Therapeutic effect of epigallocatechin-3-gallate in a mouse model of colitis

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Abstract

Epigallocatechin-3-gallate (EGCG), a green tea catechin, has been shown to inhibit signaling pathways involved in inflammation, including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which are important inducers of pro-inflammatory mediators. Aim of our study was to evaluate the therapeutic efficacy of EGCG in experimental colitis, which was induced by rectal administration of trinitrobenzenesulfonic acid (TNBS) in C57/BL6 mice. Mice were treated twice daily with vehicle or with EGCG (10 mg/kg) intraperitoneally, and sacrificed on days 1, 3, and 7 after TNBS administration. After induction of colitis, vehicle-treated mice experienced bloody diarrhea and loss of body weight. A remarkable colonic damage with hemorrhage, ulcers, and edema was observed and was associated with neutrophil infiltration as evaluated by myeloperoxidase (MPO) activity. Elevated plasma levels of tumor necrosis factor α, interleukin (IL)-6, IL-10 and keratinocyte-derived chemokine were also found. These events were paralleled by increased DNA binding of NF-κB and AP-1 in the colon of the vehicle-treated group. In contrast, the EGCG-treated mice experienced a very mild diarrhea and no weight loss. Damage of the colon was characterized by edema and hyperemia only. Tissue levels of MPO were also significantly reduced when compared to vehicle-treated mice. These beneficial effects of EGCG were associated with a significant reduction of NF-κB and AP-1 activation. However, treatment with EGCG did not reduce plasma cytokine levels. Our data demonstrate that EGCG may be beneficial in colitis through selective immunomodulatory effects, which may be mediated, at least in part, by inhibition of NF-κB and AP-1.

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1. Introduction

Green tea has been reported to possess remarkable anti-inflammatory and cancer chemopreventive effects in many animal tumors, cell culture systems, and epidemiological studies (Yang et al., 2002; Wheeler and Wheeler, 2004). These biological effects of green tea are mediated by tea polyphenols, known also as tea catechins. Epigallocatechin-3-gallate (EGCG) is the major polyphenol present in green tea. Apart from its antioxidant properties, EGCG inhibits several signaling pathways involved in inflammation, including the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Ahmad et al., 2002; Yang et al., 2001; Aneja et al., 2004; Na and Surh, 2006). These transcription factors regulates the expression of genes encoding proinflammatory cytokines, chemokines, immune receptors, and adhesion molecules that play a key part in inflammatory related injury (Zingarelli et al., 2003a; Na and Surh, 2006).

Ulcerative colitis and Crohn’s disease are chronic inflammatory bowel diseases, whose exact etiology and pathogenesis remain obscure (Fiocchi, 1998). However, there is substantial evidence that the intestinal inflammation is likely to depend, at least in part, on the activation and nuclear translocation of NF-κB. For example, activated NF-κB has been demonstrated in colonic epithelial cells and macrophages of patients with inflammatory bowel diseases (Schreiber et al., 1998; Rogler et al., 1998; Neurath et al., 1998). These findings have suggested the
hypothesis that NF-κB may be an effective therapy target in treating the inflammation. In line with this hypothesis, in previous studies of hapten-induced murine models of colitis, inflammation has been successfully prevented with the administration of antisense oligodeoxynucleotides specific to NF-κB subunits (Neurath et al., 1996; Lawrance et al., 2003).

Several reports have also documented a role for AP-1 activation in intestinal inflammation. Gonsky and colleagues have demonstrated that activation of lamina propria T cells from normal, ulcerative colitis, or Crohn’s disease mucosa through the CD2 pathway leads to induction of AP-1 complexes (Gonsky et al., 1998). Transcriptional activation of AP-1 is enhanced in immunostimulated human colonic epithelial cells (Abreu-Martin et al., 1999). In addition, we have previously demonstrated that AP-1 DNA binding is increased during colonic inflammation (Zingarelli et al., 2003b, 2004). We also have previously reported that suppression of AP-1 binding is associated with amelioration of the inflammatory process and reduction of epithelial apoptosis (Zingarelli et al., 2004).

