Adenosine Kinase Inhibitor GP515 Improves Experimental Colitis in Mice

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ABSTRACT

Adenosine is a potent anti-inflammatory mediator. Through elevation of endogenous adenosine concentrations the adenosine kinase inhibitor GP515 might serve to down-regulate local inflammatory responses. In the present study we investigated the effect of systemic GP515 in the nonacute model of dextran sulfate sodium (DSS)-induced colitis. The clinical score, colon length, histologic score, colon cytokine production, and spleen weight from mice with DSS-induced colitis (3.5% DSS in drinking water for 11 days) receiving GP515 treatment were determined and compared with untreated control mice. Splenocytes were analyzed for phenotype, interferon-γ (IFNγ) production, and CD69 expression. First, GP515 treatment resulted in a significant improvement of clinical score (weight loss, stool consistency, and bleeding) and of histologic score. Second, colon shortening, an indirect parameter for the degree of inflammation, was decreased, consistent with a decreased IFNγ concentration in the colonic tissue. Third, spleen weight was reduced in GP515-treated DSS mice. And fourth, IFNγ synthesis and CD69 expression, as a marker for early cell activation, of ex vivo-stimulated splenocytes were suppressed in the GP515-treated DSS mice. These studies show that GP515 is effective in the therapy of DSS-induced colitis. One potential mechanism of action is the suppression of IFNγ synthesis and CD69 expression. Adenosine kinase inhibition forms a pharmacologic target that should be further investigated for chronic inflammatory bowel disease.

It has been hypothesized that the chronic inflammation in inflammatory bowel disease (IBD) is due to a disturbed balance of pro- and anti-inflammatory cytokines. Mucosal biopsies of patients with IBD have demonstrated an increased expression of proinflammatory cytokines, chemokines, and adhesion molecules such as tumor necrosis factor-α (TNF), interleukin (IL)-1, IL-2, IL-6, IL-8, interferon-γ (IFNγ), intercellular adhesion molecule 1, vascular adhesion molecule 1, and E selectin (Jones et al., 1995; Eigler et al., 1997b; Monteleone et al., 1997; Parronchi et al., 1997; Baugh et al., 1999). Furthermore, different clinical trials have shown that the severity of IBD can be attenuated through suppression of T-helper cells type 1 (Th1) activity. Treatment effects in experimental animal models and patients have also demonstrated the efficacy of antibodies against TNF, a classic Th1 cytokine, and against IL-12, a cytokine central to the development of Th1 cells (Neruth et al., 1995; Targan et al., 1997; Sandborn and Hanauer, 1999).

Adenosine exerts anti-inflammatory properties in a variety of systems: it inhibits the synthesis of Th1 cytokines (i.e., TNF, IFNγ) and down-regulates neutrophil functions in vitro and in vivo, including superoxide production, degranulation, and adhesion to and destruction of endothelial monolayers (Schrier and Imre, 1986; Cronstein et al., 1992; Sullivan et al., 1995; Bouma et al., 1997; Eigler et al., 1997a, 2000). The therapeutic application of adenosine and its analogs, however, is limited by its short half-life and the occurrence of adverse side effects such as hypotension and bradycardia (Belardinelli et al., 1989; Moser et al., 1989). As an alternative strategy, agents that increase endogenous adenosine concentrations at the site of inflammation might present a therapeutic approach. One such strategy is the inhibition of the enzyme adenosine kinase, which catalyzes the phosphorylation of adenosine to adenosine monophosphate. Inhibition of this enzyme raises intracellular adenosine levels, leading to an increase in adenosine transport out of the cell. The released adenosine then acts upon adenosine receptors on...
cells in the local environment. Indeed, adenosine kinase inhibitors have been found to exert beneficial effects in inflammation models in vitro and in vivo (Firestein et al., 1994; Rosengren et al., 1995).

