The simultaneous blockade of chemokine receptors CCR2, CCR5 and CXCR3 by a non-peptide chemokine receptor antagonist protects mice from dextran sodium sulfate-mediated colitis

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Abstract

Chemokine receptors CCR2, CCR5 and CXCR3 are involved in the regulation of macrophage- and T cell-mediated immune responses and in the migration and activation of these cells. In order to determine whether blockade of these chemokine receptors modulates intestinal inflammation, we investigated here the effect of a non-peptide chemokine receptor antagonist, TAK-779 (N,N-dimethyl-N-[4-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]carbonyl]amino]benzyl]-tetrahydro-2H-pyran-4-aminium chloride), in mice with dextran sodium sulfate (DSS)-induced experimental colitis. C57BL/6 mice were fed 5% DSS in their drinking water for up to 7 days with or without the administration of TAK-779. The severity of inflammation in the colon was assessed by clinical signs and histological examination. Infiltration of inflammatory cells into the mucosa was analyzed by immunohistochemistry, and the expression of cytokine and chemokine mRNAs in tissues was quantitated by reverse transcription–PCR. During DSS-induced colitis, the recruitment of monocytes/macrophages into the colonic mucosa and the induction of proinflammatory cytokines correlated with the severity of intestinal inflammation. The onset of clinical signs and histopathologic features were delayed in animals treated with TAK-779. The expression of CCR2, CCR5 and CXCR3 mRNAs was inhibited in the TAK-779-treated mice. Consistent with these results, infiltration of monocytes/macrophages into the lamina propria was almost completely inhibited and the expression of colonic IL-1β and IL-6 was significantly decreased in the TAK-779-treated mice. The blockade of CCR2, CCR5 and CXCR3 prevents murine experimental colitis by inhibiting the recruitment of inflammatory cells into the mucosa. Therefore, chemokines and their receptors may be therapeutic targets for the treatment of inflammatory bowel disease.

Introduction

Inflammatory bowel disease (IBD) is a chronic, relapsing and remitting inflammatory disorder that exhibits a variety of autoimmune features. The evidence suggests that disruption of immune regulatory networks may lead to IBD (1). Abnormalities in intestinal epithelial cell barrier function, excessive production of either Th1 or Th2 cytokines and/or dysregulated activation of inflammatory cells by normal luminal bacteria may contribute to the pathogenesis of IBD (2). In addition to cytokine expression, chemokines and their receptors also contribute to the regulation of intestinal immune responses and mucosal inflammation. For example, they appear to be critical elements in regulating the trafficking of cells to inflammatory sites (3, 4). The CC chemokine receptors CCR2 and CCR5 are involved in both monocyte- and macrophage-mediated immune responses, and in the regulation of T cell migration and activation. CCL2 is a potent

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Chemoattractant and an activator of monocytes (5). CCR5-binding chemokines (CCL3, CCL4 and CCL5) recruit memory and activated CD4+ and CD8+ T cells (6). Importantly, intestinal epithelial cells can rapidly produce CCL2 and CCL5 upon exposure to inflammatory mediators (7, 8). Increases in the expression of CCL2 and CCL7, both of which bind CCR2, have been observed in the colonic mucosa of IBD patients, suggesting that these chemokines may contribute to the inflammatory process in the affected mucosa (9, 10). Increases in the expression of CCL3 and CCL5 in the intestinal mucosa of IBD patients has also been reported (11). Furthermore, it is reported that CXCR3+ cells are enriched in the normal colon and the inflamed intestine of patients with ulcerative colitis or Crohn’s disease (12). It has been demonstrated that CXCL9 and CXCL10 are expressed in the normal colon and by intestinal epithelial cells stimulated by IFN-γ (13, 14). These chemokines and chemokine receptors are considered to be important factors in the pathogenesis of IBD and, therefore, may represent a viable therapeutic target for the treatment of these diseases.

A non-peptide, synthetic CCR5 antagonist, TAK-779 (N, N-dimethyl-N-[4-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]carbonyl]amino]benzyl]-tetrahydro-2H-pyran-4-aminium chloride), was initially developed for the treatment of HIV infection (15). TAK-779 appears to selectively inhibit CCR5 and, to a lesser extent, CCR2b (15). Chemokine binding to CCR1, CCR3, CCR4 or CXCR4 was not inhibited by TAK-779 (15). Interestingly, Gao et al. (16) recently reported that TAK-779 blocks ligand binding to murine CCR5 (mCCR5) and CXCR3 (mCXCR3). In this study, we demonstrated that TAK-779 efficiently antagonized murine CCR2 (mCCR2). The specificity of TAK-779 for certain murine chemokine receptors presents a unique opportunity for the efficient blockade of macrophage and, to a lesser extent, T cell migration to inflammatory lesions. In order to evaluate this possibility, we examined the effects of TAK-779 in the dextran sodium sulfate (DSS)-induced mouse model of colitis.

