutes with gentle shaking, followed by a 30-minute wash in developing buffer (50 m*M* Tris HCl [pH 8.0], 5 m*M* $CaCl_2$, and 0.02% NaN_3). The gels were incubated overnight in fresh developing buffer at 37°C, stained in Coomassie brilliant blue R250, and then destained using a solution of 7% acetic acid and 5% methanol. Images of the digested regions representing MMP activity were captured using the Quantity One 1-D image analyzer (Bio-Rad) and analyzed using UN-SCAN-IT software.

Preparation of nuclear extracts. To study the effect of EGCG on IL-1β–induced NF-κB activation, RA synovial fibroblasts (2×10^6/well) were grown to confluence in 10-cm dishes and treated with EGCG (10 μ*M* and 20 μ*M*) for 12 hours with and without IL-1β stimulation for 30 minutes. Cytoplasmic and nuclear fractions were prepared as previously described (17). Upon termination of the reaction, cells were washed twice with ice-cold PBS (pH 7.4), scraped, collected in Eppendorf tubes, and centrifuged at 1,500*g* for 5 minutes at 4°C. The pellet obtained was suspended in 400 μl buffer A (10 m*M* HEPES buffered saline [pH 7.9], 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* dithiothreitol [DTT], and 0.5 m*M* phenylmethylsulfonyl fluoride PMSF), gently mixed, and placed on ice for 15 minutes. Twenty-five microliters of cold 10% Nonidet P40 was added to individual samples, and the samples were vortexed and centrifuged at 14,000*g* for 30 seconds. Supernatant (cytoplasmic fraction) was collected, and the nuclear pellet obtained was suspended in 50 μl buffer C (20 m*M* HEPES buffered saline [pH 7.9], 0.4*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT, and 1 m*M* PMSF) and rocked for 45 minutes at 4°C. Samples were centrifuged for 15 minutes at 14,000*g* at 4°C. The supernatant (nuclear fraction) was collected and stored at −80°C. Nuclear cell lysate (15 μg) was used to detect NF-κB p65 by Western blotting.

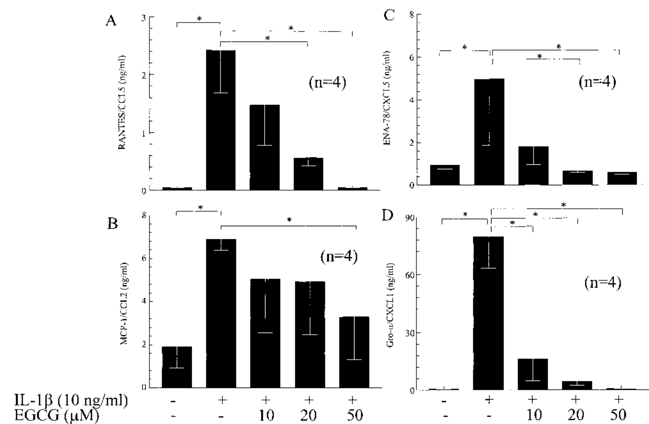


Figure 1. Inhibition of interleukin-1β (IL-1β)–induced production of **A,** RANTES (CCL5), **B,** monocyte chemoattractant protein 1 (MCP-1/CCL2), **C,** epithelial neutrophil–activating peptide 78 (ENA-78/CXCL5), and **D,** growth-regulated oncogene α (GROα/CXCL1) by epigallocatechin-3-gallate (EGCG) in rheumatoid arthritis (RA) synovial fibroblasts. RA synovial fibroblasts (2×10^4/well) were incubated with EGCG (10–50 μ*M*) for 12 hours, followed by stimulation with IL-1β (10 ng/ml) for 24 hours. Production of RANTES, MCP-1, ENA-78, and GROα in culture supernatants was measured using a commercially available enzyme-linked immunosorbent assay kit. Values are the mean and SEM. $* = P < 0.05$ versus treatment with IL-1β alone.

