Anti-melanin-concentrating hormone treatment attenuates chronic experimental colitis and fibrosis.

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Running head: MCH and intestinal fibrosis

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Abbreviations: MCH, melanin concentrating hormone; MCHR1, MCH receptor1; IBD, inflammatory bowel disease; Ab, antibody; AU, arbitrary units; TNBS, 2,4,6-trinitrobenzene sulfonic acid; DSS, dextran sulfate sodium.
ABSTRACT

Fibrosis represents a major complication of several chronic diseases, including inflammatory bowel disease (IBD). Treatment of IBD remains a clinical challenge, despite several recent therapeutic advances. Melanin-concentrating hormone (MCH) is a hypothalamic neuropeptide shown to regulate appetite and energy balance. However, accumulating evidence suggests that MCH has additional biological effects, including modulation of inflammation. In the present study we examined the efficacy of an MCH blocking antibody in treating established, DSS-induced experimental colitis. Histological and molecular analysis of mouse tissues for inflammation and fibrosis revealed that mice receiving anti-MCH had accelerated mucosal restitution and lower colonic expression of several proinflammatory cytokines as well as fibrogenic genes, including COL1A1. In parallel, they spared collagen deposits seen in the untreated mice, suggesting attenuated fibrosis. These findings raised the possibility of perhaps direct effects of MCH on myofibroblasts. Indeed, in biopsies from patients with IBD, we demonstrate expression of the MCH receptor MCHR1 in alphaSMA(+) subepithelial cells. CCD-18Co cells, a primary human colonic myofibroblast cell line, were also positive for MCHR1. In these cells, MCH acted as a profibrotic modulator by potentiating the effects of IGF-1 and TGFbeta on proliferation and collagen production. Thus, by virtue of combined anti-inflammatory and anti-fibrotic effects, blocking MCH might represent a compelling approach for treating inflammatory bowel disease.

Keywords: melanin-concentrating hormone, inflammatory bowel disease, Crohn’s disease, ulcerative colitis, experimental colitis, chronic DSS colitis, intestinal fibrosis, myofibroblasts
INTRODUCTION

In many organs, including the intestine, chronic inflammation results in impaired healing and fibrosis, a process characterized by excessive synthesis and deposition of extracellular matrix components, including collagen. In parallel, there is an inhibition of degradation of extracellular matrix, resulting from an imbalance between the various matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) (38, 47). In inflammatory bowel disease (IBD), the manifestation of fibrosis reflects the distinct pathological characteristics of each subtype; however, in both Crohn’s disease (CD) and ulcerative colitis (UC), fibrosis represents a serious clinical concern, even after the remission of acute inflammation. In Crohn’s disease (CD), transmural granulomatous inflammation results to the formation of strictures, which can require surgical intervention. Fibrosis in ulcerative colitis is less pronounced and limited to the submucosa, leading to severe disruption of tissue architecture and failure of epithelial regeneration (37). TGFbeta, a profibrogenic factor, is upregulated in the intestine of patients with Crohn’s disease, in particular in the mucosa overlying strictures, along with TIMP1, and MMPs, in particular MMP3 and MMP12 (3, 13). TGFbeta signaling results to phosphorylation of Smad2 and Smad3, which in turn form a complex with Smad4 and translocate to the nucleus where they regulate transcription of several genes, including procollagen and fibronectin, extracellular matrix degrading enzymes (MMPs) and their inhibitors (TIMP1, TIMP6) as well as regulators of mesenchymal cell proliferation and apoptosis (17). Besides TGFbeta, IGF-1 and TNFalpha have also been shown to stimulate type I collagen synthesis and their expression is increased in collagen deposition areas in the intestine of patients with Crohn’s disease (36, 41).

Myofibroblasts are alpha-smooth muscle actin (alpha-SMA)(+), vimnetin (+) and desmin(-) mesenchymal cells in the lamina propria of the gut which secrete extracellular matrix components, along with cytokines and growth factors (35, 37). Though transient appearance of myofibroblasts contributes to mucosal healing, their sustained proliferation and activation in response to chronic inflammation leads to intestinal fibrosis. Indeed,
increased numbers of myofibroblasts were found in the affected mucosa of patients with Crohn’s disease and in mouse models of IBD (36).

