Mesalamine Restores Angiogenic Balance in Experimental Ulcerative Colitis by Reducing Expression of Endostatin and Angiostatin: Novel Molecular Mechanism for Therapeutic Action of Mesalamine

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ABSTRACT

Mesalamine (5-aminosalicylate acid, 5-ASA) is an effective treatment for ulcerative colitis (UC). The mechanisms of its actions are not fully understood. Because angiogenesis is critical for healing UC, we examined whether 5-ASA alters the angiogenic balance between angiogenic factors [e.g., vascular endothelial growth factor (VEGF)] and antiangiogenic factors (e.g., endostatin and angiostatin) in the colon in experimental UC. Rats were treated with saline or 5-ASA (100 mg/kg) twice daily and euthanized 3 or 7 days after iodoacetamide-induced UC. Clinical signs (e.g., lethargy, diarrhea) and UC lesions were measured. Expression of VEGF, endostatin, angiostatin, tissue necrosis factor α (TNF-α), and matrix metalloproteinases (MMPs) 2 and 9 was determined by Western blots, enzyme-linked immunosorbent assay, and zymography in the distal colon. 5-ASA treatment reduced lethargy and diarrhea and significantly decreased colonic lesions (by ~50%) compared with saline treatment in UC (both, \( P < 0.05 \)). 5-ASA did not reverse the increased levels of VEGF, but it significantly reduced expression of endostatin and angiostatin in UC compared with vehicle treatment (both, \( P < 0.05 \)). Furthermore, 5-ASA treatment significantly diminished increased activity of TNF-α and MMP9 in UC. This is the first demonstration that 5-ASA treatment reverses an imbalance between the angiogenic factor VEGF and antiangiogenic factors endostatin and angiostatin in experimental UC. The effect of 5-ASA in UC may be caused by the down-regulation of expression of endostatin and angiostatin by modulation of MMP2 and MMP9 via inhibition of TNFα. The inhibition of antiangiogenic factors may represent a novel molecular mechanism of the therapeutic action of 5-ASA.

Angiogenesis—new blood vessel formation from existing vessels—is an essential component of ulcer healing because it ensures delivery of oxygen and nutrients to the healing site (Szabo et al., 2001; Folkman 2006). Previous studies demonstrated increased serum and tissue levels of vascular endothelial growth factor (VEGF), the most potent angiogenic growth factor in patients with active ulcerative colitis (UC) and in animal models of UC (Griga et al., 1998, 1999; Kanazawa et al., 2001; Sandor et al., 2006; Tolstanova et al., 2009). However, there was no rational explanation why the healing of UC-related mucosal injury is impaired despite increased expression of VEGF.

In our recent study, we examined the expression of angiogenic factor, VEGF, and antiangiogenic factors, endostatin and angiostatin, in two rat models of experimental UC (Sandor et al., 2006). We demonstrated that, in addition to increased VEGF, the levels of endostatin and angiostatin were also significantly increased (2- to 3-fold) in colonic mucosa at the early stage of experimental UC. This was the first demonstration that colitis triggers an imbalance between angiogenic factors VEGF and antiangiogenic factors endostatin and angiostatin in experimental UC.

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ABBREVIATIONS: VEGF, vascular endothelial growth factor; 5-ASA, mesalamine, 5-aminosalicylate acid; IA, iodoacetamide; UC, ulcerative colitis; MMP, matrix metalloproteinase; MC, methylcellulose; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tissue necrosis factor α; PPAR-γ, peroxisome proliferator-activated receptor-γ; ELISA, enzyme-linked immunosorbent assay.
increase in angiostatin and endostatin levels in the colon. Angiostatin and endostatin are 20- and 50-kDa fragments cleaved from plasminogen and collagen XVIII, respectively, by proteinases such as matrix metalloproteinases (MMPs) 2 and 9 (O’Reilly et al., 1994, 1997; Distler et al., 2003; Heljasaarva et al., 2005). Because they play antiangiogenic roles by inhibiting endothelial cell proliferation and migration, and by inducing apoptosis in proliferating endothelial cells (Claesson-Welsh et al., 1998; Taddei et al., 1999), the increased levels of endostatin and angiostatin may explain why the healing of colonic mucosal lesions decreased despite increased levels of VEGF, which promotes mucosal angiogenesis and healing of injury. This finding may provide a novel and mechanistic insight into UC.

