Published in final edited form as:

Gastroenterology. 2011 October ; 141(4): 1283–1292.e4. doi:10.1053/j.gastro.2011.06.035.

Matrix Metalloproteinase-9 Inhibition Reduces Inflammation and Improves Motility in Murine Models of Post-Operative Ileus

Beverley A. Moore^{*,†}, Carl L. Manthey^{*}, Dana L. Johnson^{*}, and Anthony J. Bauer[†]

^{*}Johnson & Johnson Pharmaceutical Research Division, Spring House, PA

[†]University of Pittsburgh, Pittsburgh, PA

Abstract

Background & Aims—Matrix metalloprotease (MMP)-9, a member of the gelatinase family of MMPs, mediates leukocyte migration during inflammation. Inflammation contributes to development of post-operative ileus (POI), which is caused by physical disturbances to the bowel during abdominal surgery. We evaluated the role of MMP-9 in POI and investigated whether disruption of *MMP-9* or administration of an inhibitor of MMP-9 activity reduced cellular inflammation and bowel dysmotility in rat and mouse models of POI.

Methods—Mice and rats underwent laparotomy and bowel manipulation; bowel tissues were collected 3–24 hr later and analyzed by real time reverse transcriptase PCR, immunoblot, in situ zymography, and functional analyses.

Results—Bowel manipulation resulted in a time-dependent increase in *MMP-9* expression within the intestinal muscularis; increases in *MMP-9* mRNA were iNOS-dependent. Immunoblot analyses confirmed the presence of the pro-enzyme and the catalytically active form of MMP-9. Administration of MMP-2/MMP-9 II, a dual active-site inhibitor, reduced the number of myeloperoxidase-positive immune cells that infiltrated the muscularis and prevented the surgically induced reduction in bowel smooth muscle contractility. Zymography analysis, performed in muscularis whole mounts in situ, indicated that MMP-9 and not MMP-2 mediated the gelatinase activity observed in infiltrating cells. MMP-9 knockout mice were protected from the inflammation and dysmotility associated with POI.

Conclusions—MMP-9 mediates cellular inflammatory responses within the intestinal muscularis in mouse and rat models of POI. Inhibition of MMP-9 activity reduced recruitment of immune cells to the intestinal muscularis, preventing loss of smooth muscle contractility. Induction of *MMP-9* expression requires iNOS.

Keywords

gastrointestinal surgery; GI; complication; intestine

© 2011 The American Gastroenterological Association. Published by Elsevier Inc. All rights reserved.

Corresponding Author: Beverley A. Moore, Johnson & Johnson, PRDUS, Welsh & McKean Roads, Spring House, PA 19477, Ph: 215-628-5659.

No financial benefit will be derived from the materials used in this manuscript. All authors confirm that no conflict of interest exists.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Disruption of the extracellular matrix (ECM) is required for immune cell migration into tissues during inflammatory events. This is accomplished through the activity of matrix metalloproteinases (MMPs) which cleave components of the matrix.¹ Because MMPs have considerable potential for widespread destruction of the ECM, they are translated as pro-enzymes in a catalytically inactive state until cleavage of the pro-domain, or by other interactions that expose the active site. Activity of the catalytic form may be further regulated by interaction with tissue inhibitors of metalloproteases (TIMPs), which compete for substrate binding and form complexes with MMPs to position them for destruction by proteolytic degradation.

The gelatinases, MMP-9 and MMP-2, are recognized as having important roles in the turnover and degradation of ECM proteins during cellular recruitment in inflammation.² Their biology has been studied extensively in tumor invasion, where both are known to be present at the membrane of invading cells.³ Although similar in their substrate selectivity, MMP-9 and MMP-2 are differentiated by tissue specificity. In normal tissues, MMP-2 is constitutively expressed in fibroblasts, endothelial cells and epithelial cells, and may be modestly up- or down-regulated by inflammatory stimuli.⁴ In contrast, MMP-9 expression is observed primarily in leukocytes including monocytes, macrophages and neutrophils.⁵ It is highly induced in response to chemokines and cytokines, and enhanced expression has been linked to a variety of inflammatory pathologies including autoimmunity,⁶ arthritis,⁷ ischemia/reperfusion injury,⁸ transplant rejection,⁹ and inflammatory bowel disease.¹⁰ The role of MMP-2 in inflammatory cell migration is less clear; however some evidence suggests it may complement MMP-9 in the activation and cleavage of specific integrins during cell attachment to and release from substrate.^{11, 12}