In light of the pharmacological profile of EGCG, and its reported inhibitory properties on NF-κB and AP-1, the purpose of this study was to investigate whether EGCG may afford therapeutic effects in experimental colitis in mice and to determine whether these effects are associated with in vivo inhibition of NF-κB and AP-1 activation.

2. Materials and methods

2.1. Animals

C57BL/6 mice (20-22 g, Charles River Laboratories, Wilmington, MA) were housed in a room with controlled temperature (22 °C) and 12 h light/dark cycle. The animals were food-fasted 24 h before experimentation and allowed food and water ad libitum after the induction of colitis.

2.2. Induction of colitis

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23 revised 1996) and commenced with the approval of the Institutional Animal Care and Use Committee. Colitis was induced by using a technique of hapten-induced colonic inflammation as previously described (Zingarelli et al., 1999). A 3.5F catheter was inserted into the colon of fasted mice via the anus until approximately the splenic flexure (4 cm from the anus). 2,4,6-Trinitrobenzene sulfonic acid (TNBS, 1 mg/mouse) was dissolved in 50% ethanol (v/v) and injected (0.2 ml) into the colon via the rubber cannula. Animals were then kept in a vertical position for 30 s and returned to their cages. After the induction of colitis the mice were immediately treated with EGCG (10 mg/kg) or vehicle (normal saline) intraperitoneally (i.p.) twice a day after the induction of colitis. Control groups of mice received EGCG or vehicle alone but were not subjected to TNBS administration. Mice were killed at 1, 3, and 7 days after TNBS administration and a segment of the colon 4 cm long was excised for the evaluation of macroscopic damage. Tissue segments 1 cm in length were then fixed in 10% buffered formalin or immediately frozen in liquid nitrogen and stored at −70 °C for the histological, immunohistochemical and biochemical studies described below.

2.3. Evaluation of colonic damage

After removal, the colon was gently rinsed with saline solution, opened by a longitudinal incision and immediately examined under microscope. The visible colonic damage was assessed by a semi-quantitative scoring system (Zingarelli et al., 1999, 2004). The following morphological criteria were taken under consideration: score 0, no damage; score 1, localized hyperemia without ulcers; score 2, linear ulcers, with no significant inflammation; score 3, linear ulcers with inflammation at one site; score 4, two or more major sites of ulceration and/or inflammation; score 5, two or more sites of inflammation and ulceration extending >1 cm along the length of the colon; score 6–8, 1 point is added for each cm of ulceration beyond an initial 2 cm. All measurements of damage were performed by two to four observers blinded to the experimental protocol.

2.4. Histopathological analysis

For microscopic histological evaluation, formalin-fixed tissues were embedded in paraffin and 5 μm sections were stained with hematoxylin and eosin and evaluated by light microscopy by a pathologist blinded to the experimental protocol.

2.5. Determination of neutrophil infiltration

Myeloperoxidase (MPO) activity was determined as an index of neutrophil accumulation (Krawisz et al., 1984). Colonic tissues were homogenized in a solution containing 0.5% hexa-decyl-trimethyl- ammonium bromide dissolved in a 10 mM potassium phosphate buffer (pH 7), and centrifuged for 30 minutes at 20,000 × g at 4 °C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H₂O₂. The rate of change in absorbance was measured by spectrophotometry at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 umol of peroxide/min at 37 °C and was expressed in milliunits per 100 mg weight of tissue.

2.6. Plasma levels of cytokines

Plasma levels of tumor necrosis factor-α (TNFα), interleukin (IL)-6, IL-10 and keratinocyte-derived (KC) chemokine were evaluated by commercially available solid-phase enzyme-linked immunosorbent assay kit (R&D Systems Minneapolis, MN), using the protocols recommended by the manufacturer.

2.7. Nuclear protein extraction

Tissue samples from colons were homogenized with a Polytron homogenizer in a buffer containing 0.32 M sucrose, 10 mM tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM NaN₃, 10 mM β-mercaptoethanol, 20 μM leupeptin, 0.15 μM
pepsatin A, 0.2 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate, 0.4 nM microcystin. The homogenates were centrifuged (1,000 g × 10 min) and the pellets were solubilized in Triton buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 20 μM leupeptin A, 0.2 mM PMSF). The lysates were centrifuged (15,000 g × 30 min, 4 °C), and the supernatant (nuclear extract) was collected to evaluate the nuclear binding of NF-κB and AP-1.

