Epigallocatechin-3-gallate Impairs Chemokine Production in Human Colon Epithelial Cell Lines

Debora Porath, Christoph Riegger, Juergen Drewe, and Joseph Schwager

DSM Nutritional Products Ltd., Research and Development, Human Nutrition and Health, Kaiseraugst, Switzerland (D.P., C.R., J.S.); and Institute of Clinic Pharmacology and Toxicology, Department of Pharmacy, University of Basel, Basel, Switzerland (J.D.)

Received May 30, 2005; accepted August 16, 2005

ABSTRACT

A major component in green tea, epigallocatechin-3-gallate (EGCG), is reported to interfere with different steps of a number of inflammatory pathways. After oral administration, EGCG is retained in the gastrointestinal tract, where it is thought to exert preventive functions against inflammatory bowel disease and colon cancer. In this study, the human colon adenocarcinoma cell lines HT29 and T84 were used to investigate the effect of EGCG on intestinal inflammation. HT29 and T84 cells were stimulated with tumor necrosis factor (TNF)-α to induce the inflammatory condition and to trigger the inflammatory cascade in vitro and treated with EGCG to study its effect on inflammatory processes. The secretion of the chemokines interleukin (IL)-8, macrophage inflammatory protein (MIP)-3α, and prostaglandin E2 (PGE2) was determined by enzyme-linked immunosorbent assay. The gene expression level was measured by quantitative real-time polymerase chain reaction. Treatment of TNF-α-stimulated HT29 cells with EGCG dose-dependently inhibited the synthesis of IL-8, MIP-3α, and PGE2. Treatment with EGCG also inhibited the production of IL-8 and MIP-3α in TNF-α-stimulated T84 cells. Gene expression analysis in both HT29 and T84 cells revealed that EGCG down-regulates genes involved in inflammatory pathways. This study shows that EGCG acts broadly on the production of chemokines and PGE2 in the chemokine and eicosanoid pathways of colon epithelial cells. Therefore, EGCG might prove useful for the prevention and/or attenuation of colonic disorders.

The constant exposure of the intestinal epithelium to diverse types of nutrients and microorganisms present in the natural flora leads to a permanent stress state for the enterocytes. Mucosal surfaces of the intestinal tract form one of the main routes for microbial pathogens to enter a host and are important sites of microbially induced diseases. The intestinal mucosa becomes compromised in some food allergies or after invasion of the epithelium by pathogenic bacteria. This might lead to acute inflammation, characterized by an excessive production of inflammatory mediators. These are, in part, responsible for the recruitment of specific cell types, for example, macrophages/monocytes, lymphocytes, or neutrophils (Kagnoff, 1996).

During intestinal inflammation, polymorphonuclear leukocytes (PMLs), including neutrophils, migrate into the mucosa. In the intestine, an exacerbation of destructive processes occurs due to different proinflammatory and chemotaxtactant molecules. The latter are chemokines, a large family of small proteins, which are involved in innate immune and inflammatory responses by chemotaxtacting to and activating leukocytes at the site of inflammation and up-regulating adhesion molecules important for leukocyte trafficking (Baggiolini and Moser, 1997; Dwinell et al., 1999). Biological actions of chemokines are mediated through G-protein-coupled receptors that are present on the surface of target cells (Balkwill, 1998; Murphy et al., 2000). These receptors exhibit overlapping specificity for chemokines within each subfamily, and the cellular expression of respective receptors determines which cell types respond to a given chemokine. Based on the arrangement of their N-terminal cysteine residues, chemokines are subdivided into four subfamilies, including CXC and CC. Cells responsive to chemokines recognize concentration gradients and migrate toward the source of chemokine secretion. IL-8, MIP-2, and growth-regulated oncogene (GRO)-α and -γ are CXC chemokines that