Methods

Animals

Specific pathogen-free, male C57BL/6J mice were purchased from CLEA Japan (Shizuoka, Japan). All of the mice were treated humanely according to the Institutional Animal Care and Use Committee guidelines. All animal experiments were approved by the Institutional Animal Care And Use Committee of Tokyo Medical University.

TAK-779 treatment

A non-peptide chemokine receptor antagonist, TAK-779, was kindly provided by Takeda Chemical Industries, Ltd (Osaka, Japan) (15). TAK-779 was dissolved in water and further diluted with sterile PBS solution to 5μg μl⁻¹. Mice received daily intra-dermal injections of TAK-779 (50 mg kg⁻¹) at the base of the tail.

Thioglycollate-induced peritonitis

For the induction of peritonitis, Brewer’s thioglycollate (2 ml, 4% solution in distilled water; Difco, Detroit, MI, USA) was injected intra-peritoneally (i.p.). After 96 h, a peritoneal lavage was performed with ice-cold PBS containing 1 mM EDTA. The recovered cells were counted and monocytes/macrophages were identified as CD11b+ (M1/70; BD Pharmingen, San Diego, CA, USA) and F4/80+ (eBioscience, Inc., San Diego, CA, USA) cells by flow cytometry analysis using an EPICS ELITE ESP cell sorter (Beckman Coulter, Hialeah, FL, USA).

Chemotaxis assay

Chemotaxis assays were performed in ChemoTx plates (Neuroprobe, Gaithersburg, MD, USA) with polycarbonate filters (5-μm pore size) according to the manufacturer’s instructions. In brief, thioglycollate-elicited peritoneal cells were suspended at 2 × 10⁶ cells ml⁻¹ in RPMI 1640 (GIBCO/Invitrogen, Grand Island, NY, USA) containing 0.5% BSA (Sigma Chemical Co., St Louis, MO, USA) and 20 mM HEPES (GIBCO/Invitrogen). Thirty microliters of cell suspension was loaded on the membrane plate and placed onto a flat-bottomed 96-well microtiter plate containing 29 μl cocktail of CCL2 (10⁻⁸ M) and indicated doses of TAK-779. Cells were incubated at 37°C in an atmosphere of 5% CO₂ for 90 min, and cells that migrated through and adhered to the bottom were counted. Percent chemotaxis was calculated by dividing the number of the cells that migrated into the lower chamber in the presence of TAK-779 by that in the absence of TAK-779. The effect of TAK-779 on CCL2-dependent chemotaxis was presented as percent inhibition of chemotaxis (percent inhibition of chemotaxis = 100 – % chemotaxis). Each experiment was performed in triplicate. Data are presented as the mean ± SD.

Induction and general assessment of colitis

Colitis was induced in an 8-week-old mice (20–25 g) with 5% (w/v) DSS (MW 50,000; Bio Research Corporation of Yokohama, Yokohama, Japan) in the drinking water. This treatment results in clinical symptoms of colitis within 3 days. In order to investigate the therapeutic effects of TAK-779 in DSS-induced colitis, we compared four groups of mice: untreated (Water/PBS; drinking water and PBS injection), water/TAK-779 (drinking water and TAK-779 injection), DSS/PBS (oral DSS treatment and PBS injection) and DSS/TAK-779 (oral DSS treatment and TAK-779 injection). Mice were treated with DSS for 7 days and subsequently sacrificed. The daily consumption of DSS-containing water was measured in each group and the mean consumption was equivalent in all groups throughout the duration of the study. TAK-779 was administered intra-dermally (50 mg kg⁻¹ per day in PBS) 2 h prior to DSS treatment and daily thereafter. Total body weight was measured at the same time each day prior to TAK-779 injection. Feces were collected from each mouse daily and the presence of occult blood reaction was determined by the guaiac method (NACALAI TESQUE, Inc., Kyoto, Japan) according to the manufacturer’s instruction. Scoring for weight loss (0–4), diarrhea (0–4) and fecal blood (0–4) was performed as previously reported (Table 1) (17). All data were collected in a double-blind fashion and all of the experiments were performed with four mice per group at each time point. The data presented are representative of at least three individual experiments.
Histological examination and immunohistochemistry

