An Oral Drug Delivery System Targeting Immune-Regulating Cells Ameliorates Mucosal Injury in Trinitrobenzene Sulfonic Acid-Induced Colitis

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ABSTRACT

Control of immune-regulating cells in the colonic mucosa is important in the treatment of patients with inflammatory bowel disease (IBD). The aim of study was to examine the therapeutic effect of dexamethasone (DX) microspheres on 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats, a model for human Crohn’s disease. DX microspheres and DX alone were administered orally to rats with TNBS-induced colitis. The macroscopic score, histological score, myeloperoxidase (MPO) activity, nitric oxide (NO) production, and gene expressions of proinflammatory cytokines and COX-2 in the colonic tissue were determined. Proliferating cell nuclear antigen (PCNA) staining and expression of nuclear transcription factor (NF)-κB in colonic tissues were also investigated. Macroscopic score, histological score, MPO activity, and NO production in rats treated with DX microspheres were significantly lower than in those treated with DX alone. The gene expression of proinflammatory cytokines and COX-2 in rats treated with DX microspheres was down-regulated, compared with that in rats treated with DX alone. The number of PCNA-positive cells in the DX microsphere group was larger than in the group treated with DX alone. DX microspheres suppressed NF-κB activation in TNBS-induced colitis more strongly than DX alone. Oral administration of DX microspheres appears to ameliorate mucosal injury in TNBS-induced colitis. This drug delivery system could be an ideal therapy for human IBD.

Ulcerative colitis (UC) and Crohn’s disease (CD) are the two major forms of chronic human inflammatory bowel diseases (IBD). Although the etiology of this disease remains unclear, several studies have indicated that active monocytes, such as macrophages and T-cells, play an important role in the pathogenesis of IBD (Wilders et al., 1984; Allison et al., 1988; Seldenerijk et al., 1989; Okazaki et al., 1993; Probert et al., 1996). Manipulation of the immune response by these cells appears essential for the treatment of patients with IBD. Recently a new drug delivery system was developed that consists of poly-D,L-lactic acid (PDLLA) microspheres containing dexamethasone (DX) microspheres and targets immune-regulating cells (Nakase et al., 2000). Since DX microspheres were mainly taken up in the inflamed colon and phagocytosed by macrophages, oral administration of DX microspheres ameliorated mucosal injury in a dextran sulfate sodium (DSS)-induced colitis model more efficiently than DX alone. Moreover, serum levels of DX did not elevate after oral administration of DX microspheres, hence DX microspheres were considered to have fewer systemic side effects than DX alone.

Several animal models of intestinal inflammation have been described (Okayasu et al., 1990; Kühn et al., 1993; Mombaerts et al., 1993; Sadlack et al., 1993; Powrie et al., 1994; Holländer et al., 1995; Rudolph et al., 1995). Among them, hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS)/ethanol-induced colitis has been well standardized (Morris et al., 1989). The TNBS-induced colitis model resembles human CD in terms of its histopathological features and T-helper profile of cytokines, including interferon (IFN)-γ,
whereas DSS-induced colitis resembles human UC (Okayasu et al., 1990; Fuss et al., 1996; Parronchi et al., 1997). Moreover, various experimental trials using antibodies to interleukin (IL)-12, IL-4 gene transfer, and antisense oligonucleotides against nuclear factor-κB (NF-κB) have indicated that the TNBS-induced colitis model is useful to test new therapeutic strategies for human CD (Neurath et al., 1996; Hoggaboam et al., 1997; Fuss et al., 1999).

In this study, the effects of oral administration of DX microspheres on TNBS colitis in rats were examined. The therapeutic effects of DX microspheres and DX alone were compared with special reference to colonic cell proliferation and intracellular signaling of NF-κB. The present study shows that TNBS-induced colitis can be treated successfully by oral administration of DX microspheres, which inhibit the activation of NF-κB more strongly than DX alone, and have fewer adverse effects on colonic cell proliferation.

Materials and Methods

Induction of Colitis. Male Wistar rats (200–250 g; Japan SLC, Inc., Shizuoka, Japan) were used for all experiments. The rats were housed in standard cages and fed with standard laboratory chow and tap water ad libitum. In this study, for induction of sustained colonic inflammation, we modified the original protocol described by Morris et al. in 1989. Colitis was induced by intracolonial administration of 50 mg of TNBS (Sigma Chemical, St. Louis, MO) in 0.25 ml of 50% (v/v) ethanol.