ABBREVIATIONS: PML, polymorphonuclear leukocyte; IL, interleukin; MIP, macrophage inflammatory protein; GRO, growth-regulated oncogene; PGE2, prostaglandin E2; COX, cyclooxygenase; EGCG, epigallocatechin-3-gallate; TNF, tumor necrosis factor NF-κB, nuclear factor κB; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; LDH, lactate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PCS-T, PBS containing 0.05% Tween 20; RT, real time; PCR, polymerase chain reaction; RANK, receptor activator of NF-κB; ΔC1, (threshold cycle reference genes) — (threshold cycle unknown gene); LOX, lipoxygenase; RANTES, regulated on activation normal T cell expressed and secreted; IBD, inflammatory bowel disease.
attract and activate neutrophils, whereas the CC chemokines, such as MIP-3α, activate leukocyte populations, including monocytes, T lymphocytes, dendritic cells, and, to a lesser extent, neutrophils (Ajuebor and Swain, 2002). Chemokines contribute to the perpetuation of inflammatory processes, increasing chronic intestinal inflammation and mucosal destruction. Secreted chemokines and their specific chemokine cell surface receptors play a crucial role in the final composition of leukocytes present in the inflamed intestine (MacDermott, 1999; Banks et al., 2003).

Prostaglandins are also involved in the regulation of a variety of physiological and pathological processes in the immune response and in inflammation. PGE2 and other prostanoids are generated through two bifunctional enzymes, cyclooxygenases (COXs)-1 and -2 (Kim et al., 1998). In general, COX-1 is constitutively expressed in a wide range of tissues, including the gastrointestinal tract and plays a role in the tissue homeostasis, e.g., maintenance of gastrointestinal integrity (Singer et al., 1998). The inducible form, COX-2, which regulates prostaglandin synthesis, is overexpressed in several epithelial cancers and at sites of inflammation (Park et al., 1997; Kim et al., 1998; Singer et al., 1998; Poligone and Baldwin, 2001; Martel-Pelletier et al., 2003). The expression of this enzyme is induced by various stimuli, e.g., TNF-α and IL-1β, in a variety of cell lines.

Natural compounds, such as components of green tea, influence inflammation and cancer (Chen et al., 2003). EGCG, the major catechin of green tea, has many biological functions, including anti-inflammatory and chemopreventive effects (Lin and Lin, 1997; Ahn et al., 2004; Park and Suh, 2004). In China, Korea, and Japan, where a large proportion of the population consumes green tea daily, the incidence of colon cancer is relatively low compared with that found in Western societies. EGCG interferes in several steps of inflammatory processes, e.g., in the synthesis of eicosanoids and in the chemokine-mediated recruitment of PMLs to the injured site (Chen et al., 2002). Furthermore, EGCG was shown to suppress the maturation of murine dendritic cells through the inhibition of extracellular signal-regulated kinase, p38 kinase, c-Jun NH₂-terminal kinase, and the NF-κB signaling pathway (Ahn et al., 2004). In vivo studies show that green tea polyphenols decrease inflammation in animal models (Variile et al., 2001).

Several reports have shown the benefits of EGCG in a variety of inflammatory conditions. In the present study, we evaluate the anti-inflammatory effects of EGCG in vitro. Human colon adenocarcinoma cell lines HT29 and T84 were stimulated with TNF-α, and the potential of EGCG to inhibit the expression of cytokines and chemokines, was studied. Here, we show that EGCG effectively modulates a number of mediators involved in different inflammatory diseases on protein and gene expression level. Our results suggest that EGCG may be of preventive and, furthermore, therapeutic value to treat intestinal inflammation. An abstract containing part of these data has been presented (Porath et al., 2004).

Materials and Methods

Reagents

Recombinant human TNF-α was purchased from PeproTech (Rocky Hill, NJ). Recombinant human IL-8, purified mouse anti-human IL-8, and biotinylated mouse anti-human IL-8 were obtained from BD Biosciences PharMingen (San Diego, CA). Recombinant human MIP-3α/CCL20, anti-human MIP-3α/CCL20, and biotinylated anti-human MIP-3α/CCL20 were purchased from R&D Systems Europe (Oxford, UK). Cell culture reagents were obtained from InVitrogen (Carlsbad, CA). EGCG (trade name: Teavigo) was obtained from DSM Nutritional Products (Basel, Switzerland). Primers and probes were purchased from Sigma-Genosys (The Woodlands, TX).