Recruitment of leukocytes to the bowel muscularis is central to the development of postoperative ileus (POI), which is defined as the transient impairment of gastrointestinal motility following open abdominal surgery. Data recently summarized in a comprehensive review by Boeckxstaens & de Jonge, points to inflammation triggered by incursion into the abdominal cavity and physical handling of the bowel as the primary underlying mechanism.¹³ Key players in this process are resident macrophages present within the bowel wall. These cells become activated during laparotomy and bowel manipulation, releasing pro-inflammatory mediators that initiate the recruitment of leukocytes (neutrophils, monocytes and mast cells) to the manipulated region. Both resident and recruited leukocytes release interleukins, reactive oxygen species, proteases, prostaglandins, and nitric oxide, which have direct inhibitory effects on neuromuscular communication, giving rise to generalized gastrointestinal hypomotility. Reduced cellular inflammation has been linked to improved GI function and more rapid recovery from POI.^{13, 14} We hypothesized that MMP-9 plays a central role in cellular inflammation in POI and that MMP-9 gene deletion or treatment with an MMP-9 active site inhibitor would modulate the cellular inflammatory response and reduce dysmotility.

Portions of the data presented in this manuscript were previously published in abstract form at the American Motility Society Meeting, 2004.

Results

Age-matched male C57Bl/6 mice (Charles River Laboratories), MMP-9 KO mice on the FVB background, iNOS KO mice on the C57Bl/6 background (The Jackson Lab), or Sprague-Dawley rats (Charles River) were used. Study protocols in animals were reviewed and approved by the University of Pittsburgh and Johnson & Johnson Pharmaceutical R&D

Institutional Animal Care and Use Committees. Animals were housed in specific pathogen-free facilities and maintained on a 12 hour light/dark cycle with access to standard rodent chow and water *ad libitum*. Post-operative ileus was induced in rats and mice by laparotomy and gentle compression of the small bowel or colon. The reader is referred to the online Supplemental Materials for a detailed description of experimental methods.

MMP Gene Expression

To determine whether gelatinases and their endogenous inhibitors play a role in postoperative ileus, expression of the genes for MMP-9, MMP-2, and TIMP-1 through 4 were evaluated in intestinal and colonic muscularis by real time RT-PCR. Tissues were harvested from C57Bl/6 mice 3, 6, 12, and 24 hr after surgical manipulation of the small bowel or colon (n=4 to 6 per group)¹⁵. Primer sources and sequences are provided in Supplemental Table S1. A time-dependent increase in MMP-9 and TIMP-1 gene expression relative to control was observed, with peak expression occurring in both organs between 12 and 24 h postoperatively (Fig. 1). MMP-2 gene expression in the colon was not significantly altered; however expression in the small bowel showed a time-dependent decrease. TIMP-2, TIMP-3 TIMP-4 gene expression was not affected by surgical manipulation (data not shown). Baseline ΔC_T values from control animals of different strains are provided in Supplemental Table S2.

MMP Protein Expression

MMP-9 is expressed as an inactive pro-enzyme that undergoes proteolytic cleavage to generate the active species¹⁶. We next sought to examine small bowel and colonic muscularis lysates harvested 14 hours after induction of postoperative ileus for the presence of both the pro- and active forms of MMP-9 by Western blot analysis. Blots were probed with mouse anti-human MMP-9 monoclonal antibody (mAb) L51/82 (UC Davis/NJMH NeuroMab Facility Antibody Incorporated), which detects both the pro-enzyme and the catalytically active form of human MMP-9, and rabbit polyclonal antibody (pAb) 1246, which is specific for the human catalytically active form¹⁷ (see Supplemental Material for details of antibody generation). Both antibodies have good cross-reactivity with rat MMP-9, but not mouse; thus expression of MMP-9 was investigated in the rat. Standards were generated by combining 2 ng of recombinant rat pro-MMP-9 with 2 ng of rat MMP-9 converted to the active form by the action of recombinant catalytic MMP-3. MMP-9 in the samples was estimated based on the ratio of the signal intensity of unknowns vs standard. MMP-9 was minimally detected in small bowel muscularis of control rats, however expression was induced >100-fold 14 hr following surgical manipulation. MMP-9 detected with mAb L51/82 was primarily in a 100 kD molecular weight form, corresponding to pro-MMP-9 (Fig 2A). L51/82 did not yield clear evidence of the 82 kD active form of MMP-9, therefore replicate blots were probed with pAb 1246. The active form was evident only in rats having undergone surgical manipulation (Fig 2B). By comparing the band intensities of the active standard vs. the band intensities of the unknowns, it is evident that only a minor fraction of total MMP-9 was present in the active form (Table 1).