Mice (four mice per group) treated with 5% DSS and either TAK-779 or PBS were sacrificed at the time points indicated for tissue harvesting. The entire colon was removed and total colon lengths were recorded. Freshly isolated tissue from the descending portion of the large intestine was fixed in PFA, washed with PBS, embedded in Tissue-Teck OCT compound (Miles, Elkhart, IN, USA) at −80°C and sectioned with a cryostat at 6 μm. Sections were stained with H&E for histological examination and were scored for severity and extent of inflammation and ulceration. Lesion severity was graded using a previously defined scoring system (18) with a scale of 0–3: 0, normal; 1, mild; 2, moderate, and 3, severe (Table 2). Ulcers were areas of the mucosa in which the epithelial lining was missing. The extent of ulceration (percent ulceration) was determined by measuring the percentage of the colon that had microscopic evidence of ulceration. The reliability and reproducibility of this semi-quantitative evaluation were assessed by comparing the scores of two independent investigators (H.T. and K.K.). Sections were also pre-incubated with Block-ace (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) to block non-specific binding for immunohistochemistry and were incubated for 30 min at 37°C in the following biotin-conjugated mAbs from BD PharMingen: hamster anti-mouse CD3 (145-2C11), rat anti-mouse CD4 (RM4-5), rat anti-mouse CD8 (53-6.7), rat anti-mouse NKG2A/C/E (20d5), rat anti-mouse B220 (RA3-6B2), rat anti-mouse Gr-1 (RB6-8C5), rat anti-mouse I-A/I-E (2G9) and rat anti-mouse CD11b (M1/70). The sections were subsequently rinsed in PBS (3x) and incubated with avidin–biotin peroxidase (Vectorstain ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA). The color reaction was developed using 3,3-diaminobenzidine (Vector Laboratories, Inc.) according to the manufacturer’s instructions. Endogenous peroxidase activity was blocked by incubation in 0.3% H2O2 and 0.1% NaN3 in distilled water for 10 min at room temperature. Control sections were incubated with isotype-matched normal hamster or rat IgG. The sections were counterstained with hematoxylin for routine microscopy.

The numbers of CD11b+, CD4+, CD8+, NKG2A/C/E+ and B220+ cells per high-powered-microscope (original magnification: ×200) field (h.p.f.) were counted by two independent observers. A total of eight randomly selected h.p.f. per animal were counted with four animals analyzed per group. The width and length of the microscopic area were measured using calibration slide and the surface area on microscope slide was 0.12 mm².

Table 2. Scoring of lesion severity

<table>
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<th>Score</th>
<th>Severity of inflammation</th>
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<tr>
<td>0</td>
<td>Normal</td>
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<tr>
<td>1</td>
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<td>2</td>
<td>Moderate</td>
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<tr>
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<td>Severe</td>
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Colons were opened longitudinally. Each section was scored for the severity of inflammation.

Quantitative reverse transcription–PCR

Whole colonic RNA was isolated using TRizol (Invitrogen). RNA from four mice per group (1 μg of each) was pooled, reverse transcribed into cDNA and amplified as described previously (19). The expressions of CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10, CCR2, CCR5, CXCR3, IFN-γ, IL-4, IL-10, tumor necrosis factor (TNF)-α, IL-1β and IL-6 were determined by real-time quantitative PCR using the ABI7700 sequence detector system (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) using primers and probes as described previously (20). The amounts of each mRNA species were normalized to the level of GAPDH at each time point. The normalized mRNA values were multiplied by 10 000 and were presented as an mRNA index. PCR was performed in triplicate for each experiment and the PCR products were also examined by electrophoresis on 1.8% agarose gels and visualized with ethidium bromide.

Statistical analysis

The non-parametric Wilcoxon rank test (i.e. the Mann–Whitney U-test) was used to evaluate the correlation between the DSS/PBS and DSS/TAK-779 data. Statistical analyses were performed with the JMP software, version 5.01a (SAS Institute Inc., Cary, NC, USA). P < 0.05 was considered to be indicative of a statistically significant difference on a local level.

Results

TAK-779 prevents thioglycollate-induced peritonitis by inhibiting the recruitment of inflammatory cells into the peritoneal cavity

To examine whether TAK-779 is effective in inhibiting murine chemokine receptors in vivo, we analyzed the recruitment of i.p. leukocytes elicited in response to i.p. administration of thioglycollate with or without the concomitant administration of TAK-779 (50 mg kg⁻¹ body weight). Thioglycollate-elicited cells were obtained by peritoneal lavage 96 h after i.p. injection of 2 ml of 4% Brewer's thioglycollate broth. While the
total number of i.p. cells and resident macrophages was unaffected by TAK-779 treatment, significantly fewer thioglycollate-elicited cells were recovered from TAK-779-treated mice than from control mice (Fig. 1). The reduction in peritoneal exudate cells was largely accounted for by a significant decrease in the number of CD11b+/F4/80+ macrophages (Fig. 1). These results suggest that TAK-779 efficiently inhibits the recruitment of inflammatory cells in vivo. Because the thioglycollate-induced accumulation of macrophages into the peritoneal cavity is dependent upon activation of CCR2 (21), TAK-779 is suggested to block mCCR2, in addition to mCCR5 and mCXCR3.

