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Phenyl methimazole suppresses dextran sulfate sodium-induced murine colitis

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ABSTRACT

Ulcerative colitis is an autoimmune-inflammatory disease characterized by abnormally increased expression of Toll-like receptor-4 (TLR4) in colonic epithelial cells, increased production of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6, IL-12), chemokines (e.g., IP-10), and endothelial cell adhesion molecules (e.g., VCAM-1), plus enhanced leukocyte infiltration into colonic interstitium. Previously, we have shown that phenyl methimazole (C10) markedly decreases virally-induced TLR-3 expression and signaling and potently inhibits both TNF-α-induced VCAM-1 expression and the resultant leukocyte-endothelial cell adhesion. In this study we probed the hypothesis that C10 is efficacious in a TLR-4- and VCAM-1-associated murine model [the dextran sulfate sodium (DSS) model] of human colitis. C10 was administered intraperitoneally coincident with or after DSS treatment was initiated. Macroscopic colon observations revealed that C10 significantly reversed DSS-induced shortening of the colon ($P<0.05$) and reduced the presence of blood in the colon. Histological analyses of colonic tissues revealed that C10 distinctly attenuated both DSS-induced edema as well as leukocyte infiltration in the colonic mucosa and resulted in pronounced protection against DSS-induced crypt damage ($P<0.001$). Northern blot analyses and immunohistochemistry of colonic tissue revealed that C10 markedly diminished DSS-induced expression of pertinent inflammatory mediators: TNF-α, IL-1β, IL-6, IL-12, IP-10, TLR-4 and VCAM-1. Most importantly, C10 significantly improved survival and protected mice against DSS-induced colitic-death: 75% by comparison to 12.5% with identical treatment with DMSO-control (log rank test: $P=0.005$). These results provide direct evidence that C10 suppresses DSS-induced colitis by inhibiting expression of key inflammatory mediators and leukocyte infiltration, and is a potentially attractive therapeutic for colitis.

1. Introduction

Ulcerative colitis is an auto-immune/inflammatory disease affecting millions of people worldwide. Neither the initiating event nor the sequence of propagating events that lead to and sustain colitis have been fully elucidated (Grisham and Granger, 1999). Nevertheless, it is increasingly clear that a dysfunctional immune-response, involving Toll-like receptor 4 (TLR4) and components of normal gastrointestinal gram-negative bacteria, play a key role in the pathogenesis of colitis (Cario and Podolsky, 2000). Thus, an early step is macrophage antigen presentation (Grisham and Granger, 1999), a process involving the CD14/TLR-4 complex, which leads to interferon (IFN) production and release, as well as T lymphocyte secretion of IL-2. IFNs activate macrophages to produce a variety of cytokines, including TNF-α, IL-1, IL-6 and IL-12 (Ashwood et al., 2004; Atreya et al., 2000; Simpson et al., 1998; Stucchi et al., 2006; Yamamoto et al., 2000) that upregulate endothelial cell adhesion molecules. Chemokines such as IP-10 are also important in colitis (Singh et al., 2003) and have been implicated in studies of the myeloid cell-specific Stat3-deficient mouse, which is one of several experimentally induced, Th1-mediated models of Crohn's disease and ulcerative colitis. The Stat3-deficient mouse model also implicated TLR-4 in disease expression, defective IL-10 signaling, and aberrant production of IL-12p40 (Kobayashi et al., 2003). Pro-inflammatory cytokines activate leukocytes and induce increased expression of endothelial cell adhesion molecules (Carlos and Harlan, 1994; Luscinskas and Gimbrone, 1996) leading to leukocyte recruitment and chemokine-mediated extravasation via the well described multi-step adhesion cascade (Springer, 1994). Thus, in sum, the aberrant immune/inflammatory response is

Abbreviations: C10, phenyl methimazole (compound 10); DMSO, dimethyl sulfoxide; DSS, dextran sulfate sodium; IL-1β, interleukin-1β; IL-2, interleukin-2; IL-6, interleukin-6; IL-12, interleukin-12; IP-10, interferon inducible protein-10; PBS, phosphate buffered saline; TLR-3, toll like receptor-3; TLR-4, toll like receptor-4; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

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characterized by increased expression of TLR-4, pro-inflammatory cytokines, chemokines, endothelial cell adhesion molecules and enhanced leukocyte infiltration into colonic interstitium.