It is estimated that 1.4 million patients suffer from IBD in the US alone, a chronic condition characterized by periods of remission and relapses. The clinical management of IBD, despite the revolutionizing use of biological therapies, remains problematic (30). For instance, treatment with anti-TNFalpha, the prototype of this class of therapeutics significantly improves the disease free time and the quality of life of patients with IBD. Meta-analyses of large clinical trials however indicate that less than 40% of patients respond to anti-TNFalpha monotherapy (9). Therefore, the need to identify novel molecular pathways modulating the inflammatory and fibrogenic processes in IBD that may be targeted to develop more effective therapies and with fewer side effects, is greater than ever. One such promising candidate is melanin-concentrating hormone (MCH).

MCH is a 17-19 amino acid peptide highly conserved in evolution. It was originally identified in fish as a hormone secreted by the pituitary and causing, as its name indicates, skin paleness in response to an environmental threat (22). MCH is also expressed in the mammalian hypothalamus where its primary role is in the regulation of appetite and energy balance (34). In addition to brain, the gut represents a significant source of neuropeptides (18). Studies in the rat revealed numerous MCH containing cells, some of them enterochromaffin cells, in gastric antrum and corpus, duodenum and colon (19). Our own group has demonstrated MCH and MCHR1 immunoreactivity in the rat myenteric and submucosal plexus (24), while others have shown that vagal afferent neurons in both human and rat express MCH and its G-protein coupled seven transmembrane receptor (MCHR1) (6). Moreover, when tested in an in situ ligated loop experiment, MCH stimulated Na⁺ and K⁺ in proximal colon; water, Na⁺ and K⁺ in duodenum; and bicarbonate absorption in the jejunum (20).
Besides the still elusive roles of MCH in gastrointestinal physiology, we have found a significant upregulation of MCH expression in the affected mucosa of patients with IBD, suggesting involvement of this molecule in the pathogenesis of IBD (24). Indeed, MCH-deficient mice appear to be less susceptible to TNBS-induced acute colitis, pointing to proinflammatory effects of MCH. However, the significance of MCH in chronic experimental colitis and its associated fibrosis, which better recapitulate the human condition, has not yet been investigated.

In the present study, we evaluated the efficacy of an anti-MCH antibody in treating established DSS-induced experimental colitis. Among the available models, mice treated with multiple cycles of DSS develop chronic colitis characterized by transmural inflammation, muscularis overgrowth and collagen deposition which starts in the lamina propria and can extend up to muscularis propria (37). It has been claimed that this type of experimental colitis shares features with Crohn’s disease based on T- and B-cell involvement and histological hallmarks, though it lacks the development of strictures and intestinal obstruction (37). In our experiments, treatment of mice with anti-MCH resulted in accelerated mucosal healing associated with reduced markers of inflammation and fibrosis. The effects of MCH on fibrosis were found to be, at least in part, inflammation-independent. Indeed, we describe that human myofibroblasts express MCHR1, the activation of which, in combination with additional factors, promotes cell proliferation and collagen production.

MATERIALS AND METHODS

Mice

Protocols of studies involving animal research were submitted to, and approved by, the Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center.
Eight-week-old CD1 male mice were purchased from Charles River Laboratories and used for the experiment four weeks later (body weight 30-35g). Chronic colitis was induced by exposure to 3% (w/v) dextran sulfate sodium (DSS, M.W. 36,000-50,000, MP Biomedicals) dissolved in drinking water as previously described (31). DSS was given *ad libitum* for 5 days per cycle, followed by 5 days of normal drinking water. Beginning on the day after the third DSS cycle, mice were treated i.p. with 1 mg/kg/day anti-MCH antibody (kindly provided by Dr. Eleftheria Maratos-Flier) (n=12) or control IgG (n=11) for a total of 7 days (Fig. 1A). No mouse death occurred during any of the treatments. Control mice received no DSS and no antibody treatment (n=7). MCH has identical sequence in mouse, human and rat and the anti-MCH antibody was raised in rabbits against the whole peptide. The IgG fraction of the anti-MCH serum was used in our studies, along with control IgG, isolated likewise from pre-immune rabbit serum. The specificity of this antibody has been previously confirmed in neuronal mapping studies of human and rat brain slices in combination with MCH mRNA detection by in situ hybridization (16), as well as in hypothalamic sections of transgenic mice overexpressing MCH (28). In an in vitro functional assay, the anti-MCH antibody could block the MCH-mediated inhibition of cAMP upregulation (23). In vivo treatments of mice with the same antibody resulted in prevention of TNBS-induced acute experimental colitis (24) and of *C. difficile* toxin A-mediated enteritis (23).