**TAK-779 efficiently inhibits macrophage chemotaxis mediated by CCL2–CCR2**

To examine whether TAK-779 can block mCCR2, an in vitro chemotaxis assay was performed. Consistent with in vivo results, TAK-779 inhibited thioglycollate-elicited (96-h post-i.p. injection) peritoneal cell migration in response to CCL2 (10^{-8} M) in a dose-dependent manner (Fig. 2). Importantly, inhibition of CCL2 to mCCR2 was achieved at the 50% inhibitory concentration (IC_{50}) of 24 nM, suggesting that TAK-779 antagonizes mCCR2 10 times more efficiently than mCCR5 or mCXCR3 (IC_{50}: 236 nM for mCCR5 and 369 nM for mCXCR3) (16).

**TAK-779 protects mice from DSS-induced colitis**

In order to evaluate the effect of TAK-779 chemokine receptor blockade on the etiology of colitis, mice were administered DSS (5%) in the drinking water with or without the concomitant administration of TAK-779. A preliminary study established the optimal dose of TAK-779 to be 50 mg kg^{-1} body weight (data not shown). There was a significant amelioration in the severity of colitis in mice treated with this dosage (Fig. 3). Mice injected with TAK-779 without DSS treatment (Water/TAK-779 group) did not exhibit significant clinical symptoms, suggesting that TAK-779 was not toxic at this dose (data not shown). On the other hand, DSS alone (DSS/PBS group) induced severe hemorrhagic colitis and caused diarrhea and bloody stools within 2 days of treatment (Fig. 3C and D). There was a significant difference in disease activity index between the DSS/PBS and DSS/TAK-779 groups until day 5 (P < 0.01 on days 3 and 4, P < 0.05 on day 5, Fig. 3A). After 5 days of DSS treatment, the DSS/PBS mice lost 16% of their body weight (83.9 ± 4.6 of their original weight, Fig. 1B). In contrast, the TAK-779-treated mice (DSS/TAK-779 group) remained active and their body weight was 90.0 ± 6.1% of their original weight on day 7. These results suggest that TAK-779 delays the onset and reduces the severity of DSS-induced colitis (Fig. 3A).

**Macrosopic examination of the colon**

Four mice per group were sacrificed on days 1, 3, 5 and 7. Their colons were removed and examined macroscopically. DSS treatment caused severe hemorrhagic colitis and a shortening of the colon (Fig. 4). Colonic involvement was diffuse in the DSS/PBS group as evidenced by both gross

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**Fig. 1.** In vivo inhibitory effect of TAK-779 on thioglycollate-induced peritonitis. Mice (three mice per group) received i.p. injections of thioglycollate (2 ml, 4% solution) to induce peritonitis. Recruitment of leukocytes into the peritoneal cavity was analyzed by peritoneal lavage 96 h after i.p. injection of thioglycollate with or without concomitant injection of TAK-779. The total number of leukocytes recovered from mice were counted, and cells were then stained with antibody against CD11b and F4/80, followed by flow cytometry analysis. CD11b+/F4/80+ cells were identified as macrophages and the percentage of CD11b+/F4/80+ cells was multiplied by total cell number to obtain the number of peritoneal macrophages. Results are expressed as the mean ± SD. *P < 0.05.

**Fig. 2.** TAK-779 inhibits chemotaxis in response to CCL2. Thioglycollate-elicited peritoneal cells were harvested 96 h after thioglycollate injection and incubated with increasing concentrations of TAK-779. The number of cells migrating in response to CCL2 (10^{-8} M) was determined in triplicate, and percent chemotaxis was calculated by dividing the number of the cells that migrated into the lower chamber in the presence of TAK-779 by that in the absence of TAK-779 (percent inhibition of chemotaxis = 100− % chemotaxis). Data are presented as the mean ± SD.
examination and an analysis of multiple sections from different colonic regions. Colon length progressively shortened and luminal bleeding was observed in the DSS/PBS-treated mice over the course of the study (Fig. 4). Though there was no statistical significance, the severity of changes in the gross appearance and in luminal bleeding was significantly decreased and the colon length was maintained in the DSS/TAK-779-treated mice, suggesting that TAK-779 ameliorated DSS-induced inflammation in the colonic mucosa (Fig. 4).

Histological examination of the colon

Histological examination confirmed that TAK-779 treatment diminished the severity of colonic inflammation and ulceration. A semi-quantitative histological analysis of DSS-induced colitis is summarized in Fig. 5. The colonic mucosa of TAK-779-treated mice was characterized by a diminished inflammatory infiltrate and ulceration on day 3 and day 5 (P < 0.01, Fig. 5A and B). The difference between the TAK-779-treated and control mice was statistically significant on days 3 and 5, whereas the TAK-779-treated mice developed severe inflammation and ulceration comparable to that of control mice by day 7 (Fig. 5).