Cell Culture

HT29 and T84 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, 50 μg/ml streptomycin, L-glutamine, and nonessential amino acids (InVitrogen). Cells were maintained in a water-saturated atmosphere of 95% O₂ and 5% CO₂ at 37°C. Cells were used between passages 20 to 50. For experiments, cells were seeded into 12-well plates at 1 × 10⁶ cells per well and used after 3 or 4 days of preculture. They were starved in DMEM containing 0.25% FBS for 18 h before the experiments. Cells were stimulated with TNF-α (100 ng/ml) in phenol-free DMEM containing 0.25% FBS. EGCG was dissolved in DMSO and added to the culture medium concomitantly with the TNF-α stimulus. Where appropriate, DMSO was added to the cell culture at a final concentration of 0.5% (v/v).

Cytotoxicity

Released lactate dehydrogenase (LDH) was measured in culture supernatants immediately after harvesting using LDH enzyme controls as standard (Sigma-Aldrich, St. Louis, MO). In a microtiter plate, 20 μL of undiluted culture supernatants or standard was mixed with β-nicotinamide adenine dinucleotide solution (172 mM) and Tris acetate buffer (13.6 g/l Tris base, 12.8 g/l KCl, 5.08 g/l L-lactate, and 1 g/l Na₃P₂O₆, pH 9.3). The kinetics of the reaction was measured at 340 nm at 30°C for 5 min. To determine the total LDH contents, cells were lysed in the presence of a buffer containing 0.1 M NaCl, 1 mM EDTA, 10 mM Tris/HCl, 1% Triton X-100, and protease inhibitors. This protocol was adapted from Korzeniewski and Callewaert (1983).

Enzyme-Linked Immunosorbent Assay (ELISA) and Enzyme Immunoassay

PGE2 Assay. The amount of PGE2 in culture supernatants was assayed with enzyme immunoassay kits (obtained from Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

IL-8 Assay. Nunc Maxisorp 96-well microtiter plate (Fisher Scientific Co., Pittsburgh, PA) was coated with 3 μg/ml goat anti-human IL-8 antibody (BD Biosciences PharMingen) in 50 μl of binding buffer (0.1 M NaH₂PO₄, pH 9.0) overnight at 4°C. After washing with PBS containing 0.05% Tween 20 (PBS-T), wells were blocked with PBS containing 10% FBS and incubated for 2 h at room temperature. After further washing with PBS-T, 100-μl aliquots of culture supernatant or recombinant IL-8 were added and incubated overnight at 4°C. To detect bound IL-8, 0.25 μg/ml biotinylated anti-human IL-8 antibody (BD Biosciences PharMingen), mixed in blocking buffer, was added for 1 h at room temperature. Streptavidin and alkaline phosphatase (DakoCytomation Denmark A/S, Glostrup, Denmark), diluted 1:50 in PBS, were preincubated for 30 min at room temperature to form conjugates. These conjugates were then added to the plate and incubated at 37°C for 1 h. After washing, 50 μl of p-nitrophenyl phosphate substrate (1 mg/ml) (Sigma Chemie, Deisenhofen, Germany) was added to each well and incubated at 37°C for 20 min. The optical density at 405 nm was read using a microtiter plate photometer (Molecular Devices, Sunnyvale, CA). All determinations were performed in triplicate.

MIP-3α Assay. Nunc immunosorb microtiter plate (Fisher Scientific Co.) was coated overnight at 4°C with 2 μg/ml goat anti-human IL-8 antibody. Released MIP-3α was assayed by incubating the microtiter plate in recombinant human MIP-3α, biotinylated anti-human MIP-3α, and streptavidin-alkaline phosphatase conjugate, according to the manufacturer’s instructions.
MIP-3α/CCL20 antibody (R&D Systems Europe) diluted in 50 μl of carbonate coating buffer, pH 9.6. After washing with PBS-T, wells were blocked with PBS, containing 1% bovine serum albumin, 5% sucrose, and 0.05% NaCl for 1 h at room temperature. After further washing with PBS-T, 50-μl aliquots of culture supernatants or recombiant MIP-3α were added for 2 h at room temperature. To detect bound MIP-3α, 0.5 μg/ml biotinylated anti-human MIP-3α antibody (R&D Systems Europe), mixed in blocking buffer, was added, and the plate was incubated for 1 h at room temperature. Streptavidin-biotin alkaline phosphatase complexes (DakoCytomation, Denmark A/S) were formed, and immune complexes were visualized and measured as described above. All determinations were performed in triplicate.