Preparation of PDLLA Microspheres Containing DX. PDLLA microspheres were synthesized by polycondensation of DL-lactic acid at 180°C under reduced pressure without any catalyst as described previously (Tabata et al., 1996). DX phosphate (Decadron) was kindly supplied by Banyu Pharmaceutical Co. (Tokyo, Japan). PDLLA microspheres containing DX were prepared by the solvent evaporation method with double emulsion. In brief, 60 μl of DX phosphate aqueous solution (W1) were poured into 1 ml of methylene chloride containing 200 mg of PDLLA microspheres (O), followed by emulsification by probe sonication to form a W1/O emulsion. The emulsion was added to 2 ml of 1% polyvinyl alcohol (molecular weight = 5400, degree of saponification = 79.85 mol %) aqueous solution (W2), which had been saturated with methylene chloride at room temperature and agitated on a vortex mixer to form a double emulsion. The W1/O/W2 double emulsion was stirred with an impeller (200 rpm) at room temperature until the methylene chloride had evaporated completely. The microspheres were collected by centrifugation (5000 rpm, 5 min, 4°C), washed three times with cold distilled water, and finally lyophilized. After hydrolysis of PDLLA microspheres containing DX, the concentration of DX incorporated in the microspheres was determined by high-performance liquid chromatography (HPLC) at 245 nm (Tabata and Ikada, 1990).

Treatment of Rats. Fifty rats with TNBS-induced colitis were divided into five groups (10 rats each; groups A–E) and treated 1 week after TNBS administration as follows: group A, no medication; group B, PDLLA microspheres (0.1 mg/kg/day) alone; group C, free DX (10−4 mg/kg/day) alone; group D, PDLLA microspheres (0.1 mg/kg/day) + free DX (10−4 mg/kg/day) (the mixture of DX and microspheres); and group E, PDLLA microspheres containing DX (DX microspheres; 0.1 mg/kg/day, which contained about 10−2 mg/g/day of DX). Another 10 rats were used as the normal control (group N).

Macroscopic Assessment of Colonic Damage. The distal 10 cm of the rat colon and rectum were excised, opened longitudinally, and washed in saline. Macroscopic damage was assessed by the scoring system of Wallace and Keenan (1990), which takes into account the area of inflammation and the presence or absence of ulcers. The criteria for assessing macroscopic damage and the numerical rating score were as follows: 0, no ulcer, no inflammation; 1, no ulcer, local hyperemia; 2, ulceration without hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; and 5, ulceration extending more than 2 cm. After macroscopic observation, full thickness specimens from inflamed tissue adjacent to the ulcerated area were subsequently excised for microscopic observation of damage, measurements of myeloperoxidase (MPO) activity, nitric oxide (NO) production, and mRNA expressions of cytokines and cyclooxygenase (COX) in the colonic tissues.

Microscopic Assessment of Colonic Damage. The colon was fixed in 3.3% formalin in phosphate-buffered saline (PBS) overnight and stained with hematoxylin and eosin. The degree of inflammation on microscopic tissue sections was scored as follows: 0, no leukocyte infiltration; 1, low level of leukocyte infiltration; 2, moderate level of leukocyte infiltration; 3, high vascular density and thickening of the colon wall; and 4, transmural leukocyte infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall (Fuss et al., 1996). Grading was done in a blind manner.

Assessment of MPO Activity. MPO activity was measured according to the method of Bradley et al. (1982). Tissue samples were homogenized three times in hexadecyltrimethylammonium bromide buffer in a Polytron homogenizer (Brinkman Instruments, Rexdale, ON, Canada). The homogenate was centrifuged and MPO activity in the supernatants measured. One unit of MPO activity was defined as the amount required to degrade 1 mM H2O2 in 1 min at 25°C.

Assessment of NO Production. Tissue from the proximal third of the colon was homogenized for 15 s in HEPES buffer solution (40 mM, pH 7.4) containing sucrose (320 mM) (Boughton-Smith et al., 1993). The combined concentration of nitrites and nitrates, and the

![Fig. 1. Macroscopic appearance of the distal colon from DX and DX microsphere-treated rats.](image-url)
degradation products of NO in the supernatants (10,000g for 20 min at 4°C) were determined by the Griess reaction after nitrate reduction as described previously (Salzman et al., 1995). Total nitrate and nitrate production are described as NO production.