Statistical analysis. Student's *t*-tests were performed to calculate statistical differences between the different variables. *P* values less than 0.05 were considered significant.

RESULTS

Effect of EGCG on RA synovial fibroblast viability. The results of an MTT-based viability assay using samples obtained from 3 different donors showed that EGCG (10–60 μ*M*) had no significant effect on the viability of cultured RA synovial fibroblasts (data not shown). The highest concentration used in the viability assay (60 μ*M*) showed a mean ± SEM 10 ± 4.0% loss in viability, which was not statistically significant ($P > 0.05$).

Effect of EGCG on IL-1β–induced chemokine production. IL-1β is a potent inducer of chemokine production in RA synovial fibroblasts (18). Excessive production of chemokines by RA synovial fibroblasts has been shown to induce proliferation of these cells and facilitate invasion into the adjacent tissues (7). In the present study, stimulation of RA synovial fibroblasts with IL-1β (10 ng/ml) for 24 hours resulted in a 50-, 3.6-, 5.2-, and 120-fold induction of RANTES, MCP-1, ENA-78, and GROα production, respectively, by RA synovial fibroblasts as compared with untreated controls ($P < 0.05$) (Figures 1A–D). Treatment with EGCG caused a significant, but differential, inhibition of IL-1β–

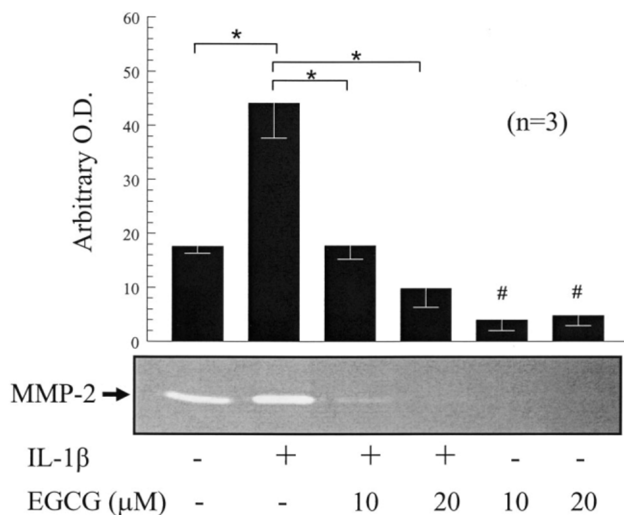
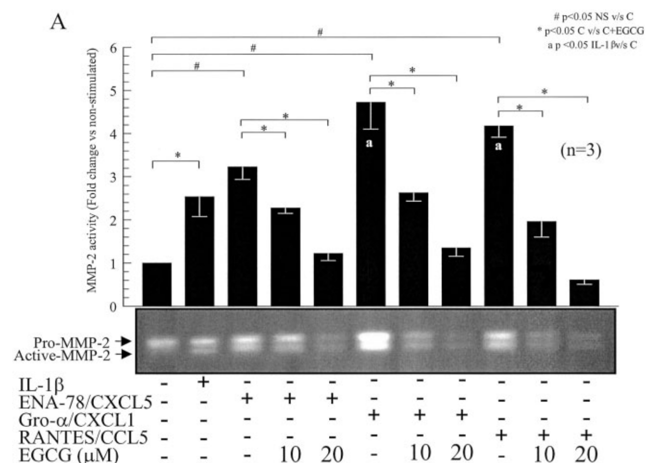


Figure 2. Inhibition of constitutive and IL-1β–induced matrix metalloproteinase 2 (MMP-2) activity by EGCG in RA synovial fibroblasts. RA synovial fibroblasts (2×10^5/well) were incubated with EGCG (10 μM or 20 μM) for 12 hours, followed by no stimulation or stimulation with IL-1β (10 ng/ml) for 24 hours. MMP-2 activity in the cell-free supernatants from different treatment combinations was estimated by gelatin zymography. Values are the mean and SEM. $* = P < 0.05$ versus treatment with IL-1β alone; $\# = P < 0.05$ versus untreated control. OD = optical density (see Figure 1 for other definitions).