RNA Isolation and Reverse Transcription

Total RNA was isolated using the RNeasy Mini Kit from QIAGEN GmbH (Hilden, Germany). Extraction was performed according to the manufacturer's directions. Matrix-bound RNA was eluted with 30 μl of RNase-free water. The quantity and purity of RNA were determined by measuring the optical density at 260 and 280 nm. Subsequently, 1.5 to 3.5 μg of total RNA was converted to first strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and 50 ng/μl random hexamers (Microsynth, Balgach, Switzerland). The conditions for the cDNA synthesis were: 5 min at 70°C for random primer annealing followed by cooling on ice; 10 min at room temperature, 50 min at 42°C, and 15 min at 70°C for the denaturation step; and 20 min at 37°C for the RNase H digestion. The cDNA was subsequently diluted to 100 μl in water and stored at −80°C.

Quantitative Real-Time (RT)-PCR Analysis

The analysis of mRNA expression profiles was performed with multiplex quantitative RT-PCR. In a 50-μl PCR reaction, 3 μl of cDNA (corresponding to 30–50 ng of total RNA input) was amplified in an ABI Prism 7700 Sequence Detector (Applied Biosystems), using 2× Taqman Universal PCR Master Mix (Applied Biosystems), 50 nM primers and 100 nM probe for the 18S rRNA reference gene, and 300 nM primers and 100 nM probe for genes of interest. The probe for 18S rRNA was fluorescently labeled with VIC on the 5’ end and 5-carboxytetramethylrhodamine on the 3’ end (Applied Biosystems), whereas probes for the genes of interest were labeled with 6-carboxy-fluorescein (5-carboxyfluorescein) on the 5’ end and 5-carboxytetramethylrhodamine on the 3’ end (Applied Biosystems). The PCR amplification conditions consisted of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 and 60 sec at 95°C and 60°C, respectively.

mRNA abundance was defined using the ΔCt method according to the manufacturer's protocol (ABI Prism 7700 Sequence Detection System). Briefly, the ΔCt for the gene of interest was determined as the difference between the Ct values for the gene of interest and 18S rRNA, where the Ct value is the cycle threshold. The S.D. was obtained from ΔCt of cDNA samples assayed in duplicate, where the upper and lower errors were defined as 2^(-ΔCt + S.D.) and 2^(-ΔCt - S.D.), respectively. ΔΔCt was determined as the difference in ΔCt of unstimulated cells compared with that of each treatment group. The mRNA level for the gene of interest was determined as 2^(-ΔΔCt) and, therefore, reflects changes relative to unstimulated cells. Each cDNA sample was assessed in duplicate.

Statistical Analysis

The upper and lower limits of mRNA expression levels were calculated as described above. All other values are presented as mean ± S.D., and all statistics were evaluated double-sided using a two-sample equal variance Student’s t test, assuming independent variance. p < 0.05, p < 0.01, and p < 0.001 were considered to be significant.

Impact of EGCG on the Viability of Intestinal Epithelial Cells

The cytotoxicity of cell treatments and EGCG was evaluated by measuring the LDH activity in cell culture supernatants. Table 1 shows percentages of LDH released in HT29 and T84 cells. For HT29, the amount of released LDH was similar in the different treatments, indicating that TNF-α and EGCG did not affect the cell viability to a higher extent than TNF-α. This, however, might induce apoptosis (Aggarwal, 2000). T84 cells treated with 25 μM EGCG displayed higher LDH release than untreated cells, suggesting that EGCG might induce apoptosis.