MMP-2 and TIMP-1 protein expression were quantified by ELISA (R&D Systems, DMP200, RTM100, respectively) in the same tissue lysates. Consistent with the mRNA expression data, TIMP-1 protein was induced strongly following surgical manipulation. In contrast, MMP-2 was moderately expressed in control tissues and increased <2-fold following surgical manipulation.

In Situ Zymography

The relative contributions of MMP-9 and MMP-2 to the gelatinase activity observed in muscularis wholemounts were determined by DQTM-gelatin (Invitrogen) *in situ* zymography

in the presence of MMP-2 or MMP-9 active site neutralizing antibodies (Fig. 3; see Supplemental Materials).¹⁸⁻²⁰ Disruption of the DQ (dye quenched)-gelatin-fluorescein isothiocyanate (FITC) complex by gelatinase activity unmasks the quenched fluorescent signal. With this technique, localized points of gelatinase activity can be identified to the resolution of individual cells. Rat small bowel muscularis whole mounts were harvested 14 hr postoperatively and pretreated for 30 min with anti-human MMP-9 (Calbiochem, clone 6-6B) or MMP-2 (Millipore, clone CA-4001) active site neutralizing antibodies, or PBS. Colonic tissue was not readily penetrated by antibodies; therefore these studies were performed only on small bowel muscularis. Tissues were imaged on an Olympus IX80 inverted microscope fitted with fluorescence optics, using SlideBook™ imaging software (Intelligent Imaging Innovations, Inc., Philadelphia, PA; version 5.0). No gelatinase activity was detected in control tissues (Fig. 3A), whereas numerous infiltrating gelatinase-positive cells were observed within the intestinal muscularis after bowel manipulation (Fig. 3B). An enlargement of the highlighted region in Fig. 3B (Fig. 3C) shows that gelatinase activity appears to be distributed near points of contact with the extracellular matrix (arrows). Gelatinase activity was completely abolished by incubation of muscularis whole mounts with anti-human MMP-9 antibody (Fig. 3D), whereas treatment with anti-human MMP-2 active site antibody had no detectable effect (Fig. 3E).

Effects of iNOS deletion on MMP-9 and TIMP-1 mRNA expression

iNOS derived nitric oxide (NO) is reported to play an important role in the inflammatory processes associated with postoperative ileus¹⁵, and there is evidence to suggest that NO may regulate the expression of MMP-9 and TIMP-1 under inflammatory conditions.²¹⁻²⁴ To determine whether iNOS derived NO has a role in modulating MMP-9 and TIMP-1 expression in POI, we evaluated the effects of iNOS deletion on MMP-9 and TIMP-1 gene expression. Time course analysis of iNOS mRNA expression showed that iNOS was transiently induced 6 and 24 postoperatively in the small bowel and 3 hr postoperatively in the colon (Fig. 4A). In iNOS KO mice, MMP-9 expression at 24 hr was reduced by 80% in the small bowel, and 90% in the colon (Fig. 4B). Baseline MMP-9 ΔC_T values for the C57Bl/6J WT and KO mice were similar (Supplemental Table S2) indicating that deletion of the iNOS gene did not affect baseline expression.