Mesalamine (5-aminosalicylate acid, 5-ASA) is an effective drug for the treatment and maintenance of remission of UC (van Bodegraven and Mulder, 2006). The proposed mechanisms of anti-inflammatory action of 5-ASA currently include the inhibition of cyclooxygenase and lipoxygenase pathways, thus reducing production of prostaglandins and leukotrienes, respectively, and the reversion of the antiproliferative effects of tissue necrosis factor α (TNF-α), resulting in the disruption of the effect of cytokines by reducing intestinal cell transcription of inflammatory mediators (Kaiser et al., 1999). However, reduction of prostaglandin by 5-ASA cannot entirely explain its action, because nonsteroidal anti-inflammatory drugs, which inhibit synthesis of prostaglandin, exacerbate UC and impair healing. Recently, 5-ASA has been identified as a ligand of peroxisome proliferator-activated receptor-γ (PPAR-γ), and its therapeutic action in UC is mediated by PPAR-γ (Rousseaux et al., 2005). PPARs are members of the nuclear receptor superfamily. They are activated by fatty acids and are involved in the transduction of metabolic and nutritional signals into transcriptional responses (Walczak and Tontonoz 2002). Among these transcription factors, PPAR-γ plays an important role in the maintenance of mucosal integrity in the intestine (Dubuquoy et al., 2002). Several investigations have demonstrated that vascular endothelial cells express PPAR-γ mRNA and protein, which inhibits growth factor-induced proliferation of endothelial cells, induces endothelial apoptosis, increases plasminogen activator inhibitor-1 expression, and suppresses endothelin-1 secretion (Delerive et al., 1999; Xin et al., 1999; Panigrahy et al., 2002). Moreover, some data showed that activation of PPAR-γ decreased expression and activity of MMP2 and/or MMP9 in age-related macular degeneration and cultured mouse celiac macrophages (Hertzlich et al., 2008; Yao et al., 2009). The precise mechanisms and molecular targets of therapeutic actions of 5-ASA on inflammatory bowel disease are still not completely elucidated (Van Staa et al., 2004).

In this study, we aimed to determine whether 5-ASA could exert its therapeutic effect on UC through modulation of angiogenic (VEGF) and antiangiogenic factors (angiostatin and endostatin) and the potential mediators (e.g., TNF-α, MMP2, and MMP9) that may be involved in generation of angiostatin and endostatin. We investigated the expression of these factors in colon in a rat model of UC, and examined the effect of 5-ASA on the expression of these factors in development and healing of the experimental UC.
for 30 min followed by incubation at 37°C over night in Zymogram Developing Buffer (50 mM Tris base, 50 mM Tris acid, 0.2 mM NaCl, 5 mM CaCl₂, and 0.02 mM Brij). Substrate gels were stained in Coomassie Brilliant Blue (0.25%) in methanol/acetic acid/water (50: 10:40), and destained in the same solution without dye. Proteolytic activity was visualized as clear bands of lysis on a blue background of undigested gelatin. The molecular mass of the enzymes was determined by comparison with protein standards (Bio-Rad Laboratories) on the same gel.

Data Analysis. The statistical significance of differences among groups was calculated by use of the nonparametric Mann-Whitney U test. The data were shown as mean ± S.E.M. We selected a P value of <0.05 as statistically significant.

Results

Pooled results from several experiments demonstrated that 5-ASA treatment for 7 days, but not for 3 days, effectively accelerated colonic ulcer healing in experimental UC. 5-ASA treatment significantly reduced lethargy and diarrhea by 50% on the 7th day compared with saline treatment in UC rats (Fig. 1, A and B). The average areas of lesions and loss of rugae were significantly decreased by 5-ASA treatment ($P < 0.05$) on the 7th day after IA (Fig. 1, C and D). The average area of lesions and loss of rugae were also significantly reduced ($P = 0.007$ or $P = 0.037$, respectively) in the 5-ASA-treated groups compared with saline-treated groups (Fig. 1, E and F).