**Assessment of colonic inflammation and fibrosis**

Tissue segments taken from distal, middle and proximal part of the colon were fixed in 10% buffered formalin and paraffin embedded. Colitis grading was performed in a blinded fashion by a pathologist specializing in gastroenterology (RMN). H&E stained transverse colonic sections were evaluated (three areas per section, three sections per mouse) and an average was calculated for each mouse. Histology score (maximum score 9) is the sum of three
partial scores (0-3): epithelial damage (E), Inflammation (I) and crypt shortening and
distortion (D), where 0 represents normal histology, 1 mild, 2 moderate and 3 severe
histological abnormality.

Fibrosis was evaluated in Sirius red-stained sections and severity scored as follows: 0=no
fibrosis, 1= mild fibrosis limited to the mucosal layer, 2= mucosal and submucosal fibrosis,
and 3= fibrosis involving mural (muscularis propria) and/or serosal layers. The assigned to
each mouse fibrosis score (0-3) is based on the average of three areas per section/three
sections per mouse as in the colitis scoring.

**Cell culture experiments**

CCD-18Co cells (ATCC, passages 8-15) were maintained in Dulbecco’s modified
Eagle’s medium supplemented with 20% fetal bovine serum and 1% antibiotic/antimycotic
(Invitrogen). These cells share morphological and functional characteristics of human
subepithelial myofibroblasts (45). CCD-18Co cells at 80% confluence were stimulated with
3% DSS for 1 hour. Cells were then washed and subsequently treated with $10^{-6}$M MCH
(Bachem), or vehicle for 24 hours (Fig. 6). Collagen production by CCD-18Co cells was
determined using the Sircol collagen assay (Biocolor). To measure cell proliferation, cells
were incubated with BrdU labeling media for 18h followed by BrdU detection using a
colorimetric immunoassay (Roche Applied Science). Assays for each condition were
performed in six replicas and the experiment was repeated twice. Results are shown in
comparison to no-peptide-treated cells (control =100).

In other experiments (Fig. 7), CCD-18Co cells, at 50-70% confluence, were
incubated overnight (16h) with reduced serum media (2% FBS) and subsequently treated
with MCH ($10^{-6}$M), IGF-1 (10ng/ml), their combination or vehicle in the same media, for 4h
and cell proliferation was assessed as above. CCD-18Co cells were cultured in MEM media
(10% FBS) and treated with MCH, TGF-beta, their combination or vehicle for 36h. At the end of the experiment, collagen was measured using the Sircol assay.

For the wound scratch assay, after removal of the DSS, a linear wound was generated in the CCD-18Co monolayer by scraping a sterile 200μl pipette tip across the bottom of the well. Following three washes, cells were incubated with growth media containing MCH (10⁻⁶M) or vehicle. Results represent six replicas per condition.

Quantitative RT-PCR

From each mouse, tissue samples corresponding to the distal, middle and proximal parts of the colon were pulled for gene expression analysis. Total RNA was extracted using the RNeasy mini-kit (Qiagen), according to manufacturer's instructions. One microgram (μg) of RNA was reverse-transcribed into cDNA using the Advantage RT for PCR reagents with oligo (dT) (Clontech). Quantitative gene expression was assessed using gene-specific primers purchased from Applied Biosystems and TaqMan Universal PCR Master Mix in an ABI PRISM 7700 Sequence Detection System. Results have been normalized to expression of the TATA-binding protein (TBP) housekeeping gene and are expressed as arbitrary mRNA units (AU) relative to control group (control =100). The following groups of mice were included into the gene expression analysis: control, no-DSS (n=5); DSS+IgG (n=9); and DSS+anti-MCH (n=10).