Colitis induced by oral dextran sulfate sodium (DSS) is characterized by lymphoid hyperplasia, inflammatory cell infiltration, focal crypt damage, epithelial injury, and ulceration (Okayasu et al., 1990; Cooper et al., 1993; Dieleman et al., 1998). The pathogenetic mechanism that ultimately induces colitis involves toxic epithelial effects and phagocytosis of DSS, leading to stimulation of lamina propria cells and increased production of proinflammatory cytokines (Dieleman et al., 1998). Although differing in several aspects from human disease, DSS-induced colitis has been recommended and is a widely used preclinical model for inflammatory bowel disease (Cooper et al., 1993; Elson et al., 1995; Bennett et al., 1997).

In the present study we investigated for the first time the therapeutic efficacy of an adenosine kinase inhibitor, GP515, in an animal model of DSS-induced colitis. GP515 was administered to BALB/c mice exposed to DSS. Endpoints of the present study were the clinical score, colon length, the histologic score of the colon, and local IFN-γ expression. As parameters of systemic inflammation we determined spleen weight and characterized activation of spleen cells as assessed by CD69 expression and IFN-γ synthesis after stimulation with endotoxin or phorbol-12-myristate-13-acetate (PMA) plus ionomycin.

Materials and Methods

Mice. Female, 8-week-old BALB/c mice (Harlan Winkelmann, Borchen, Germany) weighing 20 to 22 g were used in this study. The animals were housed in rooms at a controlled temperature and light/dark cycles. They were fed standard mice chow pellets, had access to tap water supplied in bottles, and were acclimatized to the conditions before they were studied in experiments. Mice were killed by cervical dislocation under isoflurane anesthesia (Forene; Abbott GmbH, Wiesbaden, Germany). Both animal handling and clinical and histologic scoring of colitis were performed as treatment-blinded assessments. All experiments were approved by the regional animal study committee and are in agreement with the guidelines for the proper use of animals in biomedical research.

Reagents. GP515 (4-aminomethyl-5-oxo-1-p-ribofuranosyl-3-bromo-pyrazolo[3,4-d] pyrimidine), synthesized by Dr. Howard Cottam, University of California San Diego School of Medicine, La Jolla, CA, was dissolved in distilled water to a final concentration of 0.03 mg/ml GP515. The solution was frozen into aliquots of 2 ml and stored at −80°C until use. GP515 is specific for adenosine kinase and does not inhibit other enzymes involved in adenosine metabolism, including adenosine deaminase and AMP deaminase, and it does not bind to A1 and A2 receptors or the nitrobenzylthioinosine-sensitive adenosine transporter (Firestein et al., 1994). Its structural relation to adenosine has been described in detail by Firestein et al. (1994).

Induction of Colitis and Treatment. Mice were fed 3.5% DSS (molecular weight 30–40 kDa; ICN, Eschwege, Germany) dissolved in sterile, distilled water ad libitum throughout the experiment (days 1–11). Either 0.9% NaCl or GP515 were injected twice daily intra-peritoneally with an injection volume of 200 μl (0.6 mg/kg of body weight/day). The dose was chosen due to previous experience in vivo (Firestein et al., 1994). To test the therapeutic efficacy of GP515, DSS was administered for 11 days, starting at the same day as the therapeutic injections. Control mice were offered tap water ad libitum and were injected equally with either 0.9% NaCl or GP515 twice daily.

Determination of Clinical Score, Colon Length, and Histologic Score. Body weights were determined daily, as well as stool consistency and occult blood or the presence of gross blood per rectum. The clinical score was assessed independently by two investigators blinded to the protocol, as described previously in detail (Hartmann et al., 2000). Briefly, no weight loss was scored as 0 points, weight loss of 1 to 5% as 1 point, 5 to 10% as 2 points, 10 to 20% as 3 points, and more than 20% as 4 points. For stool consistency, 0 points were given for well formed pellets, 2 points for pasty and semiformal stools that did not stick to the anus, and 4 points for liquid stools that remained adhesive to the anus. Bleeding was scored 0 points for no blood in hemoccult, 2 points for positive hemoccult, and 4 points for gross bleeding from the rectum. These scores were added and divided by 3, resulting in a total clinical score ranging from 0 (healthy) to 4 (maximal activity of colitis). Post mortem the entire colon was removed from the caecum to the anus and the colon length was measured as an indirect marker of inflammation. Rings of the transverse part of the colon were fixed in 10% formalin and embedded in paraffin for histologic analysis. Sections (4 μm) were stained with H&E and histologic scoring performed. For cell infiltration of inflammatory cells, rare inflammatory cells in the lamina propria were counted as 0; increased numbers of inflammatory cells, including neutrophils in the lamina propria as 1; confluence of inflammatory cells, extending into the submucosa as 2; and a score of 3 was given for transmural extension of the inflammatory cell infiltrate. For epithelial damage, absence of mucosal damage was counted as 0, discrete focal lymphoepithelial lesions were counted as 1, mucosal erosion/ulceration was counted as 2, and a score of 3 was given for extensive mucosal damage and extension through deeper structures of the bowel wall. The two subscores were added and the combined histologic score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage).