In the small bowel, surgical manipulation did not have a significant impact on TIMP-1 gene expression baseline (Fig. 4C), and baseline TIMP-1 ΔC_T values were similar in the WT and iNOS KO (Supplemental Table S2). In the colon, however, postoperative induction of TIMP-1 expression appeared to be substantially reduced in the iNOS KO. Baseline TIMP-1 ΔC_T values were found to be significantly lower in the KO (7.42 ± 0.26) compared to WT (11.93 ± 0.42) suggesting that TIMP-1 mRNA was present at higher baseline copy number, which would result in a smaller fold change in response to surgical manipulation. Recalculating the KO postoperative ΔC_T values relative to the WT baseline increased the relative fold change 72.0 ± 9.78 (dotted outline) suggesting that the actual postoperative TIMP-1 mRNA copy numbers in the WT and KO are similar (ΔC_T : 5.65 ± 0.52 and 5.09 ± 0.20 , respectively).

Effects of MMP-9 deletion on Postoperative Ileus

The effects of MMP-9 deletion on the cellular inflammatory response and intestinal dysmotility associated with postoperative ileus were evaluated in FVB wild type and MMP-9 KO mice (Fig 5). Cellular inflammation within the small bowel muscularis was evaluated 24 hr postoperatively by myeloperoxidase (MPO) histochemistry (Supplemental Materials). GI transit was determined by calculating the distribution (Geometric Center, GC) of the orally fed fluorescein isothiocyanate-dextran conjugate (FITC-dextran, 70,000 MW). Digital photomicrographs (Fig 5A) and graphical representations (Fig 5B) show that the

number of infiltrating MPO-positive cells is markedly reduced in MMP-9 KO mice. In addition, MMP-9 KO mice were partially protected from intestinal dysmotility (Fig. 5C), where gastrointestinal transit in the KO mice was significantly less delayed.

Effects of MMP-9/MMP-2 inhibition on POI

To determine whether pharmacological inhibition of MMP-9 would intervene in POI, the effects of a dual MMP-9/MMP-2 inhibitor (MMPi) were evaluated in C57Bl/6 mice 24 hr after small bowel or colonic manipulation (n=4; Fig. 6). MMPi treatment significantly reduced the magnitude of the cellular inflammatory response within the colonic muscularis (Fig. 6A). The surgically induced impairment of small bowel and colonic smooth muscle contractility was evaluated in organ bath experiments *in vitro* and *in vivo* (described in Supplemental Materials). Representative traces showing the contractile response of colonic smooth muscle strips exposed to 100 μ M bethanechol are provided in Fig. 6B. In control tissues, bethanechol induced a prolonged tonic contraction overlain by multiple phasic contractions. After colonic manipulation (CM), the magnitude of the tonic contraction was reduced and phasic contractions were lost. Contractile responses in tissues harvested from mice treated with MMPi were similar to control. The integrated contractile responses to increasing concentrations of bethanechol (1 to 300 μ M) are shown in Fig. 6C. Contractile responses in tissues from vehicle treated animals were reduced by approximately 50%, with marked improvement in tissues from animals treated with MMPi. The effects of MMPi on the peak contractile response (100 μ M) for both the small bowel and colon are summarized in the histogram. The impaired contractile response was significantly improved in both organs by treatment with MMPi. Evidence that increased contractility correlated with improved intestinal motility is provided in Fig. 6D where the reduction in GC associated with intestinal manipulation (IM) is significantly increased in MMPi treated animals.

Statistical Analysis

Data were expressed as mean $\pm$ SEM, with $P < 0.05$ being considered statistically significant. Data were compared where appropriate by unpaired Student's t-test, or one-way analysis of variance (ANOVA) followed by Bonferroni or Tukey post hoc testing for multiple comparisons as appropriate.

Discussion

During inflammatory events, the transcription of MMP-9 and its endogenous inhibitor TIMP-1 is induced by pro-inflammatory mediators (products of arachidonic acid metabolism, integrins, plasminogens, TNF α , IL1 β , TGF β , nitric oxide).^{5, 25} Many of these same mediators have been implicated in the cellular inflammation that forms the basis for the pathology associated with POI.¹³ We observed that MMP-9 plays a central role in this process, and that deletion of the MMP-9 gene, or inhibition of MMP-9 activity attenuated POI.