Immunostaining

Human colonic tissue samples derived from surgical resection specimens were obtained as frozen sections from the Ardais/Beth Israel Deaconess Medical Center Biomaterials and Information for Genomic Research Tissue Library (Boston, MA). The panel included areas of active disease from patients with IBD (five with Crohn’s disease and four
with ulcerative colitis) as well as histologically normal tissue from patients undergoing surgery for non-inflammatory conditions (n=3).

Slides were fixed in 4% paraformaldehyde and incubated with anti-alphaSMA mouse anti-human monoclonal antibody (clone 1A4, dilution 1:50, Dako) and with a rabbit polyclonal antibody against human/rat/mouse MCHR1 (24) (dilution 1:200) for 2 hours at room temperature followed by incubation with FITC- and Texas Red- labeled secondary antibodies, respectively. As negative controls, either or both of the primary antibodies were omitted from the staining procedure. Sections were treated with Prolong Gold antifade with DAPI (Invitrogen) mounting media and viewed under a Zeiss LSM510 META confocal microscope.

Statistical Analysis

Results are expressed as group mean +/- SE. Data were analyzed in STATView using the non-parametric Mann-Whitney U-test or repeated measures ANOVA as appropriate. A p-value less than 0.05 was considered statistically significant.

RESULTS

Treatment with an anti-MCH antibody attenuates chronic intestinal inflammation and fibrosis

To evaluate the therapeutic potential of targeting MCH in chronic experimental colitis, mice were exposed to three cycles of DSS treatment, followed by daily injections of anti-MCH or control antibody for 7 days (Fig. 1A). Body weight curves were indistinguishable between the two treatment groups throughout the study (Fig. 1B). Histological analysis of
H&E stained mouse colonic sections revealed more severe epithelial damage, based on areas of necrosis, crypt abscesses and mucosal ulcers in mice treated with control antibody compared to mice that received anti-MCH treatments (Fig. 2A). Likewise, architectural distortion, another marker of chronic epithelial injury was also less pronounced in mice treated with anti-MCH. Mice treated with control antibody, and in contrast to anti-MCH treated mice, also had significant ongoing inflammation. Furthermore, the study of mouse colonic sections stained with Sirius red (Fig. 2B) demonstrated extensive collagen deposits in mice with DSS colitis treated with control IgG, in contrast to mice treated with anti-MCH. Overall, the histology score was lower in mice receiving anti-MCH (1.75±0.69 vs. 4.54±1.13; anti-MCH vs IgG; p=0.043; Fig. 3A), as well as their fibrosis score (0.58±0.19 vs. 1.64±0.39; anti-MCH vs. IgG; p=0.024; Fig. 3B). Despite the chronic inflammation and injury, none of the mice developed colonic tumors.

Corroborating the histology findings, mice treated with anti-MCH had significantly lower colonic levels of several proinflammatory cytokines (TNFalpha: 274.7±65.4 vs. 127.7±31.5; p=0.014; IL-1beta: 4104.8±1811.1 vs. 588.6±239.5, p=0.036; KC: 2174.2±1397.4 vs. 481.8±176.9, p=0.037; expressed as arbitrary mRNA units, control IgG vs. anti-MCH, respectively, Fig. 3C). These findings further support the role of MCH as a modulator of intestinal inflammation, as previously described by our group (23, 24).

**Molecular analysis of fibrosis markers in mice with chronic colitis treated with anti-MCH**

We did not find any differences in TGFbeta mRNA expression, a major drive of fibrotic processes, among the two treatment groups (Fig. 4A). In addition to TGFbeta, IGF-1 and TNFalpha have also been shown to regulate type I collagen synthesis by myofibroblasts (14, 36, 42). Along with lower TNFalpha mRNA expression in mice treated with anti-MCH (Fig. 3C), we found a similar trend for IGF-1 expression, though it did not reach statistical
significance (344.7±86.5 vs. 227.1±29.1 IGF-1 mRNA arbitrary units, control IgG vs. anti-MCH; p=0.110; Fig. 4A). Since TGFbeta mRNA levels might not accurately reflect its activity, we subsequently measured expression of SMAD2, SMAD3, and SMAD4, the primary downstream effectors of TGFbeta receptor I signaling (17). Indeed, SMAD3 was almost two fold upregulated in mice with colitis treated with control antibody, as compared to mice with no DSS treatment, while it was similar to baseline levels in mice with colitis treated with anti-MCH antibody (171.9±13.3 vs. 101.5±9.8 arbitrary mRNA units, control IgG vs anti-MCH, p=0.0005; Fig. 4B). There was a similar trend for SMAD2 expression (177.2±36.7 vs. 111.9±18.4 arbitrary mRNA units, control IgG vs. anti-MCH, p=0.0661), while changes in SMAD4, though significant, were of a lesser magnitude (128.9±10.6 vs 98.2±19.2 arbitrary mRNA units, control vs. anti-MCH, p=0.0411; Fig. 4B).