Colon Cytokine Extraction. Strips (about 4 cm) of colon from DSS-exposed and from non-DSS mice with or without GP515 treatment were weighed, vigorously vortexed for 1 min in 100 μl of 0.01 M PBS (Roche, Ingelheim, Germany), and centrifuged at 10,000 g for 15 min. IFN-γ was quantified in the eluate with a commercial enzyme-linked immunosorbent assay kit (Endogen, Woburn, MA) according to the manufacturer’s instructions. The lower limit of detection of the assay is 50 pg/ml.

Cell Culture and Flow Cytometry. At day 11 spleens were removed aseptically, weighed, and cell suspensions were prepared according to the standard procedures (Coligan et al., 1992). Cells were washed twice in RPMI-1640, resuspended in medium containing 10% fetal calf serum, and cultured at 2.5 × 10^6/ml in 48-well plates. Cultures were incubated for 20 h in the presence or absence of lipopolysaccharide (LPS; 100 ng/ml) or PMA (25 ng/ml) plus ionomycin (500 ng/ml) at 37°C in a humidified atmosphere with 5% CO₂. At the end of the incubation period one part was frozen at −70°C until cytokine measurement. The other part was used for flow cytometry analysis (FACS Calibur; Becton Dickinson, Heidelberg, Germany). Flow cytometry followed routine procedures using 5 × 10^6 splenocytes/sample. To measure the expression of CD69, CD45R, CD3, and Mac, cells were labeled with either fluorescein isothiocyanate- or phycoerythrine-labeled antibody (Becton Dickinson).

Statistical Analysis. Data are expressed as means ± S.E.M. Statistical significance of differences between treatment and control groups was determined by factorial ANOVA analysis and a Bonferroni-Dunn procedure as post hoc test. Differences were considered statistically significant for p < 0.05. Statistical analyses were performed using StatView 4.51 software (Abacus Concepts, Calabasas, CA).

Results

Clinical Score. Mice fed with DSS developed signs of colitis as evidenced by a clinical score of >0.5 starting at day 4 (Fig. 1). Intraperitoneal injection of GP515 in a dose of 0.3
Either GP515 or 0.9% NaCl showed no clinical signs of colitis. Scores are GP515-treated DSS mice compared with 3.5 until the end of experiment on day 11 (score 2.5 each group). The difference in the clinical score persisted n 0.9% NaCl-treated DSS group; (n development of colitis compared with the 0.9% NaCl-treated DSS group (treated DSS group versus 3.3 6 p 0.003); stool consistency score 1.9 0.3 in GP515-treated DSS group versus 3.5 0.2 in the 0.9% NaCl-treated DSS group (p 0.003); and rectal bleeding score 2.4 0.4 in the GP515-treated DSS group versus 3.8 0.1 in the 0.9% NaCl-treated DSS group (p 0.001).

Colon Length. The colon in the GP515-treated DSS group was significantly longer (11.7 ± 0.5 cm) than in DSS-fed mice given 0.9% NaCl (9.7 ± 0.2 cm; n = 15, p = 0.002). In the non-DSS groups, the colon length in mice treated with GP515 (15.7 ± 0.3 cm) was not different from that in mice injected with 0.9% NaCl (15.4 ± 0.3 cm; n = 10; Fig. 2).