TNF-α Induces the Secretion of Chemokines. TNF-α is an important proinflammatory mediator involved in gastrointestinal inflammation and able to activate the synthesis of chemokines in epithelial cells (MacDermott, 1999). Therefore, we verified its effects on the production of IL-8 and MIP-3α in HT29 and T84 cell lines. Cells were cultured in the presence or absence of TNF-α for 24 h, and the secretion of these chemokines was examined. Unstimulated cells did not produce significant levels of IL-8 and MIP-3α. In contrast, TNF-α-activated cells demonstrated time- and dose-dependent synthesis of IL-8 and MIP-3α in HT29 (Fig. 1, A and C). Similar data for the dose-dependent secretion of both chemokines were also obtained in T84 cells (data not shown). The time-dependent production of IL-8 and MIP-3α in TNF-α-activated T84 cells is shown in Fig. 1, B and D. Compared with T84 cells, the production of IL-8 in activated HT29 cells was 1.5-fold increased after 24 h. However, no significant differences were observed on the MIP-3α production between both cell lines (Fig. 1).

EGCG Inhibits the Production of IL-8 and MIP-3α in Intestinal Epithelial Cells

The effects of EGCG on the IL-8 and MIP-3α secretion were investigated after 24 and 72 h in TNF-α-stimulated HT29 and T84 cells. EGCG significantly reduced the production of IL-8 at 24 h (Fig. 2A) and virtually abolished it at 72 h (Fig. 2B). Increasing concentrations of EGCG gradually reduced IL-8 secretion in activated cells, indicating that the secretion of IL-8 from the cell to the culture medium was more affected in the presence of EGCG in HT29 than in T84 cells (Fig. 2C). This indicates a difference in the sensitivity of these cells to EGCG.

To investigate the impact of EGCG on MIP-3α secretion, TNF-α-activated cells were incubated with varying concentrations of EGCG for 24 h. EGCG dose-dependently de...

### Table 1

<table>
<thead>
<tr>
<th>Cell Viability</th>
<th>HT29</th>
<th>T84</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of LDH contents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>2.5 ± 0.0</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>TNF-α (100 ng/ml)</td>
<td>5.8 ± 1.7</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>EGCG (50 μM)</td>
<td>5.1 ± 1.3</td>
<td>0.6 ± 0.2*</td>
</tr>
<tr>
<td>EGCG (25 μM)</td>
<td>2.3 ± 1.0</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>TNF-α + EGCG (50 μM)</td>
<td>4.6 ± 0.4</td>
<td>1.9 ± 0.0</td>
</tr>
<tr>
<td>TNF-α + EGCG (25 μM)</td>
<td>6.3 ± 0.4</td>
<td>6.2 ± 0.7*</td>
</tr>
</tbody>
</table>

* P < 0.05.
increased the synthesis of MIP-3α in both cell lines (Fig. 3A). The production of MIP-3α was also investigated after 72 h of culture. As expected, TNF-α strongly increased the synthesis of MIP-3α in both HT29 and T84 cells. This increase was significantly reduced in the presence of EGCG at a concentration of 50 μM (Fig. 3B).

**EGCG Inhibits the Production of PGE2 in Epithelial Cells.** Since it is known that intestinal epithelial cells produce PGE2 (Eckmann et al., 1997), we decided to investigate the influence of EGCG on its synthesis in activated HT29 cells. Unstimulated cells produced low amounts of PGE2. Upon TNF-α stimulation, a significant increase in the PGE2 secretion was observed. Figure 4A shows that TNF-α dose-dependently augmented the secretion of PGE2 after 24 h. The highest amount of PGE2 was obtained in cells stimulated in the presence of 100 ng/ml TNF-α. In cells treated with 50 μM EGCG, PGE2 synthesis was diminished by 80 ± 6% (data not shown). EGCG dose-dependently reduced the production of PGE2 (Fig. 4B).