Disease progression correlated well with changes in crypt morphology and with the extent of inflammation. There were no pathological changes in the colons of the Water/TAK-779-treated mice (Fig. 6A, b and e), suggesting that TAK-779 was not toxic at the dose employed. A loss of the basal one-third of the crypts was observed by day 3 in the DSS/PBS-treated animals (Fig. 6A, c). Furthermore, there was a segmental loss of epithelial cells accompanied by a mild inflammatory infiltrate of neutrophils, lymphocytes and monocytes/macrophages into the lamina propria and sub-mucosa (Fig. 6A, c). The pathologic changes became more evident by day 5 with the appearance of erosions, the loss of entire crypts and diminished surface epithelium (Fig. 6A, f). Both the lamina propria and the sub-mucosa were characterized by severe infiltration of inflammatory cells. In contrast, the crypts were relatively patent and few inflammatory cells were observed in the lamina propria of TAK-779-treated mice. Although ulcers and erosions were observed by day 5, many areas exhibited normal morphology and the percentage of ulcerated tissue was significantly less in the TAK-779-treated mice in comparison with that in the DSS/PBS-treated animals (Fig. 6A, d and g).

Immunohistochemical examination of the colon

TAK-779 inhibits ligand binding to CCR2, CCR5 and CXCR3, all of which are expressed during mucosal inflammation and modulate the mucosal inflammatory immune response.

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**Fig. 3.** Effects of TAK-779 on DSS-induced colitis. Mice (20 mice per group) received DSS in their drinking water for 7 days (5%, w/v). We compared four groups of animals: untreated (Water/PBS; drinking water and PBS injection, data not shown), Water/TAK-779 (drinking water and TAK-779 injection, data not shown), DSS/PBS (oral DSS treatment and PBS injection) and DSS/TAK-779 (oral DSS treatment and TAK-779 injection). The severity of the disease was scored blindly as the disease activity index (A). Colitis severity was assessed using the following parameters: (B) body weight, (C) diarrhea score and (D) fecal blood score. Parameters are reported as the mean ± SE. *P < 0.05 and **P < 0.01. The data presented are representative of three individual experiments.
Therefore, we examined whether or not the diminished severity of DSS-induced colitis in TAK-779-treated mice reflected alterations in the composition of the immune cell populations in the intestinal mucosa. DSS-induced colitis in mice is associated with an influx of monocytes/macrophages into the intestinal mucosa. Therefore, we assessed monocytes/macrophages infiltration into the lamina propria by immunohistochemistry (Fig. 6B). DSS-induced mucosal inflammation is characterized by a significant increase in the number of CD11b+ cells, whereas the infiltration of these cells into the mucosa is markedly inhibited in the TAK-779-treated mice, an observation consistent with the mild clinical symptoms (Fig. 6B, f and g). Additional analyses were performed using antibodies directed against CD4, CD8, NKG2A/C/E and B220 to further characterize lymphocytes within the intestinal mucosa (Fig. 8). CD11b+ cells were the most prominent in the intestinal mucosa of DSS-treated mice. This sub-population of cells was also positive for anti-mouse Gr-1, but not for anti-mouse I-A/I-E antibodies, suggesting that they were CD11b+ monocytes/macrophages (data not shown).

Quantitative morphometric analysis of the number of CD11b+ monocytes/macrophages infiltrating into the colonic mucosa
was performed by counting 100 h.p.f.'s (original magnification: 3200) representing 0.12 mm² of colonic mucosa (Fig. 7). TAK-779 significantly reduces the number of recruitment of CD11b+ monocytes/macrophages into the colonic mucosa by day 3 and day 5 after DSS treatment, as measured by quantitative morphometric analysis [32.5 ± 13.3 (day 3) and 172.0 ± 16.9 (day 5) in DSS/PBS mice versus 12.1 ± 3.8 (day 3) and 16.0 ± 7.6 (day 5) in DSS/TAK mice, P < 0.01]. These data suggest that TAK-779 inhibited the recruitment of CD11b+ monocytes/macrophages into the colonic mucosa. In addition to CD11b+ cells, the recruitment of CD4+ cells into the mucosa was inhibited by TAK-779 (P < 0.01, Fig. 8). In contrast, the recruitment of CD8+, NKG2A/C/E+ and B220+ cells was not inhibited by TAK-779 (Fig. 8).