The above observations have led to therapeutic approaches that seek to diminish colitis (and related diseases) by attenuating the immune/inflammatory response. Indeed, in various experimental models of acute and/or chronic colitis (i) blockade of TLR-4 function (Fort et al., 2005), (ii) specific inhibition of TNF-α expression (Assi et al., 2006), (iii) chronic suppression of endothelial cell adhesion molecule (e.g., VCAM-1) function (Soriano et al., 2000a), (iv) diminution of endothelial cell adhesion molecule (e.g., VCAM-1) expression at transcriptional level (Conner et al., 1997), and (v) attenuation of leukocyte-endothelial interactions (Zhang et al., 2001) separately provide a beneficial effect.

Previously, we have shown that phenyl methimazole (compound 10; C10), a novel tautomeric cyclic thione (Kohn et al., 2002a; Singer et al., 1996), markedly reduces virally induced TLR-3 expression and signaling (Harii et al., 2005) and potently inhibits TNF-α-induced VCAM-1 expression at transcriptional level and resultant leukocyte-endothelial cell adhesion under *in vitro* conditions (Dagia et al., 2004). However, no *in vivo* studies have been performed to assess the anti-inflammatory role of C10. The *in vitro* efficacy of C10 in reducing pathological inflammation, the fact that TLRs, TNF-α, and VCAM-1 have been implicated in the pathogenesis of colitis (Grisham and Granger, 1999; Soriano et al., 2000a), combined with the observation that therapies uniquely targeting these molecular effectors and/or the inflammatory adhesion cascade have proved quite successful in reducing the severity of experimental colitis (Assi et al., 2006; Conner et al., 1997; Fort et al., 2005; Soriano et al., 2000a; Zhang et al., 2001), led us to probe the use of C10 as a therapeutic for colitis.

For this study, we used the DSS-induced murine model of acute colitis, a model which is well-recognized and known to mimic the pathologic features of human colitis (Lange et al., 1996). This model is characterized by dysregulated inflammatory response indicated by presence of edema, infiltration of inflammatory cells, and extensive mucosal damage (Lange et al., 1996). We established this model in our laboratory (Sakhalkar et al., 2005) and used it to assess the efficacy of C10 on the gross pathology of colitis as well as on the expression of key receptors, cytokines, chemokines and endothelial cell adhesion molecules that have been implicated in the pathogenesis of colitis.

2. Materials and methods

2.1. Induction of colitis and C10 treatment

Male C57BL/6J mice (6 weeks of age, weighing 18–22 g) were obtained from Jackson Laboratories (Bar Harbor, Maine) and maintained in conventional housing conditions. All experiments were carried out in accordance with "Guide for Care and Use of Laboratory Animals" (NIH Publication No. 85-23, revised 1985) and with approval of Ohio University Animal Care and Use Committee. Colitis was induced in mice by giving 3% (wt/vol) DSS (MW 30–40 kDa; ICN Biomedicals, Aurora, OH) in drinking water ad libitum as reported by others (Soriano et al., 2000a; Lange et al., 1996; Bendjelloul et al., 2000) and established in our laboratory (Sakhalkar et al., 2005). Importantly, 30–40 kDa DSS was utilized in this study for it is known to induce more severe colitis than 5 kDa DSS and 500 kDa DSS (Kitajima et al., 2000). DSS-induction of colitis was manifested with increases in clinical disease activity index associated with weight loss, rectal bleeding, diarrhea and presence of blood in feces (Sakhalkar et al., 2005) and not shown). DSS-induced colitis was assessed by macroscopic and histological analyses of the colon (described below). To probe the efficacy of C10, a group of mice were given daily intraperitoneal (i.p.) injections of 1 mg/kg or 2.5 mg/kg or 5 mg/kg or 10 mg/kg C10 (Ricerca Inc., Cleveland, OH). Preliminary experiments revealed that C10 administered at 5 mg/kg was most effective (data not shown). Accordingly, all subsequent experiments (described in this report) were performed with 5 mg/kg C10. As a control for C10 treatment, a separate group of mice received daily i.p. injections of 2.5% DMSO (Sigma Aldrich). In some experiments, groups of mice received daily i.p. injections of methimazole (Sigma Aldrich, St. Louis, MO), prednisolone (Sigma Aldrich), or phosphate buffered saline (PBS; Biofluids, Rockville, MD; carrier control for methimazole and prednisolone).