2.8. Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were performed as previously described (Zingarelli et al., 2004). Oligonucleotide probes corresponding to NF-κB consensus sequence (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) or AP-1 consensus sequence (5′-CGC TTG ATG ACT CAG CCG GA-3′) were labeled with γ-[32P]ATP using T4 polynucleotide kinase and purified in Bio-Spin chromatography columns (BioRad, Hercules, CA). Ten μg of nuclear protein were preincubated with EMSA buffer (12 mM HEPES pH 7.9, 4 mM Tris-HCl pH 7.9, 25 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly [d(I-C)], 12% glycerol v/v, and 0.2 mM PMSF) on ice for 10 min before addition of the radiolabeled oligonucleotide for an additional 10 min. The specificity of the binding reactions was determined by co-incubating duplicate nuclear extract samples with 100-fold molar excess of unlabeled oligonucleotides (competitor assay). Protein-nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide:bisacrylamide) and run in 0.5X TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM orthovanadate, 20 μg/ml pepstatin A, 0.2 mM PMSF). The homogenates were centrifuged (1,000 g ×10 min) and the pellets were solubilized in Triton buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 20 μM leupeptin A, 0.2 mM PMSF). The lysates were centrifuged (15,000 g × 30 min, 4 °C), and the supernatant (nuclear extract) was collected to evaluate the nuclear binding of NF-κB and AP-1.

2.9. Materials

Oligonucleotide probes for NF-κB and AP-1 consensus were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other chemicals, reagents and EGCG were from Sigma/Aldrich (St. Louis, MO).

2.10. Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean of n observations, where n represents the number of mice (n=5-10 animals for each group). Data sets were examined by one-and two-way analysis of variance, and individual group means were compared with Student’s unpaired t test. Statistical analysis of scores was performed using the Mann-Whitney U test. A P-value less than 0.05 was considered significant.

3. Results

3.1. Effect of EGCG on severity of colitis

As early as 1 day after administration of TNBS, vehicle-treated mice exhibited serious bloody diarrhea, which was associated with a reduction of weight gain (Fig. 1). Macroscopic evaluation of the distal colon and rectum after TNBS treatment revealed the presence of mucosal edema and hemorrhagic ulcerations up to 7 (Figs. 2 and 3). In contrast, EGCG-treated mice appeared healthier and exhibited a very mild diarrhea; weight loss was also less pronounced when compared to vehicle-treated mice (Fig. 1). The mucosal surface of the colon and rectum showed localized hyperemia and edema only (Figs. 2 and 3). Histological examination of the colon confirmed the macroscopic findings. Erosions, edema, hemorrhage and large stretches of denuded epithelia were present at 1 and 3 days after TNBS administration in colon of vehicle-treated mice. Alteration of colonic architecture was associated with a diffuse leukocyte infiltrate in the submucosa. At 7 days a re-epithelization process was observed in the mucosa associated with some mild infiltrate in the submucosa (Fig. 4). In contrast, in vivo treatment with EGCG markedly reduced erosions of the mucosa at 1 day after TNBS administration. At 3 and 7 days, the histological features of the colon were typical of normal or healing mucosa with an intact epithelium and no cellular infiltration (Fig. 4).
3.2. Effect of EGCG on neutrophil infiltration in the colon

The large influx of inflammatory cells observed in histological specimens was confirmed by measurement of the activity of MPO, an enzyme specific to granulocyte lysosomes and therefore directly correlated to the number of neutrophils. We found that MPO activity rose dramatically at 1 day after TNBS administration, and declined thereafter in vehicle-treated mice. In contrast, at 1 day after TNBS administration EGCG-treated mice exhibited a significant reduction in MPO activity compared with vehicle-treated mice (Fig. 5).