Chemokine and chemokine receptor expression
Reverse transcription (RT)–PCR was used to assess intestinal chemokine mRNA expression during DSS-induced colitis since the migration of inflammatory cells into the intestinal mucosa is partly regulated by chemokines (Fig. 9). DSS caused strong and equivalent induction of CCL2 mRNA in both the DSS/PBS and DSS/TAK-779 groups by day 3 (Fig. 9B). However, TAK-779 treatment (DSS/TAK-779 group) inhibited the induction of CCR2 mRNA expression in

Fig. 6. Histopathological and immunohistochemical changes in the colon of DSS-treated mice with and without TAK-779 therapy. (A) Colons were harvested on days 3 (a–d) and 5 (e, f and g), sections were prepared and stained with H&E. Photomicrographs of the colon tissue from the water/PBS (a), water/TAK-779 (b and e), DSS/PBS (c and f) and DSS/TAK-779 (d and g) groups are shown. Magnification is ×200. There was a segmental loss of surface epithelium accompanied by inflammatory cell infiltration in the DSS/PBS group (c) in comparison with the TAK-779-treated group (d) by day 3. By day 5, the DSS/PBS group exhibited a greater loss of normal surface epithelium and increased inflammatory infiltrates in the lamina propria and sub-mucosa (f), while normal intestinal mucosal morphology was maintained in the TAK-779-treated group (g). (B) Cells positive for anti-CD11b antibody are brown. Note that the epithelium is well preserved in the TAK-779-treated mice with minimal recruitment of CD11b+ cells (d and g) in comparison with the absence of luminal epithelium and marked recruitment of CD11b+ cells into the lamina propria and sub-mucosa in the DSS/PBS group (c and f).
comparison with the DSS/PBS-treated mice (Fig. 9A). DSS strongly induced the CCR5 ligands, i.e. CCL3, CCL4 and CCL5. DSS-treated mice exhibited 10-, 4- and 2.5-fold increases in CCL3, CCL4 and CCL5 mRNA expression by day 3, respectively, in comparison with untreated mice (Fig. 9D, E and F). By day 5, these mRNA levels were elevated 110-, 40- and 15-fold, respectively (Fig. 9D, E and F). Consistent with the increases in these chemokines, the level of their receptor mRNA increased 2.5-fold on day 5 (Fig. 9C). However, CCR5 mRNA expression was much more muted in the TAK-779-treated animals. These findings are consistent with diminished inflammatory cell recruitment into the colons of these mice (Figs 6, 7 and 8). Despite the fact that CXCL9 and CXCL10 mRNA levels were rapidly and strongly induced by DSS treatment, the expression of their chemokine receptor, CXCR3, was comparable to that of the control animals (Fig. 9G, H and I). TAK-779 treatment reduced the levels of CCL2, CCL3, CCL4 and CCL5 mRNAs by day 5, suggesting that a blockade of chemokine receptors (CCR2 and CCR5) perturbed the infiltration of inflammatory cells into the colonic mucosa, thus terminating the amplification cascade of intestinal inflammation and reducing the induction of their ligands, i.e. CCL2, CCL3, CCL4 and CCL5.

The effect of TAK-779 on colonic expression of cytokines

In order to examine the pattern of cytokine expression in the DSS-treated animals, we also performed RT–PCR using RNA
Fig. 9. Expression of cytokines, chemokines and their receptor mRNAs in the colons of DSS-treated mice. Twenty mice per group were treated as described in Fig. 1. Mice (four mice per group) were sacrificed at the indicated times and the colon tissues from four animals per group were harvested and pooled. Whole colonic RNA was isolated, reverse transcribed into cDNA and the expressions of CCR2, CCL2, CCR5, CCL3, CCL4, CCL5, CXCR3, CXCL9, CXCL10, IFN-γ, IL-4, IL-10, TNF-α, IL-1β and IL-6 were determined by real-time quantitative PCR. The amounts of mRNAs were normalized to the levels of GAPDH at each time point. The normalized mRNA values were multiplied by 10,000 and presented as an mRNA index. PCR was performed in triplicate for each experiment. There were no significant increases in mRNA expression in either the untreated (Water/PBS) or control (Water/TAK-779) groups (data not shown). The amount of mRNA is expressed as an increase over the control values in the DSS/PBS (filled circle) and DSS/TAK-779 (open circle) mice. The data presented are representative of three individual experiments.
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derived from whole colonic tissue. T₃₁-type cytokine (IFN-γ) mRNA expression was rapidly induced after DSS treatment. Following normalization to GAPDH levels, the DSS/PBS-treated animals exhibited 2-, 5- and 7-fold increases in IFN-γ mRNA in the intestinal mucosa after 1, 3 and 5 days of DSS, respectively (Fig. 9J). TAK-779 treatment did not diminish IFN-γ mRNA expression. In fact, an even higher level of expression was observed on days 1 and 3 (Fig. 9J). T₃₂-type cytokines (IL-4 and IL-10) were also detected, and increased 2-fold by day 5 (Fig. 9K and L). While TAK-799 transiently induced IL-4 mRNA on day 1, IL-10 mRNA expression was similar to that observed in the DSS/PBS mice (Fig. 9K and L). Interestingly, the expression of both T₃₁ (IFN-γ) and T₃₂ (IL-4) mRNA was up-regulated by TAK-779. However, the amount T₃₁ and T₃₂ cytokine mRNA was not abundant.