induced ENA-78, RANTES, and GROα production, in a concentration-dependent manner. EGCG at 10 μM blocked IL-1β–induced ENA-78, RANTES, and GROα production by 64%, 39%, and 80%, respectively. Interestingly, EGCG at 50 μM resulted in almost 88% inhibition of the production of ENA-78, and almost completely blocked RANTES and GROα production as compared with samples treated with IL-1β alone ($P < 0.05$). However, EGCG 10 μM and 20 μM had no significant inhibitory effect on IL-1β–induced MCP-1 production, whereas 50 μM EGCG caused almost 48% inhibition of MCP-1 production ($P < 0.05$).

Effect of EGCG on IL-1β–induced MMP-2 activity in RA synovial fibroblasts. MMPs execute a rate-limiting step in synovial fibroblast invasion, inflammation, and cartilage breakdown under abnormal conditions such as RA (19). The effect of EGCG on IL-1β–induced MMP-2 activity was determined using zymography, and the densitometric values of the bands were obtained for statistical analysis. RA synovial fibroblasts were found to have a detectable basal level of MMP-2 activity with no stimulation. Stimulation of RA synovial fibroblasts with IL-1β resulted in a 2.5-fold increase in MMP-2 activity ($P < 0.05$) (Figure 2). Treatment of RA synovial fibroblasts with EGCG at concentrations of 10 μM and 20 μM resulted in a mean $\pm$ SEM 58 $\pm$ 14% and 75 $\pm$ 16% inhibition of IL-1β–induced MMP-2 activity, respectively ($P < 0.05$). Interestingly, the fibroblasts treated with EGCG (10 μM and 20 μM) alone showed a marked inhibition of MMP-2 activity (76 $\pm$ 22% and 70 $\pm$ 20%, respectively), when compared with the activity observed in untreated samples ($P < 0.05$).



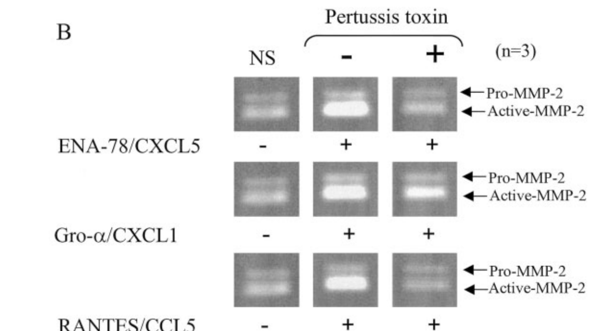


Figure 3. Inhibition of chemokine (ENA-78, GROα, and RANTES)–induced matrix metalloproteinase 2 (MMP-2) activity by EGCG in RA synovial fibroblasts. **A,** RA synovial fibroblasts (2×10^5/well) were left untreated or treated with EGCG (10 μM or 20 μM) for 12 hours, followed by stimulation with IL-1β (10 ng/ml), ENA-78 (100 ng/ml), GROα (100 ng/ml), or RANTES (100 ng/ml) for 24 hours. MMP-2 activity in the cell-free supernatants was determined by zymography using gelatin (1 mg/ml) as a substrate, after activation with APMA (10 μM) for 2 hours to separate proMMP-2 and active MMP-2 bands. Values are the mean and SEM. **B,** RA synovial fibroblasts (2×10^5/well) were left untreated or treated with pertussis toxin (0.75 μg/ml) for 2 hours, followed by stimulation with ENA-78, GROα, or RANTES (100 ng/ml each) for 24 hours. Blots in **A** and **B** are representative of 3 independent samples. NS = not stimulated; C = chemokines (see Figure 1 for other definitions).