Western blotting demonstrated that expression of VEGF was significantly increased in saline-treated group 7 days (but not 3 days) after IA versus baseline control (Fig. 2, A and B), while 5-ASA treatment did not change the levels of VEGF on both 3 and 7 days after IA (Fig. 2, A and B). ELISA confirmed the preceding results of VEGF expression in the rat colon of both saline-treated and 5-ASA-treated groups (Fig. 2, C and D). Western blotting also demonstrated that levels of endostatin and angiostatin were significantly increased in saline-treated groups both 3 and 7 days after IA compared with basic line control (Fig. 3). In contrast, in rats treated with 5-ASA after IA, expression of endostatin was significantly reduced 75% by the 7th day (Fig. 3B), and expression of angiostatin was significantly reduced 75% by the 3rd day (Fig. 3C) and 95% by the 7th day (Fig. 3D) compared with rats treated with saline ($P < 0.05$).

Because MMP2 and MMP9 cleave collagen XVIII and plasminogen to generate endostatin and angiostatin, respectively (Distler et al., 2003; Heljasvaara et al., 2005), we first investigated the expression of MMP2 and MMP9 by Western blotting in the present study. The results showed that MMP2 expression was markedly decreased (Fig. 4, A and B) and, in contrast, MMP9 expression was markedly increased in the saline-treated groups both 3 and 7 days after IA compared with baseline controls (Fig. 4, C and D). Treatment with 5-ASA reversed the changes of MMP2 and MMP9 both 3 and 7 days after IA (Fig. 4).

Fig. 1. The effect of treatment with mesalamine (5-ASA) at 3 and 7 days on ulcerative colitis induced by iodoacetamide (IA) in rats. *, $P < 0.05$. Diarrhea (A), lethargy (B), and colonic lesions (C) are the parameters reflecting the severity of ulcerative colitis. Loss of rugae (D), colon wet weight (E), and colonic thickness (F) are the parameters reflecting the edema in colonic tissue.
Fig. 2. Expression of VEGF (23 kDa) measured by Western blotting (A and B) and ELISA (C and D) in rat colon after iodoacetamide-induced ulcerative colitis treated with saline or mesalamine (5-ASA).

Fig. 3. Expression of endostatin (20 kDa) (A and B) and angiostatin (50 kDa) (C and D) measured by Western blotting in rat colon after iodoacetamide-induced ulcerative colitis treated with saline or mesalamine (5-ASA).
Furthermore, we measured proteolytic activities of these MMPs by gelatin zymography. The results showed a significantly decreased gelatinolytic activity of MMP2 on the 3rd and 7th day (Fig. 5, A and B) and significantly increased MMP9 activity on the 7th day after IA (Fig. 5D), which confirmed the changes of MMP2 and MMP9 determined by Western blotting. Treatment with 5-ASA significantly reversed these changes (Fig. 5), indicating that 5-ASA promoted activation of MMP2 and inhibited or halted activation of MMP9.

It has been demonstrated that TNF-α/H9251 is important in the pathogenesis of inflammatory bowel disease, and inhibition of TNF-α/H9251 results in alteration of MMP2 and MMP9 levels in CD patients (Podolsky, 2002; Gao et al., 2007). Therefore, we detected the expression of TNF-α/H9251 and the effect of mesalamine on TNF-α-regulated MMP2 and MMP9 in the experimental UC. The results showed that TNF-α was significantly increased in the saline-treated groups both 3 and 7 days after IA compared with baseline controls (Fig. 6, A and B), whereas 5-ASA treatment significantly reversed the increase of TNF-α on 7 days (Fig. 6B) compared with salinetreated rats with UC.

Discussion

In this study, we demonstrated that both the angiogenic factor VEGF and antiangiogenic factors endostatin and angiostatin were significantly increased in the rat colon with experimental UC. The increased levels of endostatin and angiostatin were reversed significantly (75–95%) by 5-ASA treatment, whereas 5-ASA treatment did not change VEGF expression. Because 5-ASA could inhibit angiogenesis through up-regulating expression of PPAR-γ, which is a downstream inhibitor of VEGF-induced angiogenesis (Murata et al., 2000), it was not surprising that the levels of VEGF did not change. It is possible that increased VEGF and simultaneously up-regulated endostatin and angiostatin contribute to pathologic angiogenesis. This study indicates that the effectiveness of 5-ASA on UC healing may be directly related to down-regulation of the antiangiogenic factors endostatin and angiostatin in UC.