In contrast to the minimal expression of T₃₁ and T₃₂ cytokines, proinflammatory cytokines were vigorously induced by DSS. All of the DSS-treated mice exhibited increased TNF-α, IL-1β and IL-6 mRNA expression by day 3, and these levels subsequently underwent marked increases by day 5, concomitant with the increased severity of the disease (Fig. 9M, N and O). Although TNF-α mRNA expression in the DSS/TAK-779 mice was comparable to that observed in the DSS/PBS animals, TAK-779 strongly inhibited the expression of IL-1β and IL-6 mRNAs (Fig. 9M, N and O). Perhaps more importantly, the expression of IL-1β and IL-6 mRNAs was 10-fold less in the TAK-779-treated mice compared with the DSS/PBS animals (Fig. 9N and O). Cumulatively, these data suggest that TAK-779 protects mice from severe colitis by inhibiting the induction of proinflammatory cytokines in the intestinal mucosa.

Discussion

We employed the well-established murine model of DSS-induced colitis to assess the role of chemokine receptors in the pathogenesis of this inflammatory disease. We demonstrated that the simultaneous blockade of CCR2, CCR5 and CXCR3 by a non-peptide chemokine receptor antagonist, TAK-779, was associated with an ameliorative effect on the severity of colitis by inhibiting the recruitment of monocytes/macrophages into the mucosa and, thereby, reducing the expression of proinflammatory cytokines.

Although the effect of TAK-779 on the mouse chemokine system differs greatly from that reported in humans, TAK-779 has been reported to be an effective inhibitor of T cell migration in the mouse model (22, 23). TAK-779 inhibits both mCCR5 and mCXCR3, as well as human CCR5 and CCR2 (16). However, IC₅₀ values for mCCR5 and mCXCR3 were 236 and 369 nM, respectively, suggesting that the IC₅₀ value for mCCR5 is 100-fold greater than that measured for human CCR5 (15). In this study, we demonstrated that TAK-779 inhibited mCCR2 more efficiently than mCCR5 or mCXCR3. The IC₅₀ value for mCCR2 was 24 nM, which is comparable to that of human CCR5 and CCR2b (the IC₅₀ values for human CCR5 and CCR2b were 1.4 and 27 nM, respectively). Therefore, TAK-779 is believed to inhibit monocyte/macrophage migration, rather than that of T cells and NK cells, in mouse models. However, based on the projected differences in sensitivity to TAK-799, we anticipate that much less inhibitor may be equally effective in humans.

Continuous oral administration of DSS results in colitis characterized by mucosal ulceration and the accumulation of monocytes/macrophages, neutrophils and lymphocytes in the lamina propria and the sub-mucosa (24). DSS is thought to induce colitis by interfering with intestinal epithelial cell barrier function, and/or stimulating regional colonic inflammation by up-regulating both T₃₁ cytokines and inflammatory mediators (25). Since TAK-779 blocks mCCR2, which is important for the macrophage recruitment, and mCCR5 and mCXCR3, both of which are associated with T cell migration to sites of inflammation, we examined whether or not TAK-779 can ameliorate the excessive inflammatory response in DSS-induced colitis.

DSS-induced colitis was accompanied by marked inductions of CCL2, CCL3, CCL4 and CCL5 mRNA expression and enhanced monocyte/macrophage infiltration into the colonic mucosa. These inflammatory events contribute to the induction of TNF-α, IL-1β and IL-6 in the colon and the subsequent tissue damage associated with colitis (Fig. 9). Therefore, the recruitment and activation of monocytes/macrophages appear to be a critical step in the pathogenesis of DSS-induced colitis (24). These events appear to constitute a self-amplification process wherein activated monocytes/macrophages secrete proinflammatory cytokines that recruit additional inflammatory cells, including more monocytes/macrophages, into the intestinal mucosa which, in turn, stimulates factors that cause severe tissue damage. TAK-779 treatment interrupts this inflammatory sequence by inhibiting CCR2, CCR5 and CXCR3 expression, thus ameliorating DSS-induced colitis. Our data demonstrating reduced expression of IL-1β and IL-6 on day 5 in TAK-779-treated mice substantiate our hypothesis.

Our data also suggest that although CCR2 appears to be primarily responsible for colonic mucosal injury induced by DSS, both CCR5 and CXCR3 are involved as well. As shown in Fig. 8, the level of mucosal infiltration of CD4⁺ cells in TAK-779-treated mice was reduced compared with control animals. Whereas macrophage infiltration into the lesion was extremely suppressed in TAK-779-treated animals, the clinical scores were approximately the same as control animals on day 7 (Fig. 3). This is partly because the TAK-779 could not inhibit mCCR5 or mCXCR3 as efficiently as mCCR2. The recruitment of CCR5* and CXCR3* cells was suggested by mRNA expression at later time points (Fig. 9). Consistently, IFN-γ and TNF-α mRNAs were induced in TAK-779-treated mice. CCR5 and CXCR3 may not be directly responsible for macrophage migration into the colonic mucosa, but both chemokine receptors are known to regulate the migration and differentiation of mucosal T cells (3, 4). Our data suggest that these intestinal T cells may contribute to this inflammatory response by regulating the recruitment of monocytes and other inflammatory cells into the colon mucosa and/or their subsequent activation.