2.2. Macroscopic colon assessment

At the end of DSS treatment period, mice were killed by cervical dislocation. The whole colon (i.e., including caecum, proximal colon and distal colon) was excised. The colon was macroscopically assessed by determining (a) the presence or absence of blood and (b) the longitudinal length. Subsequently, the whole colon was divided into ceacum, proximal colon and distal colon for ELISA and histological, Northern blot, and immunohistochemical analyses. The presence or absence of blood (in caecum, proximal colon and distal colon) was scored as either 1 or 0, respectively.

2.3. Histological analysis of colon

Colon biopsies were fixed in 4% formalin, dehydrated in serial alcohol, cleared in chloroform and embedded in paraffin. Sections were stained with Harris's hematoxylin (Fisher Diagnosis, Middletown, VA) and eosin, and histopathologically scored, by an investigator blinded to the treatment, for severity of inflammation (score: 0, none; 1, mild; 2, moderate; and 3, severe), extent of inflammation (score: 0, none; 1, mucosal; 2, mucosal and submucosal; and 3, transmural) and crypt damage (score: 0, none; 1, basal 1/3; 2, basal 2/3; 3, crypts lost but surface epithelium present and 4, crypts and surface epithelium lost). This scoring criteria is similar to that described by others (Mabley et al., 2003).

2.4. Northern blot analysis

Northern blot analysis was used to characterize the mRNA levels of key inflammatory mediators involved in the pathogenesis of colitis. RNA was extracted from colonic tissue sections using Trizol (Invitrogen, Carlsbad, CA) and subjected to Northern blot analysis in a manner similar to that described previously (Dagia et al., 2004; Suzuki et al., 1999). The G3PDH cDNA was from Clontech (Palo Alto, CA). mTNFα and mTLR4 cDNA were excised from pORF9-mTNFα and pUNO-mTLR4 vectors (Invivogen, San Diego, CA). Other probe sequences were synthesized by RT-PCR (Suzuki et al., 1999) using the following cDNA specific primers: human VCAM-1, forward: GACTCCGTCTCATTGACTTGCAGCACCACAG, backward: ATACTCCCGCATCCTTCAACTGGGCCTTTCG; mouse IP-10, forward: CCATCAGCACCATGAACCCAAGTCCTGCCG, backward: GGACGTCCTCCTCATCGTCGACTACACTGG; mouse IL-1β, forward: CTCATCTGGGATCCTCTCCAGCCAAGCTTC, backward: CCATGGTTTCTTGTGACCCTGAGCGACCTG.

2.5. Immunohistochemistry

Immunohistochemistry was used to characterize VCAM-1 and TLR-4 protein expression in a manner similar to that described by others (Cario and Podolsky, 2000; Ortega-Cava et al., 2003). For characterizing VCAM-1 expression, a rat mAb to mouse VCAM-1 (429MVCAMA; IgG2aκ; BD Pharmingen, San Diego, CA) and FITC-conjugated anti-rat sera (Zymed Laboratory, San Francisco, CA) were used. For characterizing TLR4 expression, a murine biotin-conjugated mAb to human TLR4 (HTA125; IgG2a; IMGENEX, San Diego, CA), extravidin peroxidase conjugate (Sigma) and DAB solution [1 mg/ml DAB (3,3′diamonobencidine, Sigma) and 1.2 μl H2O2 in PBS] were used.