MMP-9, MMP-2, and TIMP gene expression were evaluated in the mouse small intestine and colonic muscularis following laparotomy and bowel manipulation. MMP-9 gene induction commenced 3 hr postoperatively and reached peak expression by 12 to 24 hr, a time course that closely paralleled the known time of onset for leukocyte migration and the time at which peak numbers of leukocytes are actively migrating into the intestinal muscularis.²⁶ MMP-9-specific gelatinase activity was readily detected within infiltrating cells, and western blot analysis confirmed that both the pro- and active forms of the MMP-9 protein were expressed 14 hr postoperatively time when matrix degradation would be necessary for cellular migration to occur. It was clear, however, that the catalytically active

species comprised only a very small component of the total MMP-9 present in the muscularis.

Left unchecked, MMP-9 has considerable potential for non-specific matrix degradation and its activity must be tightly controlled. This is accomplished in several ways. MMP-9 is first translated as a pro-enzyme and secreted to the leading edge of actively migrating cells.²⁷ It is maintained in a catalytically inactive state by the interaction between the thiol group of the pro-domain cysteine residue and the zinc ion of the catalytic site. Disruption of this interaction by cleavage of the pro-domain or by modification of the cysteine thiol group (the so-called cysteine switch) converts pro-MMP-9 to an active protease.²⁸ The expression of TIMP-1, the endogenous inhibitor of catalytic MMP-9, is initiated by many of the same pro-inflammatory mediators that induce MMP-9.²⁹ It is reported to be co-expressed with MMP-9 in migrating cells where it binds with high affinity to the catalytic domain of activated MMP-9 inhibiting its ability to bind substrate.³⁰ MMP-9-inhibitor complexes are internalized into cells via the low-density lipoprotein receptor-related scavenger protein where they undergo endosomal degradation.³¹ Thus TIMP-1 is positioned to rapidly neutralize active MMP-9, minimizing its capacity for unwanted matrix degradation. In the current study, TIMP-1 showed a time dependent increase in gene expression in parallel with MMP-9, and TIMP-1 protein was also expressed in abundance 14 hr postoperatively. The inhibitory activity of TIMP-1 and its role in the sequestering and degradation of MMP-9 likely accounts for the low levels of catalytically active MMP-9 detected in POI, despite the abundant increase in MMP-9 protein.

Bowel surgery had little effect on MMP-2 mRNA expression. In keeping with its known constitutive expression,⁴ the ΔC_t value for MMP-2 in non-operated control animals was much lower (7.66 ± 0.28) than that for MMP-9 (14.25 ± 0.51), indicating that MMP-2 mRNA was present at higher baseline copy number. This was supported by ELISA where significant MMP-2 protein was detected in tissues from control animals. The function of endogenously expressed MMP-2 is not well understood. MMP2 and MMP-9 have similar activity for gelatin *in vitro*,³ however gelatin zymography performed on muscularis wholemounts from control animals showed only minimal gelatinase activity up to 90 min of incubation implying that little of the constitutively expressed MMP-2 was catalytically active. Following surgical manipulation, MMP-2 protein levels were only modestly increased (<2-fold). The primary activation route of MMP-2 on the cell surface is through the formation of a molecular complex containing MMP-2, MT1-MMP and TIMP-2 which localizes with integrins on the cell surface.³² Integrins have the capacity to gather several proteins into a single complex, and integrins that localize the MMP-2 complex can also recruit MMP-9 during cell attachment and release from the substrate.^{11, 12} Thus, MMP-2 may be activated only at the instant that cell attachment and release is actively in progress. The application of neutralizing antibodies to MMP-9 or MMP-2 during *in situ* zymography indicated that the gelatinase activity observed in tissue from manipulated animals was mediated predominantly by MMP-9 activity within infiltrating leukocytes.