**Immunohistochemistry of Proliferating Cell Nuclear Antigen (PCNA).** The paraffin-embedded tissues samples were cut into 4-μm sections, and each section was then dewaxed and allowed to react with 0.03% H2O2 aqueous solution to inhibit endogenous peroxidase activity. Anti-PCNA antibodies (Dako, Kyoto, Japan) were diluted 1:50 with PBS, and the sections were then incubated with diluted antibody for 1 h, followed by 1 h with Envision and peroxidase activity. Anti-PCNA antibodies (Dako, Kyoto, Japan) were reacted with 0.03% H2O2 aqueous solution to inhibit endogenous peroxidase activity. Hematoxylin was used for nuclear counterstaining. The glands were selected randomly and the number of PCNA-positive cells were counted under a microscope. The PCNA-labeling index was defined as the percentage of PCNA-positive cells in the counted crypts. Five hundred nuclei were counted (Mariadason et al., 1999).

**Measurement of Cytokine and COX mRNA Expressions.** Total RNA in the colonic tissues was isolated by the guanidium isothiocyanate method. The concentration of RNA was determined by measuring absorbance at 260 nm in relation to that at 280 nm. One microliter of reverse transcriptase (RT) product was added to 1 mM concentrations of each primer and a solution of 1 U of Taq DNA polymerase (Takara, Biochemicals, Ohtsu, Japan) in a final volume of 20 μl. The mixture underwent polymerase chain reaction (PCR) amplification for 25 cycles (1 min at 94°C, 1 min at 52°C, and 20 s at 72°C). Negative controls of cDNA-free solution were included in each reaction. The sequences of primers for the cytokine genes were: tumor necrosis factor (TNF)-α forward, 5'-AGCCTTCTTCTATTGCTGTC-3', reverse, 5'-GGTGTCTTTGAGATCCATGCC-3'; IL-1β forward, 5'-TGGATGCCATAGAGCAGC-3', reverse, 5'-GAGGAGTGCGTGATGTACAGGTT-3'; IPN-γ forward, 5'-AGGCCATCAGCAACACAT-3', reverse, 5'-TGGTCCCCTGCTTAAGCTAG-3'; COX-1 forward, 5'-GCCCTCACTCACC-3', reverse, 5'-CAGGAGCCTGTTCACGGA-3'; COX-2 forward 5'-CACACTTTCCCTCTTC-3', reverse, 5'-CTTATTTTTCCTTCACAC-3'; 3X-β actin forward 5'-TGTAAACAACCTGGGAGCATGG-3', reverse, 5'-GATCTTGATCTTCTAGGTGG-3'.

For semiquantitative RT-PCR, serial 1:2 dilutions of cDNA were amplified with increasing numbers of cycles according to the method of Wang et al. (1989). After gel electrophoresis, PCR products were visualized using a 1% gel. Densities of the bands were measured with the computer software program (1-D advanced; Advanced American Biotechnology, Fullerton, CA). Log 2 values of optical units were plotted against the number of cycles. Rates of amplification were linear between a defined number of cycles. With increasing cycle numbers, the rates did not increase further and approached a plateau. These studies of amplification kinetics were repeated for each cytokine. Dilution experiments showed that the method could resolve less than 8-fold differences in cDNA concentration.

**Electrophoretic Mobility Shift Assays (EMSA).** EMSAs were performed as described previously (Salzman et al., 1995). Briefly, frozen tissue was broken down mechanically, transferred to a 50-ml Falcon tube containing 5 ml of cold buffer A (10 mM HEPES/KOH, pH 7.9 at 4°C, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 0.6% Nonidet P40) and homogenized with a Polytron homogenizer for 1 min. Insoluble material was removed by centrifugation for 30s at 2,000 rpm and 4°C, and the supernatant was incubated on ice for 10 min before centrifugation for 5 min at 8,000 rpm at 4°C. The supernatant was discarded, and the nuclear pellet was resuspended in 100 ml of buffer B (25% glycerol, 20 mM HEPES/KOH, pH 7.9 at 4°C, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, and 5 mg/ml leupeptin) and incubated on ice for 20 min. Cellular debris was removed by centrifugation for 2 min at 14,000 rpm and 4°C, and the supernatant was snap frozen at −80°C. Protein concentrations were determined with a BCA protein assay kit (Pierce Chemical Co, Rockford, IL). The oligonucleotide probe used for EMSA for NF-κB, 5'-AGTTGAGGG-GACTTCCAGGC-3' (Promega), was labeled with [32P]adenosine triphosphate (Amersham Pharmacia Biotech UK, Ltd., Little Chalfont, Buckinghamshire, UK) using T-4 polynucleotide kinase and purified on a Bio-spin chromatography column.