Colonic mRNA levels of COL1A1 were significantly reduced in DSS-exposed mice which received treatment with anti-MCH antibody (334.2±76.5 vs. 118.6±19.9 arbitrary mRNA units, control IgG vs. anti-MCH; p=0.007; Fig. 4C), consistent with the findings on histology (Sirius red stained sections)

Collagen deposition and extracellular matrix remodelling are parts of a dynamic process regulated by various matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Mice with DSS-induced chronic colitis had increased mRNA expression of MMP-3, MMP-9, and MMP-12. Among them, MMP-3 upregulation was significantly attenuated in mice treated with anti-MCH antibody (1599.1±897.3 vs. 396.3±151.5, control IgG vs anti-MCH, p=0.033; Fig. 4D) while MMP-9 levels followed a similar trend. In parallel to the induction of MMPs, mice with chronic DSS colitis had also increased Timp-1 and Timp-6 mRNA expression, while there was a trend for lower Timp-1 levels in mice treated with anti-MCH (685.0±215.9 vs. 347.1±79.2, control IgG vs anti-MCH, p=0.106, Fig. 4E).

**Upregulation of MCHR1 expressing myofibroblasts in patients with IBD**
Areas of collagen deposition in patients with IBD were identified by Masson’s Trichrome staining (Fig. 5A). Sequential sections were stained for alphaSMA, a marker for myofibroblasts and MCHR1. Confocal analysis revealed that the majority of myofibroblasts from the inflamed mucosa of patients with IBD were MCHR1 positive. Pictures of representative patients from each group are shown in Fig. 5B).

**Direct effects of MCH on myofibroblasts**

CCD18-Co primary human intestinal sub-epithelial myofibroblasts were also found to express MCHR1 (Fig. 6A). In these cells, we examined the effect of MCH treatment on cell proliferation, collagen production and cell migration, at baseline conditions and after exposure to DSS-induced cell injury (15). Following DSS treatment, we observed a moderate increase in cell proliferation in response to MCH, as measured by a BrdU incorporation assay (135±11 vs. 100±5, relative units, MCH vs vehicle, p=0.004; Fig. 6B). Furthermore, under similar conditions, MCH promoted collagen production, though to a lesser extent, as measured by a biochemical assay (107.3±1.4 vs. 100±0.6 relative units, MCH vs. vehicle, p=0.008, Fig. 6C). However, under the conditions tested, no effects of MCH were apparent in the absence of DSS treatments. This suggested that perhaps MCH modulates the effects of factors secreted in response to myofibroblast activation (2). To further explore this possibility, CCD18-Co cells were treated with IGF-1, alone or in combination with MCH. Indeed, we observed a potentiation of the IGF-1 effects on cell proliferation in the presence of MCH (97.05±3.0 vs 110.0±2.9 relative BrdU units, IGF-1 vs IGF-1 plus MCH, p=0.025; Fig. 7A). Likewise, MCH enhance TGF-beta1 induced collagen production in the same cells (118.5±7.3 vs 160.3±8.3 relative units, TGF-beta1 vs TGF-beta1 plus MCH, p=0.01; Fig. 7B).
Effects of MCH on wound healing

To determine the role of MCH in wound healing, a scratch was made into a confluent monolayer of CCD-18Co myofibroblasts (=100%), and closure of the wound was monitored at different time points. No differences in migration were observed between MCH and vehicle treated cultures without prior stimulation with DSS. In cells that had been pre-treated with DSS, differences between MCH and vehicle treated myofibroblasts became apparent at 24 hours post-wounding. At 48 hours, 74.1±3.5% of the wound area had been closed in MCH treated cells vs 62.2±1.7% in vehicle treated (p=0.0117; Fig. 8).