Effect of EGCG on chemokine-induced MMP-2 activity in RA synovial fibroblasts. Chemokines facilitate a unique mechanism of tissue invasion in the diseased joint by enhancing RA synovial fibroblast proliferation and MMP up-regulation (7). Regulation of MMP-2 activity is of therapeutic value since it plays a predominant role in processes such as angiogenesis, inflammation, and tissue invasion (19). In the present study, we found that ENA-78, GROα, and RANTES increased RA synovial fibroblast MMP-2 activity by 3.9-, 5.4-, and 4.9-fold, respectively, as compared with untreated controls ($P < 0.05$) (Figure 3A). MMP-2 activity induced by ENA-78, GROα, and RANTES was 1.1-, 1.5-, and 1.3-fold higher, respectively, than that observed in IL-1β–stimulated samples. Each sample was activated with 10 μM APMA for 2 hours to separate proMMP-2 and active MMP-2 by gelatin zymography. Treatment of RA synovial fibroblasts with EGCG markedly inhibited the ability of chemokines to stimulate MMP-2 activity, in a concentration-dependent manner ($P < 0.05$). EGCG at 10 μM and 20 μM blocked MMP-2 activity induced by ENA-78 by 33% and 75%, by GROα by 52% and 80%, and by RANTES by 55% and 84%, respectively ($P <$ 0.05). In another set of experiments, RA synovial fibroblasts treated with pertussis toxin (0.75 μg/ml) showed a significant decrease in MMP-2 activity (Figure 3B), which suggests that the chemokines have a direct effect on MMP-2 activation.

Role of PKCδ and NF-κB in IL-1β–induced chemokine production and MMP-2 activation. To study the signaling pathways involved in IL-1β–induced production of chemokines and MMP-2 activation, RA synovial fibroblasts were pretreated with 10 μM Rottlerin (PKCδ inhibitor), 10 μM Gö 6976 (PKCα inhibitor), or 200 μM PDTC (NF-κB inhibitor) for 2 hours, followed by IL-1β stimulation for 24 hours. The culture supernatants were used to estimate ENA-78, GROα, and RANTES production and MMP-2 activation. The PKCδ inhibitor almost completely blocked IL-1β–induced ENA-78, GROα, and RANTES production and MMP-2 activation ($P < 0.001$) (Figures 4A and B). In contrast, PDTC treatment caused almost complete inhibition of MMP-2 activity, but induced only partial inhibition of the chemokines studied. The PKCα inhibitor moderately decreased ENA-78 and RANTES production and MMP-2 activity, with little effect on GROα production.

Effects of EGCG on chemokine production and MMP-2 activation. In light of these observations, we evaluated the effect of EGCG treatment on IL-1β–induced PKC isoform phosphorylation and NF-κB activation. Densitometric analysis of protein bands showed