**Table 1**

<table>
<thead>
<tr>
<th>PCNA-Labeling Index (%)</th>
<th>Normal colonic mucosa</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Group E</th>
<th>Group N</th>
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<tr>
<td></td>
<td>18.1 ± 6.8</td>
<td>45.6 ± 3.3, p &lt; 0.01, compared with normal control.</td>
<td>48.2 ± 4.3, p &lt; 0.05, compared with groups A and B.</td>
<td>21.3 ± 4.8, p &lt; 0.05, compared with normal control.</td>
<td>24.2 ± 6.7, p &lt; 0.05, compared with normal control.</td>
<td>35.2 ± 9.5, p &lt; 0.05, compared with normal control.</td>
<td>10.0, for each group).</td>
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*P < 0.01, compared with groups A and B.

**Fig. 2.** Effects of treatments with DX on colonic damage in TNBS-induced colitis. A. colonic macroscopic scores. B. microscopic scores. Data are expressed as the means ± S.E. (n = 10, for each group). A, group A; B, groups B and D; C, group C; D, group E. *P < 0.05, compared with groups A and B.
of an excess of unlabeled NF-κB oligonucleotide. The DNA-protein complex was separated from free oligonucleotide on a 6% nondenaturing polyacrylamide gel. After electrophoresis, the gel was vacuum-dried at 80°C for 30 min and exposed.

Statistical Analysis. Student’s t test and Mann-Whitney U test were used where appropriate for statistical analysis. One-way analysis of variance was used to compare the transcript levels of cytokines and COXs in colonic tissues of each group. The data are presented as the means ± S.E. A two-tailed P value of <0.05 was considered statistically significant.

Results

Macroscopic Evaluation. Macroscopic examination of the distal colon and rectum from groups A and B revealed the presence of multiple mucosal erosions and ulcerations. Macroscopic examination of groups C and D showed a reddish, edematous mucosa, but fewer erosions and ulcerations than in groups A and B. In contrast, macroscopic examination of group E demonstrated only mild mucosal edema (Fig. 1). Macroscopic scores for groups C, D, and E were significantly lower than those for groups A and B. Moreover, the macroscopic score for group E was significantly lower than those for groups C and D. There were no statistically significant differences in macroscopic scores between groups A and B, or C and D (Fig. 2a).

Histological Evaluation. In groups A and B, histological scores were significantly higher than those for the free DX groups C, D, and E. The histological score for group E was significantly lower than those for groups C and D (Fig. 2b). There were no statistically significant differences in the histological scores between groups A and B, or C and D.

PCNA Staining. A significant increase in PCNA-labeling index in TNBS-induced colitis was observed. The PCNA-labeling indices in groups C, D, and E were significantly lower than those in groups A and B. Although there were no significant differences, the PCNA-labeling index in group E tended to be higher than those in groups C and D. There were no statistically significant differences in the labeling index between groups A and B, or C and D (Table 1; Fig. 3).

MPO Activity and NO Production. MPO activity and NO production in the colonic tissue from groups A and B were
significantly higher than those of groups C, D, and E. Moreover, MPO activity and NO production of group E were significantly lower than those in groups C and D. There were no statistically significant differences between A and B, or C and D (Fig. 4, a and b).

**mRNA Expression of Proinflammatory Cytokines and COXs.** The results of RT-PCR showed that mRNA expressions of TNF-α, IL-1β, IFN-γ, and COX-2 were all significantly up-regulated in colonic tissues after induction of TNBS colitis. On the other hand, no significant differences in the transcript levels of COX-1 were observed among all groups. Compared with groups A and B, transcript levels of TNF-α, IL-1β, IFN-γ, and COX-2 were significantly down-regulated in groups C, D, and E. Moreover, transcript levels of TNF-α, IL-1β, IFN-γ, and COX-2 in group E were also significantly lower than the respective values in groups C and D (Table 2).

**EMSA.** Nuclear levels of NF-κB were increased in colonic tissue from groups A and B when compared with normal mice. Nuclear levels of NF-κB were decreased in colonic tissue from groups C, D, and E, compared with those of groups A and B. Moreover, nuclear levels of NF-κB in group E were markedly decreased in comparison with those of groups C and D (Fig. 5).