In postoperative ileus, COX-2 derived prostaglandins and iNOS-derived NO, produced by resident and recruited macrophages, are well established as mediators that have direct inhibitory effects on smooth muscle contractility.^{15, 33, 34} Prostaglandins and nitric oxide also play a role in leukocyte recruitment, where enzyme inhibition or genetic deletion markedly inhibits cellular inflammation in POI;^{15, 33} and both mediators induce MMP-9 expression in rodent macrophages.^{25, 35} iNOS has also been shown to co-localize with MMP-9 at the leading edge of actively migrating cells²¹ where it is positioned to release nitric oxide in close proximity to MMP-9. Nitric oxide is reported to interact with the zinc ions and cysteine residues of the cysteine switch, contributing to the conversion of pro-MMP-9 to its catalytic form.^{21, 22} Furthermore, iNOS deficiency impairs MMP-9 activity

and disrupts leukocyte migration in models of cardiovascular disease²³ and hepatic ischemia/reperfusion injury.²⁴ In the current study, we evaluated the effects of iNOS deletion on MMP-9 and TIMP-1 gene expression. In wild type mice, iNOS was induced in the small bowel and colonic muscularis following surgical manipulation. In the iNOS KO mouse, surgically induced MMP-9 gene expression was reduced by ~90% in the colon, and by ~70% in the small bowel indicating that nitric oxide differentially regulates MMP-9 gene expression in these organs. This finding is consistent with earlier reports describing differences in the relative contribution of iNOS and COX-2 derived mediators to the impairment in small bowel and colonic motility,³⁶ where it was observed that both prostaglandins and nitric oxide were involved in suppressing smooth muscle contractility in the small intestine, but impaired contractility in the colon was almost entirely mediated by nitric oxide. Further evidence for regional differences in the effects of iNOS was reported in the current study, where iNOS deletion did not alter baseline or surgically induced TIMP-1 mRNA expression in the small bowel muscularis of non-operated iNOS KO mice; but resulted in elevated basal levels of TIMP-1 mRNA in the colonic muscularis. This suggests that, in the colon, baseline levels of iNOS derived nitric oxide may regulate baseline TIMP-1 gene expression. After surgical manipulation, postoperative TIMP-1 ΔC_T values in both the small bowel and colon were comparable to wild type. These findings suggest that the protective effects of iNOS inhibition in POI may be due in part to a shift to a more anti-inflammatory phenotype where the induction of MMP-9 gene expression is suppressed while leaving TIMP-1 gene expression, and therefore TIMP-1-mediated inhibitory mechanisms, intact. In the current study, a contribution from COX-2 derived prostaglandins cannot be ruled out and may account for the residual iNOS-independent MMP-9 gene expression in the small bowel muscularis of surgically manipulated iNOS KO mice. Nevertheless, the available data places iNOS-derived NO as an important regulator of MMP-9 expression in gastrointestinal tract.

The role of MMP-9 in the cellular inflammatory response and dysmotility associated with POI *in vivo* was evaluated in the mouse by MMP-9 gene deletion and by treatment with a dual MMP-2/MMP-9 active site inhibitor (MMPi). Surgical manipulation of the small bowel in wild type animals resulted in a dramatic increase in MPO-positive leukocytes within the intestinal muscularis. This cellular influx was reduced by 93% in the MMP-9 KO. A similar reduction in cellular inflammation was observed in a second series of experiments where wild type animals were treated with MMPi. Because this inhibitor was not selective for MMP-9, it was important to differentiate between the activities of the two gelatinases before concluding that the protective effects of the active site inhibitor were mediated through inhibition of MMP-9. Results from *in situ* zymography experiments demonstrated that MMP-2 activity contributed little if any gelatinase activity within the bowel muscularis. These findings, taken together with little or no induction of MMP-2 gene and protein expression, indicate that MMP-9 plays the central role in mediating cellular trafficking during postoperative ileus. The resulting reduction in cellular influx associated with MMP-9 inhibition was accompanied by a significant improvement in small bowel and colonic smooth muscle contractility resulting in an improvement in the surgically induced delay in gastrointestinal transit.

In conclusion, the current study demonstrates that MMP-9 plays a prominent role in immune cell recruitment into the postoperative intestinal muscularis and that the induction of MMP-9 was iNOS-dependent. Inhibition of MMP-9 activity attenuated bowel inflammation and dysmotility associated with POI, suggesting the prophylactic dosing with an MMP-9 inhibitor would provide benefit to patients at high risk for development of post-operative dysmotility. The relevance of MMP-9 in other human gastrointestinal inflammatory disorders is underscored by observations that both MMP-9 and MMP-2 are elevated in the urine and in mucosal tissues of patients with Crohn's disease and ulcerative colitis.^{42, 43}