Because angiostatin and endostatin could be generated by MMP2 and MMP9 through cleavage of collagen XVIII and plasminogen (Distler et al., 2003; Heljasvaara et al., 2005), we examined the expression and activities of MMP2 and MMP9. We found that 5-ASA significantly reversed the decreased MMP2 and increased MMP9 levels in experimental UC. We have previously demonstrated increased MMP9 in colon of experimental UC induced by IA in rats and interleukin-10 knockout mice (Tolstanova et al., 2007). It is relevant that a recent clinical study also showed that therapy with infliximab (anti-TNF-α monoclonal antibody) increases MMP2 and decreases MMP9 in patients with Crohn’s disease (Gao et al., 2007).

MMPs, also called matrixins, can promote a proangiogenic Milikan by releasing extracellular matrix-bound growth factors and inhibit angiogenesis by impairing vessel matura-
tion, cleaving angiogenic factors, or generating antiangiogenic factors such as angiostatin (Gao et al., 2005). MMPs involved in angiogenesis or inflammation can be released from infiltrating inflammatory cells or from endothelial cells in response to cytokines during inflammation (Rundhaug, 2005; Greenlee et al., 2006). MMPs have been implicated as one of the main factors contributing to mucosal ulceration and inflammation. Gene array analysis and in situ hybridization have shown that MMP1, MMP3, and MMP9 are upregulated in inflamed colonic mucosa (Salmela et al., 2002, 2004).

Although MMP2 and MMP9 are both gelatinases that originate from similar epithelial sources and share structural and substrate similarities, it was demonstrated recently that they have opposing effects on the development of acute colitis (Garg et al., 2006). MMP9 is abundantly expressed in pa-

Fig. 5. Proteolytic activity of MMP2 (A and B) and MMP9 (C and D) by gelatin zymography in rat colon after iodoacetamide-induced ulcerative colitis treated with saline or mesalamine (5-ASA).

Fig. 6. A and B, expression of TNF-α by Western blotting in rat colon after iodoacetamide-induced ulcerative colitis treated with saline or mesalamine (5-ASA).
Mesalamine Restores Angiogenic Balance in Ulcerative Colitis


ser et al. (1999) demonstrated that 5-ASA blocked TNF-alpha activation in mouse colonocytes. This finding implicates that restored balance between MMP2 and MMP9 by 5-ASA in UC might be mediated by reduction of TNF-alpha, as demonstrated by Gao and his colleagues that inhibition of TNF-alpha increases MMP2 and decreases MMP9 in patients with Crohn’s disease (Gao et al., 2007). In addition, another study demonstrated that stimulation of CaCO-2 cells with TNF-alpha led to a dose-dependent increase in expression and secretion of MMP9 (Gan et al., 2001). Moreover, PPAR-gamma significantly reduces secretion of MMP9 in intestinal epithelial cells and human dental pulp cells, indicating that 5-ASA may also down-regulate MMP9 level in UC through PPAR-gamma (Gan et al., 2001; Yu et al., 2009).

Our present study indicates that effectiveness of 5-ASA on UC is directly related to its action on antiangiogenic factors, angiostatin and endostatin, and their generators, e.g., MMP2 and MMP9. Angiogenesis is governed by a balance between pro- and antiangiogenic factors (Pandya et al., 2006). Patho-

logic (abnormal) angiogenesis has been implicated to play a pathogenic role in inflammatory bowel disease and increased expression of VEGF is considered to play a central, specific role in the angiogenesis. (Koutrubakis et al., 2006). In this study, although the amelioration of experimental UC by 5-ASA treatment was not shown by a reduction of VEGF as our previous study (Tolstanova et al., 2009), it was possible that angiogenesis could be blocked by 5-ASA through up-regulating PPAR-gamma to inhibit mitogen-activated protein kinase in VEGF downstream pathway, resulting in the angiogenesis inhibition (Herzlchiu et al., 2008).

In summary, our study demonstrates that 5-ASA treatment reverses the imbalance between MMP2 and MMP9 and down-regulates levels of antiangiogenic factors endostatin and angiostatin in experimental UC. Therefore, the therapeu-
tic action of 5-ASA in UC may be due, at least in part, to its down-regulation of endostatin and angiostatin production, which is mediated by modulation of MMP2 and MMP9 activities. The inhibition of antiangiogenic factors may rep-


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