![Fig. 4. Effects of treatments with DX on colonic inflammation in TNBS-induced colitis. A, MPO activity. B, NO production in colonic tissue. Data are expressed as the means ± S.E. (n = 10, for each group). □, group N; ■, group A; □, group B; □, group C; □, group D; □, group E. *P < 0.05, compared with groups A and B. #P < 0.05, compared with groups C and D. NS, not significant.](image-url)
The increased levels of MPO, NO, and gene expressions of proinflammatory cytokines in colonic tissue from TNBS-induced colitis were decreased more significantly with DX microspheres than with free-DX. NF-κB is known to regulate the transcription of various cytokines, as well as activate specific enzymes such as inducible forms of NO and COX-2 (Schottelius and Baldwin, 1999). Thus, NF-κB is considered to play a critical role at the site of inflammation. Inhibition of translocation of NF-κB into the nucleus or degradation of IκBα in the cytoplasm by glucocorticosteroids is considered to result in the amelioration of inflammation (Auphan et al., 1995; Scheinman et al., 1995). In accordance with this idea, we observed potent inhibitory effects of DX microspheres on NF-κB activity through induction of IκBα synthesis. Science (Wash DC) 270: 286–290.

Table 2: Effect of DX treatment on the transcript levels of cytokines and COXs in colonic tissues.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 4)</th>
<th>Group A (n = 5)</th>
<th>Group B (n = 6)</th>
<th>Group C (n = 6)</th>
<th>Group D (n = 7)</th>
<th>Group E (n = 7)</th>
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<tbody>
<tr>
<td><strong>NF-κB</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>TNF-α</strong></td>
<td>0.10 ± 0.03</td>
<td>0.65 ± 0.05</td>
<td>0.58 ± 0.06</td>
<td>0.40 ± 0.05</td>
<td>0.30 ± 0.05</td>
<td>0.15 ± 0.06</td>
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<tr>
<td><strong>IL-1β</strong></td>
<td>0.11 ± 0.02</td>
<td>0.43 ± 0.06</td>
<td>0.40 ± 0.07</td>
<td>0.35 ± 0.03</td>
<td>0.29 ± 0.06</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td>0.12 ± 0.05</td>
<td>0.52 ± 0.12</td>
<td>0.56 ± 0.08</td>
<td>0.38 ± 0.04</td>
<td>0.25 ± 0.06</td>
<td>0.12 ± 0.05</td>
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<tr>
<td><strong>COX-1</strong></td>
<td>0.90 ± 0.12</td>
<td>0.96 ± 0.10</td>
<td>0.93 ± 0.10</td>
<td>0.85 ± 0.08</td>
<td>0.92 ± 0.10</td>
<td>0.88 ± 0.11</td>
</tr>
<tr>
<td><strong>COX-2</strong></td>
<td>0.10 ± 0.02</td>
<td>0.85 ± 0.12</td>
<td>0.82 ± 0.10</td>
<td>0.42 ± 0.10</td>
<td>0.35 ± 0.11</td>
<td>0.12 ± 0.02</td>
</tr>
</tbody>
</table>

* P < 0.05, compared with groups A and B.

**References**


Boughton-Smith NK, Evans SM, Hawkey CJ, Cole AT, Balsitis M, Whittle BJ and Eberhart and Dubois, 1995). COX-1 is a constitutive enzyme and is thought to produce cytoprotective prostaglandins, whereas COX-2 represents the inducible form of COX leading to production of proinflammatory prostaglandins (Williams and Dubois, 1996). Therefore, the expression of COX-2 mRNA is usually up-regulated in the acute inflammatory phase of IBD (Hawkey and Rampton, 1983; Hendel and Nielsen, 1997; Lesch et al., 1999). In our study, expression of COX-2 mRNA was also up-regulated in TNBS-induced colitis and was down-regulated by treatment with DX microspheres or free DX. Notably, expression of COX-2 mRNA was inhibited more strongly in the DX microspheres group than in the free DX groups, again showing a more potent effect of DX microspheres on inhibition of mucosal inflammation.

In conclusion, our data showed a novel therapeutic effect of DX microspheres on mucosal injury in TNBS-induced colitis. Considering the ability of DX microspheres to inhibit colonic inflammation more strongly than free DX, with less inhibitory effect on regeneration of the colonic epithelium, this may represent a pivotal anti-inflammatory approach for patients with IBD.


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