These observations are supported by preclinical models of IBD where MMP-9 was shown to be elevated in the mucosa in TNBS and DSS colitis, and where MMP inhibition or MMP-9 gene deletion was protective.^{37, 38} We have also observed elevated MMP-9 protein expression in the colonic mucosa in the rat model of POI (Control: 9.7±4.9 vs POI: 240±85 ng/g tissue wet weight; $p < 0.05$) suggesting that targeting MMP-9 may be beneficial in inflammatory diseases affecting the intestinal mucosa. The development of pharmaceutical agents suitable for the clinic has been stymied by the structural uniformity of the active site among the various MMPs, resulting in agents that show poor specificity and have significant side effects. Nevertheless, MMP-9 remains a viable target for pharmaceutical intervention, awaiting more selective agents for controlling MMP-9 activity or expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported in part by National Institute of Health grants: R01-GM58241, R01-DK068610, P50-GM-53789, and DK02488.

References

1. Murphy G, Nagase H. Progress in matrix metalloproteinase research. *Mol Aspects Med.* 2008; 29:290–308. [PubMed: 18619669]
2. Malla N, Sjöli S, Winberg JO, Hadler-Olsen E, Uhlin-Hansen L. Biological and pathobiological functions of gelatinase dimers and complexes. *Connect Tissue Res.* 2008; 49:180–4. [PubMed: 18661338]
3. Björklund M, Koivunen E. Gelatinase-mediated migration and invasion of cancer cells. *Biochim Biophys Acta.* 2005; 1755:37–69. [PubMed: 15907591]
4. Huhtala P, Tuuttila A, Chow LT, Lohi J, Keski-Oja J, Tryggvason K. Complete structure of the human gene for 92-kDa type IV collagenase. Divergent regulation of expression for the 92- and 72-kilodalton enzyme genes in HT-1080 cells. *J Biol Chem.* 1991; 266:16485–90. [PubMed: 1653238]
5. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol.* 2002; 37:375–536. [PubMed: 12540195]
6. Ram M, Sherer Y, Shoenfeld Y. Matrix metalloproteinase-9 and autoimmune diseases. *J Clin Immunol.* 2006; 26:299–307. [PubMed: 16652230]
7. Tchetverikov I, Lard LR, DeGroot J, Verzijl N, TeKoppele JM, Breedveld FC, Huizinga TW, Hanemaaijer R. Matrix metalloproteinases-3, -8, -9 as markers of disease activity and joint damage progression in early rheumatoid arthritis. *Ann Rheum Dis.* 2003; 62:1094–9. [PubMed: 14583574]
8. Hamada T, Fondevila C, Busuttil RW, Coito AJ. Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury. *Hepatology.* 2008; 47:186–98. [PubMed: 17880014]
9. Kuyvenhoven JP, Verspaget HW, Gao Q, Ringers J, Smit VT, Lamers CB, van Hoek B. Assessment of serum matrix metalloproteinases MMP-2 and MMP-9 after human liver transplantation: increased serum MMP-9 level in acute rejection. *Transplantation.* 2004; 77:1646–52. [PubMed: 15201662]
10. Garg P, Vijay-Kumar M, Wang L, Gewirtz AT, Merlin D, Sitaraman SV. Matrix metalloproteinase-9-mediated tissue injury overrides the protective effect of matrix metalloproteinase-2 during colitis. *Am J Physiol Gastrointest Liver Physiol.* 2009; 296:G175–84. [PubMed: 19171847]
11. Morini M, Mottotese M, Ferrari N, Ghiorzo F, Buglioni S, Mortarini R, Noonan DM, Natali PG, Albini A. The alpha 3 beta 1 integrin is associated with mammary carcinoma cell metastasis, invasion, and gelatinase B (MMP-9) activity. *Int J Cancer.* 2000; 87:336–42. [PubMed: 10897037]