3.3. Effect of EGCG on plasma cytokine levels

A substantial increase of plasma TNFα, IL-6, IL-10 and KC chemokine was found in vehicle-treated animals at 1-3 days after TNBS administration. Treatment with EGCG did not affect plasma elevation of these cytokines (Fig. 6).
3.4. EGCG inhibits NF-κB and AP-1 DNA binding

To investigate the cellular mechanisms by which treatment with EGCG attenuates TNBS-induced injury, we evaluated the activation of NF-κB and AP-1 by EMSA, in colonic specimens. In a time course study, we found that DNA binding of both NF-κB and AP-1 increased after TNBS administration in vehicle-treated mice (Fig. 7). Administration of EGCG markedly reduced DNA binding activity of both transcription factors (Fig. 7).

4. Discussion

Several studies have evaluated the anti-inflammatory potential of green tea extracts in experimental models of colitis (Varilek et al., 2001; Mazzon et al., 2005). The present study was particularly focused on elucidating the effects of EGCG, which comprises approximately 60% of the catechins in tea. In a murine model of colitis we have demonstrated that systemic administration of EGCG exerts intestinal anti-inflammatory effects. Specifically, we have demonstrated that mice treated with EGCG exhibited a less pronounced body weight loss as well as amelioration of gross anatomic and histologic signs of damage and reduction of neutrophil infiltration in colonic tissue, when compared with vehicle-treated mice. At the molecular level, these beneficial effects of EGCG were associated with reduced activation of NF-κB and AP-1 in the colon.

Activation and accumulation of inflammatory cells is one of the initial events of tissue injury, triggering the release of oxygen free radicals, lysosomal proteases and other pro-inflammatory mediators. In our experimental model of colitis, we found that the increase in neutrophil infiltration occurred at an early stage after TNBS administration (i.e. 1 day after TNBS administration) and correlated with the severity of inflammation, while it declined at the initiation of the healing process in the vehicle-treated group. EGCG prevented this earlier influx of neutrophils. Therefore, it appears that EGCG may prevent the early oxidative stress of colitis, while it allows an earlier...
recovery as demonstrated by the histological analysis. At the later stage of the TNBS experimental model, polymorphonuclear cells are replaced mainly by mononuclear cells and, therefore, the tissue MPO activity gradually returns to baseline levels. Whether EGCG also affects the activation of mononuclear cells deserves further investigation.

Activation of NF-κB is closely linked to the inflammatory onset of colitis. Colonic macrophages appear to be anergic in normal mucosa. However, reports show that during inflammatory bowel diseases intestinal macrophages exhibit a phenotypic change, which is associated with NF-κB activation (Rogler et al., 1998). NF-κB DNA binding activity has also been found to increase in the inflamed mucosa of colon biopsies in patients with active inflammatory bowel diseases and to correlate with severity of the disease (Schreiber et al., 1998; Ardite et al., 1998). Recently, bacteria-induced experimental colitis has been shown to involve the NF-κB signaling in mucosal immune cells (Karrasch et al., 2007). Therefore, NF-κB appears to be a key regulator of the inflammatory response in colitis. In support of this hypothesis it has been reported that the anti-inflammatory effects of glucocorticoids are associated with reduction of NF-κB signaling in intestinal biopsies of patients with ulcerative colitis (Ardite et al., 1998). Furthermore, it has been shown that administration of an antisense oligonucleotide to NF-κB p65 ameliorated inflammation after induction of colitis in rodents (Neurath et al., 1996). Similarly, in a mouse model of spontaneous colitis, colonic damage was attenuated by suppression of mucosal NF-κB-DNA binding activity and IL-8 suppression (Kanauchi et al., 1999). Nevertheless, in contrast to these studies, NF-κB has been recently shown to play an important role in intestinal epithelial function and preservation of the barrier, as NF-κB deficiency led to apoptosis of colonic epithelial cells, impaired expression of antimicrobial peptides and translocation of bacteria into the mucosa (Nenci et al., 2007). Our current study demonstrates that NF-κB binding is significantly decreased in EGCG-treated animals and this decrease is associated with reduction of colon damage. Although further studies are necessary to establish the role of NF-κB, altogether these studies suggest that while chronic and permanent inhibition of NF-κB may alter intestinal homeostasis, blocking NF-κB activity may represent an attractive strategy to treat acute relapses of intestinal inflammation.