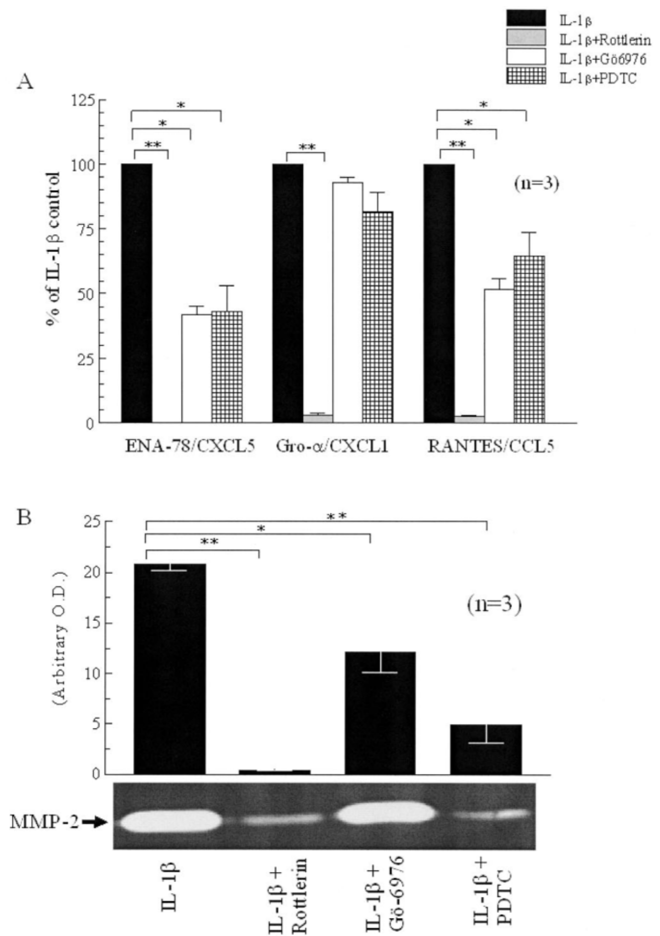


Figure 4. Involvement of protein kinase C (PKC) and NF-κB in the regulation of IL-1β–induced chemokine production and matrix metalloproteinase 2 (MMP-2) activation. RA synovial fibroblasts (2×10^5/well) were pretreated with inhibitors of PKCδ (Rottlerin; 10 μM), PKCα (Gö 6976; 10 μM), or NF-κB (pyrrolidine dithiocarbamate [PDTC]; 200 μM) for 2 hours, followed by stimulation with IL-1β (10 ng/ml) for 24 hours. **A,** Production of chemokines ENA-78, GROα, and RANTES in culture supernatants. **B,** MMP-2 activity. Values are the mean and SEM. $* = P < 0.05$; $** = P < 0.001$, versus treatment with IL-1β alone. OD = optical density (see Figure 1 for other definitions).

that IL-1β preferentially increased PKCδ phosphorylation to almost 3-fold that in untreated controls, without causing any marked activation of other PKC isoforms (Figure 5A). Treatment of RA synovial fibroblasts with EGCG (1–20 μM) resulted in concentration-dependent inhibition of the phosphorylation of PKCδ. In the present study, even the lowest concentration of EGCG (1 μM) suppressed phosphorylated PKCδ levels by 35%, whereas 20 μM of EGCG completely blocked the phosphorylation of PKCδ (Figure 5A). Interestingly, EGCG

was able to reduce the levels of phosphorylated PKCε by up to 30% at a 20 μM concentration, but did not inhibit the phosphorylation of PKCα/βII in IL-1β–treated RA synovial fibroblasts.