**EGCG Modulates Genes Involved in Inflammatory Responses.** To clarify whether EGCG modulates the expression of TNF-α-induced genes, we used quantitative RT-PCR. Since activated HT29 and T84 cells up-regulated inflammatory genes within 2 to 24 h (Fig. 5), the influence of EGCG on these genes was analyzed after 6 h of culture. Basal gene expression levels in unstimulated HT29 cells were different, with weakly (e.g., TNF-α, MIP-2, GRO-α, IL-1α, IL-1β, interferon-inducible protein-10), moderately (IL-8, MIP-3α, GRO-γ), prostaglandin E synthase, matrix metalloproteinase-14, RANK, congenic gene of inhibitor apoptosis protein, RANTES, complement factor b), and abundantly (e.g., COX-2, 5-LOX, β5 integrin, RANTES, fos-related antigen-1) expressed genes (data not shown). TNF-α induced a substantial increase in mRNA levels of cytokines and chemokines (TNF-α, IL-8, MIP-2, MIP-3α, GRO-α, GRO-γ, interferon-inducible protein-10), in addition to COX-2. However, mRNA levels of other genes, including 5-LOX, RANK, and β5 integrin, were unaltered by TNF-α stimulation. EGCG (50 μM) reduced the mRNA levels of IL-8, TNF-α, MIP-3α, MIP-2, GRO-α, GRO-γ, and COX-2 by 40 to 85% in both cell lines (Fig. 7). This effect was specific, but not restricted, to these genes. Conversely, EGCG did not augment the expression of TNF-α-induced genes. TNF-α strongly augmented the expression of IL-8 mRNA levels in HT29 and T84 cells, whereas it moderately and weakly increased the expression of TNF-α, MIP-3α, and GRO-γ mRNA (Fig. 7, A, B, and E) and COX-2 and GRO-α mRNA (Fig. 7, C and D), respectively. Notably, these genes were significantly down-regulated by EGCG, an observation that is consistent with our results of produced chemokines (IL-8 and MIP-3α) (Figs. 2 and 3).

Finally, the dose-dependent impact of EGCG on gene expression was evaluated. In HT29 and T84 cells, EGCG down-regulated IL-8, TNF-α, MIP-3α, and COX-2 mRNA levels in the concentration range of 6.25 to 50 μM (Fig. 8). In T84 cells, EGCG at 12.5 and 25 μM did not have any significant effect on IL-8 mRNA levels, although a trend for down-regulation was observed (Fig. 8B). In summary, expression levels of...
tested chemokines were significantly diminished following EGCG treatment in these cell lines.

Discussion

In this report, we present evidence that EGCG beneficially affects the inflammatory response in the colon adenocarcinoma cell lines HT29 and T84. These cell lines were used as models for intestinal mucosal diseases and have different origins and phenotypes. In vitro, differentiated HT29 cells express characteristic features of mature intestinal cells, such as microvilli, small intestinal digestive enzymes, lipid droplets, and primary and secondary lysosomes (Velcich et al., 1995). T84 is a transplantable human carcinoma cell line derived from a lung metastasis of colon carcinoma (Murakami and Masui, 1980) that possesses secretory capacity such as small intestinal crypt cells (Mun et al., 1998).

Our experimental approach dealt with the identification of the anti-inflammatory function of EGCG in TNF-α-activated colon adenocarcinoma cell lines. The effects of EGCG were determined at two levels: the expression of genes involved in inflammation and the production of chemokines (IL-8 and MIP-3α) and PGE2. EGCG showed potent effects on genes of the inflammatory pathway, including those of the cytokine/chemokine network (e.g., IL-8, TNF-α, MIP-3α, MIP-2, GRO-α, and GRO-γ). CXC chemokines, such as IL-8, MIP-2, and GRO-α and -γ, are responsible for chemotraction of neutrophils, whereas CC chemokines (e.g., MIP-3α) play a role in the recruitment of lymphocytes and dendritic cells to the inflamed tissue. Cytokines and chemokines are induced by TNF-α stimulation and are abundantly expressed and secreted in IBD (Fiocchi, 1997; Auebhr and Swain, 2002; Banks et al., 2003; Kaser et al., 2004; Papadakis, 2004). We demonstrated that EGCG markedly diminished the expression and secretion of IL-8 and MIP-3α in colon epithelial cells.