Histologic Score. Histology of rings of the transverse part of the colon in DSS-fed mice revealed multiple erosive lesions and inflammatory cell infiltrations composed of macrophages, lymphocytes, eosinophils, and occasional neutrophils. After 11 days of continuous DSS administration, GP515 decreased the histologic score to 3.2 ± 0.5 compared with 4.5 ± 0.2 in the 0.9% NaCl-treated DSS group (n = 5, p = 0.001; Fig. 3). In the non-DSS control groups no histologic signs of inflammation could be detected (1.0 ± 0.0 in the GP515-treated group and 0.8 ± 0.1 in the 0.9% NaCl-treated group).

Interferon-γ Concentration in Colon. IFNγ concentration in the colonic tissue was reduced in GP515-treated DSS mice (139 ± 5 pg/100 mg of colonic tissue) compared with the 0.9% NaCl-treated DSS group (1909 ± 50 pg/100 mg of colonic tissue; n = 8, p = 0.006; Fig. 4). The noninflamed colons of the non-DSS groups receiving either GP515 (304 ± 61 pg/100 mg of colonic tissue) or 0.9% NaCl (175 ± 75 pg/100 mg of colonic tissue) showed IFNγ concentrations comparable to those in the GP515-treated DSS group.

Spleen Weight. As a marker of systemic inflammation, the spleen weight was increased in DSS-treated mice (Fig. 5). DSS-fed mice at day 11 had larger spleens (158 ± 8 mg; n = 10) compared with control mice without DSS that had received either 0.9% NaCl (116 ± 4 mg; n = 10, p = 0.006) or GP515 (113 ± 3 mg; n = 10, p = 0.009). This increase in spleen weight was significantly reduced by concurrent GP515 treatment to 131 ± 8 mg (n = 14, p = 0.001).
IFNγ Production by Cultured Splenocytes. To evaluate whether the in vivo-administered GP515 influences IFNγ production in vitro, splenocytes at the end of the 11-day course were cultured for 20 h in the presence or absence of LPS (100 ng/ml), PMA (25 ng/ml) plus ionomycine (500 ng/ml), or without stimulus (Fig. 6). After LPS stimulation, splenocytes of the 0.9% NaCl-treated DSS group showed an IFNγ production of 102 ± 56 pg/ml (Fig. 6, top). LPS-induced IFNγ synthesis was almost completely abolished in the GP515-treated DSS group (1.1 ± 1.0 pg/ml; n = 4, p = 0.040). Interestingly, an even higher IFNγ synthesis could be measured in LPS-stimulated splenocytes of the non-DSS group, with no significant difference between 0.9% NaCl-treated mice (2155 ± 621 pg/ml) and GP515-treated mice (2417 ± 230 pg/ml; n = 3). Likewise, PMA plus ionomycine-stimulated splenocytes of the 0.9% NaCl-treated DSS group synthesized more IFNγ (20 ± 6 ng/ml) compared with IFNγ synthesis in the GP515-treated DSS group (13 ± 3 ng/ml; n = 5; Fig. 6, bottom). Maximal IFNγ synthesis could be detected in the non-DSS groups (35 ± 1 ng/ml in the 0.9% NaCl group and 33 ± 3 ng/ml in the GP515 group). There was no difference in IFNγ production of unstimulated splenocytes between any of the four experimental groups (data not shown).