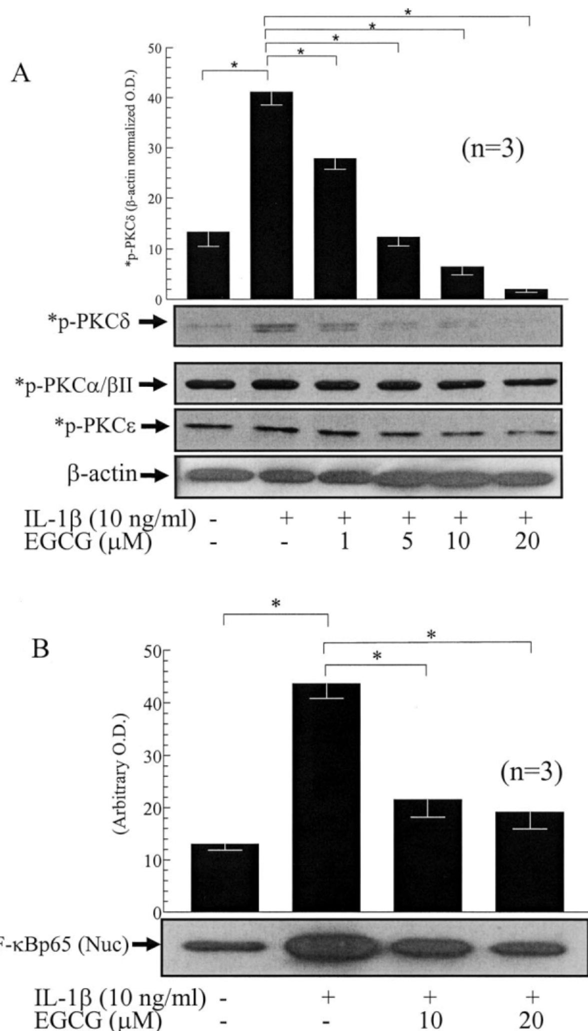


Figure 5. Preferential inhibition of IL-1β–induced protein kinase Cδ (PKCδ) phosphorylation and NF-κB activation by EGCG in RA synovial fibroblasts. **A,** RA synovial fibroblasts (2×10^5/well) were treated with EGCG (1–20 μM) for 12 hours, followed by stimulation with IL-1β (10 ng/ml) for 20 minutes. Cells were lysed in extraction buffer containing protease inhibitors, and the phosphorylation (p) of PKC isoforms in 20 μg of each sample was determined by Western blotting. A representative blot is shown. Equal loading of protein was verified by reprobing blots for β-actin. **B,** RA synovial fibroblasts (2×10^6/well) were pretreated with EGCG (10 μM or 20 μM) for 12 hours, followed by stimulation with IL-1β (10 ng/ml) for 30 minutes. The nuclear fraction was prepared as described in Materials and Methods. Nuclear protein (Nuc; 15 μg) was used to analyze nuclear NF-κB p65. Values are the mean and SEM. $* = P < 0.05$ versus treatment with IL-1β. OD = optical density (see Figure 1 for other definitions).

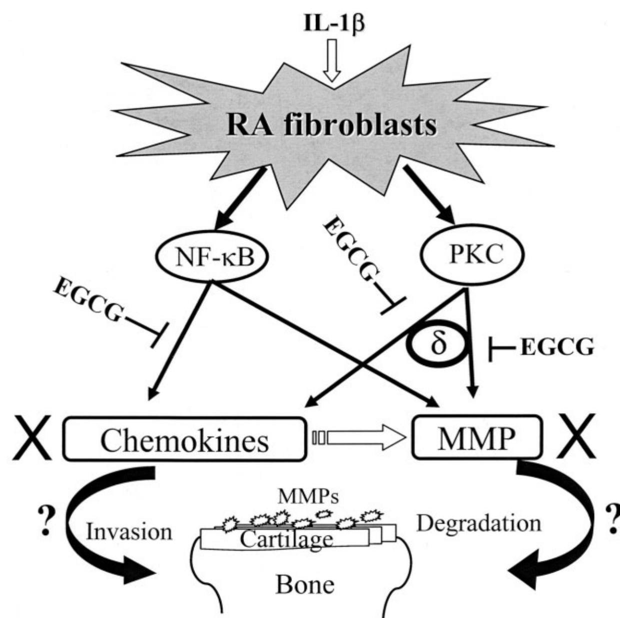


Figure 6. Schematic representation of the mechanism of EGCG in inhibiting IL-1β–induced chemokine production and matrix metalloproteinase 2 (MMP-2) activation in RA synovial fibroblasts. Findings of the present study suggest the efficacy of EGCG in regulating the stimulating effects of IL-1β on RA synovial fibroblast chemokine production and MMP-2 activation via specific inhibition of protein kinase Cδ (PKCδ) phosphorylation and NF-κB activation and nuclear translocation. See Figure 1 for other definitions.

The results of Western blotting in the nuclear lysates showed that IL-1β increased activation and nuclear translocation of NF-κB in RA synovial fibroblasts by 3.5-fold, when compared with unstimulated controls ($P < 0.05$) (Figure 5B). Treatment with 10 μM and 20 μM EGCG resulted in the inhibition of IL-1β–induced nuclear translocation of NF-κB, by 51% and 58%, respectively ($P < 0.05$). These results provide direct evidence of the signaling mechanism by which EGCG produces these regulating effects in RA synovial fibroblasts (Figure 6).