DISCUSSION

In the present study, we demonstrate not only attenuation of chronic experimental colitis by blocking MCH, but also of intestinal fibrotic processes associated with ongoing inflammation. Intestinal fibrosis represents an recurring clinical problem in inflammatory bowel disease that leads to dysmotility, bowel narrowing and obstruction, complications which often require surgical intervention (38). Though the control of fibrosis in mice with chronic DSS colitis treated with an anti-MCH antibody could be secondary to reduced inflammation, our in vitro studies suggest that additional direct effects of MCH on intestinal myofibroblasts are possible. It would be of interest to test this in other organs or myofibroblastast from these organs, including liver, lung and kidney (47).

Earlier studies from our group using MCH-deficient mice pointed to MCH as a proinflammatory mediator in experimental colitis (24). Investigating potential underlying mechanisms of such effects, we uncovered that MCHR1 expression on colonic epithelial cells is upregulated in IBD and in vitro in response to various proinflammatory stimuli. In turn, acting on colonocytes, MCH stimulates the expression of IL-8 and perhaps of additional
cytokines and chemokines (23, 24). In addition to these cells, previous studies describe expression of MCHR1 in immune organs like spleen, lymph nodes and thymus as well as in isolated populations of T-cells, B-cells, monocytes and granulocytes (11, 19, 26, 46). On the other hand, and of particular interest for IBD, MCH was found to be upregulated specifically by Th2 cytokines (32, 40). Interestingly, the Th2 type cytokines, IL-4 and IL-13, directly promote fibrosis by stimulating collagen production (4). Taken together these observations further support an immunomodulatory role for MCH, which might be of importance to IBD pathogenesis.

Extending our studies beyond inflammation, we describe here the unexpected finding that in patients with IBD, MCHR1 is present on intestinal subepithelial alphaSMA (+) cells, presumably myofibroblasts. Furthermore, we provide evidence for direct effects of MCH on myofibroblast proliferation, collagen production, and migration under proinflammatory conditions. These findings are in agreement with a previous report showing that MCH promotes migration of 3T3-preadipocytes via actin cytoskeleton rearrangements (10). Additionally, the functional antagonist of MCH, alphaMSH (44), suppresses TGFbeta1 induced collagen synthesis in human dermal fibroblasts and has anti-fibrogenic activity in vivo (5). However, in our experimental setting, the effect of MCH on myofibroblasts was moderate and required the presence of co-factors (42). This is not totally unexpected, given that in other studies, substance P induces collagen production by CCD18-Co only in the presence of TGFbeta and IGF-1 (25), bradykinin synergizes with TNFalpha to induce expression of COX-2 in the same cells (48), and VIP increases proliferation of primary colonic myofibroblasts only in the presence of PDGF (21). At the molecular level, this MCH requirement for co-stimulators could perhaps be explained by the fact that G-protein coupled receptors, like MCHR1, lack intrinsic kinase activity and thus they need to transactivate growth factor receptors to convey mitogenic and growth signals (12). For instance, it has been previously shown that neurotensin promotes the growth of colonic epithelial cells via
IGF-1R transactivation (49) and substance P the proliferation of U-373MG astrogliaoma cells via EGFR transactivation (7).

In an in vitro assay, we have shown that MCH, under certain conditions (DSS treatment), can accelerate wound healing. While this effect in highly desirable as a response to acute tissue injury, in cases of persistent inflammation, as in IBD, it might become deleterious. In patients with Crohn’s disease, chronic or excessive wound healing results in fibrostenotic lesions, a major indication for surgery, while in patients with ulcerative colitis it may cause colon shortening and dysmotility (39). As such, from a therapeutic perspective, blocking MCH might prevent such complications.

The molecular analysis of tissues from mice with colitis treated with anti-MCH provides insights into potential downstream mechanisms by which MCH modulates fibrotic processes in the context of inflammation, as for example regulation of SMAD3 expression. This finding might be of significance in IBD, given that SMAD3 not only is upregulated in the strictured areas of patients with Crohn’s disease (13), but its downregulation or genetic ablation inhibits intestinal fibrosis while accelerates re-epithelization and mucosa healing in mouse models of experimental colitis (27, 43) and in mouse skin wound healing (1). Furthermore, mouse fibroblasts null for Smad3 showed decreased production of collagen in vitro (33).