GP515 Suppresses CD69 Expression on Splenocytes. On the last day of the treatment course (day 11), spleens were removed aseptically and splenocytes were isolated as described under Materials and Methods. Splenocytes were incubated for a 20-h period in the absence or presence of PMA (25 ng/ml) plus ionomycine (500 ng/ml). At the end of the incubation period cells were examined by flow cytometry for the expression of CD69 as a marker for activation of T cells, B cells, neutrophils, and natural killer cells (Ziegler et al., 1994). The cell population under investigation consisted of 50% lymphocytes (CD45R+), 32% T cells (CD3+), and 7%
macrophages (Mac-1<sup>+</sup>). Eleven percent of the cells were negative for any of the antibodies. Figure 7 represents the flow cytometric analysis of splenocytes of one representative experiment of n = 5 experiments per treatment group. After the 20-h incubation period with PMA plus ionomycine 82% of splenocytes of the 0.9% NaCl-treated DSS group were positive for CD69 (Fig. 7A). In the GP515-treated DSS group only 50% of the splenocytes were positive for CD69 after the 20-h incubation period (Fig. 7B). Ninety-one percent of stimulated splenocytes of the 0.9% NaCl group (Fig. 7C) were CD69 positive compared with 78% in the non-DSS GP515-treated group (Fig. 7D). In contrast, less than 15% of unstimulated splenocytes were positive for CD69 without any differences between the four treatment groups (Fig. 7, A–D).

Discussion

The principal observation of our present study is that the adenosine kinase inhibitor GP515 effectively attenuates experimental colitis in vivo. Administration of DSS in the drinking water induced colitis, as assessed by clinical and histologic parameters (Figs. 1–3). GP515 and DSS were applied in parallel over an 11-day period. Even though GP515 did not delay the onset of colitis, it significantly mitigated colitis severity at later time points (Fig. 1). In agreement with the clinical and histologic score GP515 prevented colon shortening in DSS-fed mice (Fig. 2). Additionally, GP515 effectively suppressed IFN<sub>γ</sub> synthesis in the colonic tissue of DSS-exposed mice (Fig. 4). To evaluate systemic effects of GP515, the spleen weight was determined and splenocytes were isolated, characterized by flow cytometry, and stimulated: GP515 treatment led to a significant decrease in spleen weight compared with the 0.9% NaCl-treated DSS group (Fig. 5). Compared with control animals, splenocytes of GP515-treated DSS mice expressed less CD69 (Fig. 7) and synthesized less IFN<sub>γ</sub> (Fig. 6) after stimulation with LPS or PMA plus ionomycine.

The DSS model of colitis has been recommended for preclinical testing of new pharmacologic compounds for therapy of chronic inflammatory bowel disease (Cooper et al., 1993; Elson et al., 1995; Bennett et al., 1997). A number of therapeutic agents that are now in clinical use or under clinical evaluation have been tested in this model (Kojouharoff et al., 1997; Axelson et al., 1998; Tomoyose et al., 1998; Murthy et al., 1999). DSS-induced colitis has a number of advantages, including its simplicity and the high degree of uniformity and reproducibility of the colonic lesions (Elson et al., 1995). We examined several endpoints in this model: 1) clinical activity, which was quantified with a scoring system that has been described to correlate with pathologic changes (Cooper et al., 1993; Hartmann et al., 2000); 2) shortening of the colon as a morphometric measure for the degree of inflammation, which correlates with pathologic changes and proved to be a consistent marker of colitis (Okayasu et al., 1990); 3) histology, assessing the degree of infiltration by inflammatory cells in the mucosa and the degree of tissue damage; 4) spleen weight, which has been described in the literature to be a reproducible marker for systemic inflammation of mice (McComb et al., 1999); and 5) splenocyte phenotype and responsiveness served as indicators of the systemic anti-inflammatory effect of GP515.