Our previous studies have supported the hypothesis of a positive synergy between NF-κB and AP-1 in colitis (Zingarelli et al., 2003b, 2004). In line with these findings, our current study demonstrated that DNA binding activity of both NF-κB and AP-1 was increased in the inflamed colon of vehicle-treated mice. On the contrary, the beneficial effects of EGCG were associated with reduction of binding of both transcription factors. Thus, it is conceivable that the beneficial effects of EGCG may be also mediated by inhibition of AP-1 activation. Our data further confirm that EGCG may modulate several stress-induced signal transduction pathways.

Interestingly, while EGCG effect on NF-κB and AP-1 well correlated with reduction of neutrophil infiltration into the colonic mucosa, the catechin did not alter plasma levels of cytokines. Our data are in agreement with previous reports demonstrating that EGCG possesses selective immunoregulatory properties. For example, it has been shown that green tea did not affect plasma levels of TNFα in rats with endotoxin-induced liver injury (He et al., 2001), or plasma levels of TNFα, IL-6 and IL-10 in rats subjected to polymicrobial sepsis (Wheeler et al., 2007). Furthermore, EGCG appears to selectively upregulate production of TNFα and IL-12 in macrophages infected with Legionella pneumophila (Matsunaga et al., 2001) and to increase TNFα production in dendritic cells stimulated with bacterial products in a dose-dependent manner (Rogers et al., 2005). In contrast to our study, however, it has been reported that treatment with green tea extracts reduces colonic levels of TNFα and exerts beneficial effects in a chemical model of colitis (Mazzon et al., 2005). Similarly, it has been demonstrated that EGCG inhibits the production of IL-8 and MIP-3α in TNFα-stimulated colon epithelial cells (Porath et al., 2005). Although we did not measure colonic levels of cytokine, taken together with these previous studies, our data suggest that EGCG may have differential systemic and local effects on cytokine production, which may be dependent on the cell type and function and on the phase of the inflammatory process. In addition, our data are consistent with clinical reports demonstrating that plasma cytokines (such as IL-1β, IL-6, IL-8, and TNFα) do not always correlate with relapse in patients (Yamamoto et al., 2005).

Alternatively, other beneficial mechanisms of EGCG may be involved in the protection against experimental colitis. For example, tea flavanoids, especially EGCG, have previously been shown to exhibit potent antioxidative effects (Siddiqui et al., 2004). Previous investigators have shown that scavenging the superoxide anion (O2-) improves outcome in rat models of colitis (Araki et al., 2003; Segui et al., 2005). It is also possible that other immunomodulatory properties of EGCG could affect the course of inflammatory bowel disease. For example, it has been demonstrated that the catechin can inhibit the ability of CD8+ T cells to bind intracellular adhesion molecule 1 and to migrate in response to chemokines (Kawai et al., 2004). EGCG may, therefore, abrogate organ injury and improve outcome via similar mechanisms, though further studies are necessary to prove this assertion.

Previous studies have analyzed the anti-inflammatory effects of black tea or green tea extracts in experimental models of colitis when given orally as dietary supplements (Varilek et al., 2001; Maity et al., 2003). However, no epidemiological studies exist to suggest a preventive effect of green tea consumption against colitis in humans. An important observation of our study is that EGCG affords a remarkable attenuation of colon inflammation when administered at large concentration by a systemic route. The concept of acute therapeutic pharmacological effects of this catechin other than preventive dietary effect has important clinical implications to the treatment of colitis and may overcome inadequate bioavailability in patients with altered gastrointestinal absorption function. However, in our study we used an early treatment design with therapy starting the same day of TNBS administration. Such early treatment probably is not feasible in the clinical setting. Therefore, it remains to be determined whether EGCG can provide beneficial effects in animals with established colitis.
In conclusion, EGCG reduces TNBS-induced colonic damage. This beneficial effect is associated with reduction of NF-κB and AP-1 activation in the colon. However, EGCG did not affect the systemic release of cytokines. Our data further emphasizes the potential benefits of EGCG as an anti-inflammatory drug. However, it remains to be determined whether EGCG may be used as therapeutic agent in patients with colitis.

References