In the current and our previous studies, lower TNFalpha expression as a result of MCH ablation or inhibition is a common finding in mice with intestinal inflammation (23, 24). Notably, TNFalpha has been implicated not only in inflammatory but also in fibrotic processes by directly regulating collagen production (36). Indeed, myofibroblasts isolated from patients with Crohn’s disease expressed high levels of TNFalpha and treatment of these cells with an anti-TNFalpha antibody (infliximab) decreased collagen production (14). Hence, blocking MCH might be valuable as a combination, “add-on” treatment to current therapies of IBD aiming at blocking TNFα.
In conclusion, our findings suggest that targeting MCH might have dual benefit in the treatment of inflammatory bowel disease by reducing the severity of inflammation itself as well as preventing fibrosis. Interestingly, the field of IBD therapeutics can move forward by repurposing several existing MCHR1 antagonists initially developed for the treatment of obesity and the metabolic syndrome (8, 29). These candidate drugs have already been tested in early clinical trials and were found to be relative safe and well tolerated. However, they have been abandoned by pharmaceutical companies primarily due to lack of efficacy in obese individuals, and some of them are readily available for new therapeutic uses (http://www.ncats.nih.gov/research/reengineering/rescue-repurpose/therapeutic-uses/directory.html).
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Disclosures: The authors have nothing to disclose

Author contributions: DCZ, EK designed the experiments; DCZ, BGM, SM, BMG, SNF, YHT, YP performed the experiments; DCZ, RMN, EK JMN analyzed data; DCZ, BMG, YP, EK wrote the manuscript.
References


FIGURE CAPTIONS

Figure 1: Induction of chronic DSS colitis in mice

(A) After completion of three cycles of 3% DSS (5 days DSS, 5 days recovery) mice received daily injections of anti-MCH or control (anti-IgG) antibody for 7 days.

(B) Changes in mouse body weight were monitored throughout the study and were comparable in mice with colitis treated with anti-MCH or control IgG.

Figure 2: Disease attenuation in mice with DSS colitis treated with anti-MCH

Representative H&E stained (upper panel) and Sirius red stained (lower panel) colonic sections of mice with chronic DSS colitis treated with anti-MCH or control IgG. Mice with no colitis are included for comparison (200x magnification)

Figure 3: Treatment with anti-MCH attenuates inflammation and fibrosis in mice with chronic DSS colitis

(A) Histological scoring of H&E stained colonic biopsies of mice with colitis treated with anti-MCH or control IgG.

(B) Fibrosis scoring of Sirius red stained colonic biopsies from the same mice as above.

(C) Colonic mRNA expression of cytokines in mice with chronic DSS-colitis treated with anti-MCH or control IgG. Mice that were not treated with DSS were included as a baseline control (control=100). Results as expressed as arbitrary units.

*p<0.05, anti-MCH vs. IgG control treatment
Figure 4: Analysis of fibrosis markers in mice with chronic DSS colitis

Consistent with the histological findings of reduced fibrosis, mice with chronic colitis treated with anti-MCH had lower mRNA expression of procollagen α1(I) (encoded by COL1A1 gene), SMAD4 and MMP3. Results are expressed as arbitrary units (AU)(no DSS group=100).

*p < 0.05; **p < 0.01; ***p<0.001; anti-MCH vs. control IgG treatment

Figure 5: MCHR1 positive myofibroblasts in the colon of patients with IBD

(A) Masson’s trichrome staining of colonic biopsies from controls and patients with IBD, which included fibrotic areas (blue deposits).

(B) Confocal analysis of staining with αSMA (green), a marker of myofibroblasts and MCHR1 (red), revealed a large number of myofibroblasts stained positive for MCHR1 (yellow) in the subepithelium of patients with IBD but not in normal colonic tissue.

Figure 6: In vitro effects of MCH on myofibroblasts in response to cell injury

CCD-18Co cells were stimulated with 3% DSS or media alone for 1 hour. Cells were then washed and subsequently treated with MCH (10^{-6}M), or vehicle for 24 hours.