In vivo adenosine applied locally onto microcirculation beds prevents leukocyte rolling, adhesion, and emigration induced by platelet-activating factor or by ischemia. Adenosine or adenosine analogs can also inhibit an inflammatory response when administered systemically, but the clinical utility of this approach is limited by severe cardiovascular side effects (Belardinelli et al., 1989; Moser et al., 1989; Nolte et al., 1991). One strategy to circumvent this problem is the use of adenosine-regulating agents, which increase tissue concentrations of endogenous adenosine through impeding purine metabolism. The adenosine kinase inhibitor GP515 used in this study exhibits these capacities and additionally achieves in vitro and in vivo all the above-described anti-inflammatory effects for adenosine. Furthermore, the anti-inflammatory effects of GP515 in a carrageenan-induced rat paw swelling can be antagonized by an A<sub>2</sub>-receptor antagonist, suggesting enhanced adenosine formation as the mechanism of its anti-inflammatory action (Rosengren et al., 1995). Up to 10 mg/kg of body weight was orally administered and no adverse effect of GP515 on blood pressure or heart rate in rats was detected at anti-inflammatory doses, suggesting that adenosine up-regulation in inflammatory tissues did not result in relevant systemic concentrations (Rosengren et al., 1995).
In the present study we focused our investigations on two endpoints. First, we evaluated the local effect of GP515 at the site of inflammation in the colon. The anti-inflammatory effect of GP515 is demonstrated by the clinical and histologic score and by colon length. On the level of proinflammatory mediators, we were able to demonstrate the suppression of IFN\(\gamma\) generation in the colon. IFN\(\gamma\) is a Th1 cytokine upregulated in the inflamed intestinal mucosa of patients with Crohn’s disease (Parronchi et al., 1997; Monteleone et al., 1999). Second, we examined the systemic impact of GP515 on the immune system by investigating spleenocyte phenotype and function. Reduced spleen weight in GP515-treated DSS mice (Fig. 5) pointed toward altered spleenocyte function. After the 20-h incubation period with PMA plus ionomycin, a specific T-cell stimulus, the CD69 expression in the GP515-treated DSS group was reduced. CD69 is a type II membrane glycoprotein and a member of the C-lectin family (Ullman et al., 1990). It is one of the earliest cell surface antigens induced on activated T cells, thymocytes, B cells, natural killer cells, and neutrophils (Cosulich et al., 1987; Lanier et al., 1988; Risso et al., 1989; Gavioli et al., 1992; Ziegler et al., 1994).

Both CD69 expression and IFN\(\gamma\) synthesis were markedly suppressed in the GP515-treated DSS group compared with the untreated DSS group, which is consistent with the reduced IFN\(\gamma\) synthesis in the colon. This indicates that interference with purine metabolism influences the regulation of CD69 and IFN\(\gamma\). However, both results cannot be explained by a direct action of GP515 because the spleenocytes have been washed three times during the isolation process. Rather, the in vivo application of the adenosine kinase inhibitor appears to induce a sustained down-regulation of the inflammatory process.

Two observations were of particular interest in the GP515-treated or untreated non-DSS-fed mice. First, no inhibitory effect on IFN\(\gamma\) synthesis or CD69 expression by GP515 treatment in non-DSS-fed mice could be detected. GP515 exerts its anti-inflammatory effects by increasing the concentration of extracellular adenosine at sites of inflammation. Because the group of non-DSS-fed mice was without inflammation during the experimental period, the time of GP515 treatment, no anti-inflammatory effect can be observed in vitro. Second, the IFN\(\gamma\) synthesis was higher in stimulated splenocytes of non-DSS-fed mice. One might speculate that the decreased IFN\(\gamma\) synthesis and CD69 expression in DSS-fed mice is due to desensitization of spleenocytes during the systemic inflammatory response, as has been described for LPS-induced desensitization in monocyte macrophages (Ziegler-Heitbrock et al., 1997).

IFN\(\gamma\) is produced by natural killer cells and by Th1 cells. The latter are involved in the pathogenesis of chronic inflammatory bowel disease, and inhibition of IL-12, the cytokine central in the development of Th1 cells, was shown to abrogate trinitrobenzene sulfonic acid-induced colitis in mice (Neurath et al., 1995). However, whether IFN\(\gamma\) presents the key inflammatory mediator influenced by GP515 treatment in this model cannot be concluded at this point. To prove this further experiments with neutralizing anti-IFN\(\gamma\) antibodies are necessary.

We conclude that the therapeutic application of a Th1 response-suppressing agent acting preferentially at the site of inflammation presents a novel, promising pharmacologic strategy. In the current study we could demonstrate that the adenosine kinase inhibitor GP515 achieves this goal in the experimental setting. Adenosine kinase inhibition forms an attractive pharmacologic principle that warrants to be pursued for the treatment of chronic inflammatory bowel disease in the clinic.

References