DISCUSSION

This is the first study, to our knowledge, to provide evidence of the ability of EGCG, a potent antiinflammatory molecule derived from green tea, to regulate IL-1β–induced chemokine production and MMP-2 activation in cultured RA synovial fibroblasts. In particular, this study assigns EGCG a novel property of chemokine suppressor, and identifies these inhibitory effects as being mediated via specific inhibition of the PKCδ signaling pathway. In addition, EGCG inhibited the activation and nuclear translocation of NF-κB to

elicit its response. These findings indicate that EGCG, when present in concentrations that are physiologically achievable, is able to suppress the effects of IL-1β by blocking chemokine production, and hence may play a role in regulating tissue invasion by RA synovial fibroblasts. Other regulatory mechanisms, e.g., transcriptional and posttranscriptional control of messenger RNA levels of chemokines and chemokine receptors by EGCG, may also be important, and are currently under study.

Despite the identification of potent chemokine and chemokine receptor antagonists in vitro, their structural diversity, species-based potency differences, and suboptimal pharmacokinetic and toxicity profiles suggest that novel RA therapies targeting multiple chemokines and their receptors may prove to be more beneficial treatment options (20). In the present study, EGCG blocked IL-1β–induced ENA-78, GROα, and RANTES production by RA synovial fibroblasts in a concentration-dependent manner, with no effect on the viability of these cells. ENA-78 and GROα are members of the CXC chemokine subgroup that possess potent chemoattractant and neutrophil activator functions (6, 21,22). RANTES is a member of the CC subfamily and has been shown to chemoattract lymphocytes and monocytes (23). These chemokines are constitutively produced by RA synovial fibroblasts and are up-regulated upon stimulation with IL-1β (7,18). Interestingly, in animal models of arthritis, the expression of these chemokines generally preceded the onset of clinical symptoms (24–28).

Recently, EGCG was shown to inhibit TNFα-induced IL-8 and macrophage inflammatory protein 3α (MIP-3α) production in human colon epithelial cells (29). In another study, EGCG was found to block lipopolysaccharide-induced neutrophil chemotaxis of rat macrophages (30). In human keratinocytes, preincubation with EGCG has been shown to act in a synergistic manner with genistein, a dietary polyphenol, in inhibiting TNFα-induced vascular growth endothelial factor and IL-8 production (31). EGCG-mediated inhibition of chemokines in the present study is consistent with the findings of previous studies with animal models, in which treatment with neutralization-specific antibodies, polyclonal antibodies, or receptor antagonists against ENA-78, GROα, and RANTES has led to significant improvement in clinical symptoms of arthritis (27,32,33). Furthermore, the regulation of multiple chemokines by EGCG in RA synovial fibroblasts is consistent with the finding of synergistic effects with the use of antibodies directed against multiple chemokines (34–36).

MMP-2 is involved in RA pathogenesis by assisting RA synovial fibroblasts in the invasion of microvascular basement membrane and the interstitium (37,38). Chemokine-activated RA synovial fibroblasts may mediate this process by their attachment to the cartilage surface and the release of MMPs (7). Interestingly, recent findings suggest an active involvement of selective chemokines in the destructive phase of RA, in which migration, proliferation, and MMP production by RA synovial fibroblasts are characteristic features (7). Therefore, possible therapeutic strategies include impeding the production of MMPs, blocking the active sites of MMPs, increasing production of endogenous tissue inhibitors of MMPs, and preventing the activation of MMPs (39).

Recently, we showed that treatment of cultured human OA chondrocytes with EGCG significantly inhibited the expression and activities of collagenases (MMP-1 and MMP-13) (14). Also, others reported that catechins from green tea inhibited the degradation of human cartilage proteoglycan and type II collagen, and selectively inhibited the aggrecanases ADAMTS-1, -4, and -5 (40,41). The evidence from the present study that EGCG blocks constitutive and cytokine-induced MMP-2 activity is important. Previous studies have shown that the lack of efficacy of direct MMP inhibitors (trocade and BAY12-9566) was partially attributed to their impaired bioavailability and inability to control angiogenesis (42,43), which is also involved in the early and destructive phase of RA. The pharmacokinetics of EGCG in human volunteers taking a single dosage of 1,600 mg/day showed a rapid absorption, with a maximum plasma concentration value of 3,392 ng/ml; the time to reach maximum plasma concentration was 2.2 hours, and the terminal elimination half-life ranged between 1.9 and 4.6 hours (44). Interestingly, 10-day repeated administration of oral doses of EGCG of up to 800 mg per day was found to be safe and very well tolerated (45).