Andres et al. (18) reported that CCR5-deficient and, to a lesser degree, CCR2-deficient mice were protected from DSS-induced colitis. However, the lack of CCR2 or CCR5 did not reduce the DSS-induced migration of monocytes/macrophages into the colonic lamina propria (18). Sasaki et al.
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reported that the neutralization of CXCL10 protected against epithelial ulceration in mice with DSS-induced colitis without inhibiting macrophage infiltration into the intestinal mucosa (26). These reports suggest that the blockade of a single chemokine or chemokine receptor is not sufficient to inhibit the infiltration of inflammatory cells into the intestinal mucosa. On the other hand, we demonstrated that TAK-779 inhibits the recruitment and activation of monocytes/macrophages in DSS-induced colitis, suggesting that simultaneously blockade of multiple and functionally related chemokine receptors is necessary for the inhibition of this process.

DSS-induced colitis is ameliorated in CCR5- and CCR2-deficient mice due to the shift from a Th1- to a Th2-type immune response (18). However, pathogenetic contributions from T cells appear to be secondary in DSS-induced colitis since T and B cell-deficient SCID mice develop disease in response to DSS (27). In this study, we induced colitis by 5% DSS, compared with 2.5% DSS used in the study by Andres et al. (18). Direct toxic effects on colonic epithelium might be more important in our model, and this might explain why the shift from a Th1- to a Th2-type immune response was not evident, and why the disease progressed even though the recruitment of inflammatory cells was extremely suppressed.

Clinical signs of colonic injury in response to DSS were not apparent for the first 3 days of TAK-779 treatment (Fig. 3A). This indicates that treatment with TAK-779 prevents murine experimental colitis by inhibiting the recruitment of inflammatory cells. However, the severity of the disease in DSS/TAK-779 mice was similar to that observed in DSS/PBS mice by day 7 (data not shown). This may reflect the possibility that other pathways of intestinal inflammation are involved or that alternative pathways were activated in response to DSS-induced epithelial cell damage and that such events masked the inhibitory effect of TAK-779. The identification of additional therapeutic targets for intestinal immune regulation will require a better understanding of the pathogenesis of IBD.

TAK-779 could not prevent or ameliorate established colitis in our study (data not shown). However, this does not imply that TAK-779 was not effective on established colitis. It is difficult to examine the effect of TAK-779 on established colitis. Once the disease is induced by DSS, direct toxic effects on colonic epithelium are intensively involved in our model. Thus, inhibiting the recruitment of inflammatory cells by TAK-779 might not be sufficient to ameliorate the colitis. Furthermore, once colitis is established, many inflammatory cells are recruited into the mucosa and secrete many cytokines and chemokines in addition to the ligands for CCR2, CCR5 and CXCR3. Many cannot be inhibited by TAK-779. Nevertheless, we clearly demonstrated that TAK-779 inhibited the recruitment of macrophages into the colon, resulting in the reduction of the proinflammatory cytokines in the colonic mucosa. Based on these results, we believe that TAK-779 can be used to prevent the exacerbation of IBD.

In conclusion, our data demonstrate that the blockade of CCR2, CCR5 and CXCR3 signaling protects mice from severe intestinal inflammation and mucosal damage associated with DSS-induced colitis. Therapeutic strategies for IBDs, especially for Crohn’s disease, target the inhibition of individual molecular events in the pathogenesis of mucosal inflammation, including the cytokine cascade, chemokines and integrins. Recent clinical trials using neutralizing antibodies directed against TNF-α, IL-6R and a4 integrins for the treatment of Crohn’s disease appear promising (28–31). We also demonstrated that the blockade of chemokine receptors can modulate intestinal inflammation in a murine model of IBD. Therefore, in order to develop effective strategies for the treatment of IBD, the therapeutic targets must be determined by identifying each molecule responsible for dysregulating the intestinal immune response in individual patients.

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Abbreviations

DSS dextran sodium sulfate
h.p.f. high-powered-microscope field
IBD inflammatory bowel disease
IC50 50 % inhibitory concentration
i.p. intraperitoneally
RT reverse transcription
TAK-779 N,N-dimethyl-N-[4-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-yl]carbonyl]amino]benzyl]-tetrahydro-2H-pyran-4-aminium chloride
TNF tumor necrosis factor

References