IBD and colon cancer are associated with an increased activity of intestinal immune cells, which augments the production of proinflammatory cytokines, including TNF-α. Therefore, TNF-α was used to induce an inflammatory state...
in vitro. TNF-α is involved in the mediation of the sustained inflammatory response, and high amounts of this cytokine can be found in IBD patients (D’Haens, 2003). The expression and production of IL-8 were studied in macrophages isolated from normal and inflamed colonic tissue resected for IBD (Grimm et al., 1996). Macrophages from IBD expressed more IL-8 mRNA than those from the normal mucosa, and lipopolysaccharide treatment further increased it. In addition, the recruitment of macrophages may be responsible for the IL-8 secretion that leads to neutrophil attraction in IBD (Grimm et al., 1996). This suggests that in the intestine, activated macrophages migrate into the epithelium and secrete IL-8, which recruits PML to the injured site. This is supported by our macrophage in vitro model (data not shown) where RAW 264.7 cells were stimulated with LPS in the presence or absence of EGCG. In this study, we have identified EGCG as an inhibitor of IL-8. Moreover, after TNF-α stimulation, epithelial cells also produce IL-8 and, consequently, recruit PML. The inhibition of the expression and production of IL-8, either by EGCG or other natural compounds (i.e., phenolic compounds), interferes with the recruitment of PML and thus may impede progression and aggravation of inflammation (Sugimoto et al., 2002; Park and Surh, 2004). In conclusion, EGCG has the potential to reduce IL-8 production in both epithelial cells and macrophages.

from normal and inflamed colonic tissue resected for IBD (Grimm et al., 1996). Macrophages from IBD expressed more IL-8 mRNA than those from the normal mucosa, and lipopolysaccharide treatment further increased it. In addition, the recruitment of macrophages may be responsible for the IL-8 secretion that leads to neutrophil attraction in IBD (Grimm et al., 1996). This suggests that in the intestine, activated macrophages migrate into the epithelium and secrete IL-8, which recruits PML to the injured site. This is supported by our macrophage in vitro model (data not shown) where RAW 264.7 cells were stimulated with LPS in the presence or absence of EGCG. In this study, we have identified EGCG as an inhibitor of IL-8. Moreover, after TNF-α stimulation, epithelial cells also produce IL-8 and, consequently, recruit PML. The inhibition of the expression and production of IL-8, either by EGCG or other natural compounds (i.e., phenolic compounds), interferes with the recruitment of PML and thus may impede progression and aggravation of inflammation (Sugimoto et al., 2002; Park and Surh, 2004). In conclusion, EGCG has the potential to reduce IL-8 production in both epithelial cells and macrophages.

With regard to MIP-3α, it is synthesized by the human colon epithelial cells and therefore by HT29 cells, which also express CCR6, the cognate receptor for MIP-3α (Izadpanah et al., 2001). MIP-3α mRNA and protein levels were found to be increased in colonic tissues from patients with IBD (Kwon et al., 2002). In the present study, EGCG inhibited the synthesis of MIP-3α in colon cancer cell lines and also downregulated gene expression. Via MIP-3α production, intestinal epithelial cells recruit dendritic cells and memory T cells to the site of inflammation. Although the normal intestinal mucosa is home of dendritic and T cells, a specific T cell population immigrates during IBD and contributes to the generation of inflammation (Fiocchi, 1998). Therefore, an excess of these cells in the inflamed intestine should be avoided. By reducing MIP-3α production, EGCG could prevent homing of these cells and, consequently, IBD symptoms.

Furthermore, we observed that EGCG inhibits PGE2 production. In the gastrointestinal environment, prostaglandins

Fig. 4. EGCG inhibited the secretion of PGE2 in TNF-α-activated HT29 cells. HT29 cells were cultured with different concentrations of TNF-α for 24 h (A). Stimulated cells were incubated with varying concentrations of EGCG for 24 h (B). Activated cells produced 420 pg/ml PGE2. The production of PGE2 was determined by ELISA. **, p < 0.01; ***, p < 0.001. Similar results were obtained in two other series of experiments.

Fig. 5. Time-dependent up-regulation of specific genes in HT29 cells. Cells were cultured without or with 100 ng/ml TNF-α for 6 h. RNA was isolated, and gene expression levels of IL-8, TNF-α, MIP-3α, and COX-2 were measured using the quantitative RT-PCR. Values are expressed relative to unstimulated cells at arbitrary units ± S.D. Similar results were obtained in two other series of experiments.