(A) Immunofluorescence analysis confirmed the presence of MCHR1 (green) in CCD-18Co human myofibroblasts.

(B) BrdU incorporation (Roche Applied Science) was used to access proliferation of CCD-18Co human myofibroblasts in response to MCH treatment.
Collagen production by CCD-18Co cells in response to MCH treatment was evaluated using the Sircol collagen assay (Biocolor). **p<0.01, MCH vs vehicle treatment

Figure 7: MCH modulates the effects of IGF-1 and TGFβ1 in vitro

(A) CCD-18Co human myofibroblasts were treated with MCH (1 μM), IGF-1 (10ng/ml), their combination or vehicle for 4h and cell proliferation was measured by BrdU incorporation the following 16h, using a colorimetric assay. *p < 0.05; IGF-1 vs IGF-1 plus MCH

(B) CCD-18Co cells were treated with MCH (1 μM), TGF-β1 (2 ng/ml), their combination or vehicle for 36h and collagen production was evaluated using the Sircol collagen assay. Results are expressed are relative units (vehicle treated=100).

*p < 0.05; IGF-1 vs IGF-1 plus MCH

**p < 0.01; TGFβ1 vs TGFβ1 plus MCH

Figure 8: In vitro effects of MCH treatment on wound healing

Monolayers of CCD-18Co cells (human myofibroblasts) were treated with 3% DSS for 1 hour and a linear wound using a pipette tip was created. After washing, cells were treated with MCH (10^{-6}M) or vehicle for 48 hours. At the indicated time points, pictures of cell cultures were taken under an inverted microscope and the open wound area was measured using the Image Quant software. Results are expressed as % of wound closure compared to 0 time point for each condition.

**p < 0.01 by ANOVA repeated measurements; MCH vs vehicle treatment
Figure 1

A

anti-MCH antibody (1mg/kg/day i.p.)

D0  D5  D10  D15  D20  D25  D32

3% DSS 3% DSS 3% DSS

B

% weight change

no DSS  DSS+IgG  DSS+anti-MCH

Time (days)
Figure 2

- no DSS
- DSS + IgG
- DSS + anti-MCH
Figure 4

A) TGFbeta-1

B) SMAD2, SMAD3, SMAD4

C) IGF-1, COL1A1

mRNA (AU)

no DSS  DSS  DSS
+IgG  +anti-MCH

control  DSS+IgG  DSS+anti-MCH

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Figure 4

D

E

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<td>600</td>
<td>400</td>
<td>300</td>
</tr>
<tr>
<td>DSS+anti-MCH</td>
<td>400</td>
<td>200</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>mRNA (AU)</th>
<th>TIMP1</th>
<th>TIMP6</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>800</td>
<td>200</td>
</tr>
<tr>
<td>DSS+IgG</td>
<td>600</td>
<td>400</td>
</tr>
<tr>
<td>DSS+anti-MCH</td>
<td>400</td>
<td>200</td>
</tr>
</tbody>
</table>
Figure 5

A

control

inflammatory bowel disease

B

alphaSMA  MCHR1  merged
Figure 6

A  

anti-MCHR1  

neg. control

B

\[
\begin{array}{ccc}
\text{BrdU incorporation (relative units)} & & \\
\text{veh} & \text{MCH} & \text{veh} & \text{MCH} \\
\text{no DSS} & & \text{DSS} & \\
\end{array}
\]

C

\[
\begin{array}{ccc}
\text{collagen secretion (relative units)} & & \\
\text{veh} & \text{MCH} & \text{veh} & \text{MCH} \\
\text{no DSS} & & \text{DSS} & \\
\end{array}
\]

**
Figure 7

A

BrdU incorporation (relative units)

veh MCH IGF1 IGF1

B

collagen secretion (relative units)

veh MCH TGFβ TGFβ

MCH

* **
Figure 8

Comparison of wound closure between vehicle and MCH treatments at 0h, 24h, and 48h time points. The graph shows a significant increase in wound closure in the MCH group compared to the vehicle group, with a marked difference at 48h. The data is presented as mean ± SEM, and statistical significance is indicated by the asterisk symbol (**).