Traditionally, strategies for the development of antiinflammatory treatment have been broadly divided into 2 approaches: those acting outside the cell and those acting inside the cell. The first approach targets receptors and cytokines, using agents such as antibodies or cytokine traps (e.g., anti-TNFα or soluble IL-1 receptor antagonist therapies) to block ligand–cell surface receptor interactions. The second approach uses inhibitors such as cyclosporine, cyclooxygenase 2 (COX-2) inhibitors, and steroids that easily enter the cell. Such intracellular interference with signaling pathways has generated very effective and wide-ranging antiinflamma-

tory therapies, with the caveat that they also work as general immunosuppressants (20). The chemokine network lends itself to intervention using both approaches.

In autoimmune diseases such as RA, it is partly the inappropriate leukocyte recruitment accompanied by cellular activation that results in disease symptoms and progression (5). This is due largely to abnormal expression of chemokines and cytokines (2). In the present study, EGCG specifically blocked IL-1β–induced phosphorylation of the PKCδ isoform, with little or no effect on other PKC isoforms. Some of the effects of EGCG have been shown to be mediated via PKCα isoform inhibition. EGCG was shown to inhibit amyloid β toxicity in neuronal cells by blocking PKCα (46). It also modulated the gene expression profile of pro-oncogenes in prostate carcinoma LNCaP cells by completely blocking PKCα, without affecting PKCβ, PKCα, PKCζ, PKCε, and PKCδ isoforms (47). A recent study by Lee et al showed that EGCG inhibited NO-induced apoptosis by regulation of p53 gene via PKCδ in dopaminergic neurons (48). In the present study, using PKCα and PKCδ inhibitors, we demonstrated that activation of PKCδ plays a pivotal role in the regulation of IL-1β–stimulated chemokine production and MMP-2 activity in RA synovial fibroblasts. The inhibition of IL-1β–stimulated PKC activation by EGCG provides indirect evidence that EGCG may act through PKC to inhibit the IL-1β–stimulated increase in chemokine production and MMP-2 activity.

Recent studies suggest that PKCδ regulates intercellular adhesion molecule 1 expression via NF-κB activation in human umbilical vein endothelial cells as well as NF-κB–dependent gene expression in a human epithelial cell line (49). In another study, PKCδ has been shown to mediate lysophosphatidic acid–induced NF-κB activation and IL-8 secretion in human bronchial epithelial cells (50). In the present study using RA synovial fibroblasts, inhibition of MMP-2 activity and moderate suppression of chemokine production by the NF-κB inhibitor PDTC suggest that these effects are, at least in part, controlled by PKCδ. However, this relationship was not directly examined in the current study. Our earlier studies provided direct evidence of the role of EGCG in inhibiting IL-1β–induced degradation of endogenous IκBα and NF-κB activation to suppress inducible NO synthase and COX-2 expression, resulting in the reduction of NO and PGE_2 synthesis in human chondrocytes (11,13). We also showed that EGCG differentially regulated several distinct pathways and transcription factors NF-κB and activation protein 1, to attenuate the production of MMP-1 and MMP-13 in chondrocytes isolated from arthritic joints (13,14).

In conclusion, the data presented here demonstrate that, in RA synovial fibroblasts, EGCG inhibits IL-1β–stimulated increases in chemokine production and MMP-2 activation by inhibition of the PKCδ pathway and NF-κB translocation to the nucleus (Figure 6). Activation of PKCδ plays a critical role in the regulation of these downstream effectors, and the regulation of PKCδ by EGCG provides evidence of the potential of EGCG to block chemokine production and invasion in RA synovial fibroblasts.

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