Fig. 6. Expression levels of selected genes in HT29 cells. RNA of unstimulated and TNF-α-activated HT29 cells that were cultured for 6 h were isolated, and the levels of mRNA of the indicated genes were determined by quantitative RT-PCR. Values are expressed relative to 18S rRNA and are indicated as means ± S.D. of two to four experiments.
are important regulators of gastrointestinal fluid secretion. In food allergies or after invasion of microorganisms, water secretion and TNF-α-induced electrolyte production lead to an increase of Ca^{2+}-dependent PGE2 in the intestinal epithelium. Exogenous PGE2 up-regulated IL-8 gene expression and protein production in human colonic epithelial cells (Yu and Chadee, 1998). PGE2 production was increased in colonic fibroblasts and in IBD patients. Intestinal fibroblasts, in addition to epithelial cells, could be targets for PGE2 and sites of colonic prostanoid biosynthesis in vivo (Kim et al., 1998). The expression of COX-2 has also been observed after Salmonella infection in intestinal epithelial cells (Singer et al., 1998), and COX-2 protein was reported to be expressed in three of eight colon cancer cell lines, including HT29 (Parker et al., 1997). Our results demonstrated that EGCG diminished proinflammatory PGE2 production and expression in HT29 cells. Studies have shown that EGCG targets COX-2 by inhibiting the PGE2 production and possibly reduces the risk of colon cancer and inflammation in humans (August et al., 1999; Hong et al., 2001; Park and Surh, 2004). Due to differences between HT29 and T84 cells, TNF-α-stimulated T84 cells did not produce detectable amounts of PGE2 and COX-2 protein (data not shown). With regard to this observation, in HT29 cells, EGCG had shown an influence on the three parameters tested: COX-2 gene, COX-2 protein expression (data not shown), and PGE2 generation, whereas in T84 cells, only the COX-2 mRNA levels were affected by EGCG.

Quantitative RT-PCR analysis identified several genes
that were induced in TNF-α-stimulated HT29 and T84 cells, including IL-8, TNF-α, MIP-3α, MIP-2, GRO-α, and GRO-γ. These genes were down-regulated by EGCG and have NF-κB regulatory binding sites. Consequently, they are regulated by the transcription factor NF-κB. Similar to the effects observed for IL-8, the expression levels of MIP-2, GRO-α, and GRO-γ were also diminished by EGCG in both cell lines. These chemokines, like IL-8, act through the chemokine receptor CXCR2 (Murphy et al., 2000) and are also elevated after TNF-α stimulation (Fig. 7, D and E). The concerted effect of EGCG on four chemokines binding to a common receptor indicates that this flavonoid impedes most or all biological activities that are mediated by CXCR2. Furthermore, 5-LOX, an enzyme expressed in differentiated HT29 cells (Cortese et al., 1995) and responsible for the synthesis of leukotrienes, was also expressed in HT29 and T84 cell lines, but no differences were observed between unstimulated and TNF-α-activated cells (Fig. 6). In our experiments, the expression of 5-LOX was not influenced by EGCG (data not shown). A possible explanation for this observation is that TNF-α is not able to induce 5-LOX.

Collectively, we provide evidence that EGCG attenuates the inflammatory response in the colon adenocarcinoma cell lines HT29 and T84 by inhibiting the production of chemokines and PGE₂. However, this study considers the effect of EGCG in the in vitro gastrointestinal inflammation; thus, it should be followed by in vivo experiments to consolidate this statement. In addition, in vivo studies will also corroborate the use of EGCG in preventing and attenuating gastrointestinal disorders.

**Fig. 8.** EGCG dose-dependently down-regulated some inflammatory genes. TNF-α-stimulated HT29 (A) and T84 (B and C) cells were cultured in the presence of varying concentrations of EGCG for 6 h. The effect of EGCG on the mRNA levels of IL-8, TNF-α, MIP-3α, and COX-2 is given. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Representative data of one of three similar experiments ± S.D. are shown.

**References**


Address correspondence to: Joseph Schwager, DSM Nutritional Products Ltd., Building 205/209, P.O. Box 3255, Wurmisweg 576, CH-4303 Kaiseraugst, Switzerland. E-mail: joseph.schwager@dsm.com