12. Rolli M, Fransvea E, Pilch J, Saven A, Felding-Habermann B. Activated integrin α v β 3 cooperates with metalloproteinase MMP-9 in regulating migration of metastatic breast cancer cells. *Proc Natl Acad Sci U S A*. 2003; 100:9482–7. [PubMed: 12874388]
13. Boeckxstaens GE, de Jonge WJ. Neuroimmune mechanisms in postoperative ileus. *Gut*. 2009; 58:1300–11. [PubMed: 19671558]
14. Moore BA, Peffer N, Pirone A, Bassiri A, Sague S, Palmer JM, Johnson DL, Nesspor T, Kliwinski C, Hornby PJ. GLP-2 receptor agonism ameliorates inflammation and gastrointestinal stasis in murine postoperative ileus. *J Pharmacol Exp Ther*. 333:574–83. [PubMed: 20167840]
15. Turler A, Kalff JC, Moore BA, Hoffman RA, Billiar TR, Simmons RL, Bauer AJ. Leukocyte-derived inducible nitric oxide synthase mediates murine postoperative ileus. *Ann Surg*. 2006; 244:220–9. [PubMed: 16858184]
16. Ogata Y, Enghild JJ, Nagase H. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J Biol Chem*. 1992; 267:3581–4. [PubMed: 1371271]
17. Duncan ME, Richardson JP, Murray GI, Melvin WT, Fothergill JE. Human matrix metalloproteinase-9: activation by limited trypsin treatment and generation of monoclonal antibodies specific for the activated form. *Eur J Biochem*. 1998; 258:37–43. [PubMed: 9851689]
18. Gottsch ML, Van Kirk EA, Murdoch WJ. Role of matrix metalloproteinase 2 in the ovulatory folliculo-luteal transition of ewes. *Reproduction*. 2002; 124:347–52. [PubMed: 12201808]
19. Mook OR, Van Overbeek C, Ackema EG, Van Maldegem F, Frederiks WM. In situ localization of gelatinolytic activity in the extracellular matrix of metastases of colon cancer in rat liver using quenched fluorogenic DQ-gelatin. *J Histochem Cytochem*. 2003; 51:821–9. [PubMed: 12754293]
20. Radjabi AR, Sawada K, Jagadeeswaran S, Eichbichler A, Kenny HA, Montag A, Bruno K, Lengyel E. Thrombin induces tumor invasion through the induction and association of matrix metalloproteinase-9 and β 1-integrin on the cell surface. *J Biol Chem*. 2008; 283:2822–34. [PubMed: 18048360]
21. Harris LK, McCormick J, Cartwright JE, Whitley GS, Dash PR. S-nitrosylation of proteins at the leading edge of migrating trophoblasts by inducible nitric oxide synthase promotes trophoblast invasion. *Exp Cell Res*. 2008; 314:1765–76. [PubMed: 18394602]
22. Gu Z, Kaul M, Yan B, Kridel SJ, Cui J, Strongin A, Smith JW, Liddington RC, Lipton SA. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science*. 2002; 297:1186–90. [PubMed: 12183632]
23. Marcet-Palacios M, Graham K, Cass C, Befus AD, Mayers I, Radomski MW. Nitric oxide and cyclic GMP increase the expression of matrix metalloproteinase-9 in vascular smooth muscle. *J Pharmacol Exp Ther*. 2003; 307:429–36. [PubMed: 12954809]
24. Hamada T, Duarte S, Tsuchihashi S, Busuttill RW, Coito AJ. Inducible nitric oxide synthase deficiency impairs matrix metalloproteinase-9 activity and disrupts leukocyte migration in hepatic ischemia/reperfusion injury. *Am J Pathol*. 2009; 174:2265–77. [PubMed: 19443702]
25. Steenport M, Khan KM, Du B, Barnhard SE, Dannenberg AJ, Falcone DJ. Matrix metalloproteinase (MMP)-1 and MMP-3 induce macrophage MMP-9: evidence for the role of TNF- α and cyclooxygenase-2. *J Immunol*. 2009; 183:8119–27. [PubMed: 19923455]
26. Kalff JC, Buchholz BM, Eskandari MK, Hierholzer C, Schraut WH, Simmons RL, Bauer AJ. Biphasic response to gut manipulation and temporal correlation of cellular infiltrates and muscle dysfunction in rat. *Surgery*. 1999; 126:498–509. [PubMed: 10486602]
27. Owen CA, Hu Z, Barrick B, Shapiro SD. Inducible expression of tissue inhibitor of metalloproteinases-resistant matrix metalloproteinase-9 on the cell surface of neutrophils. *Am J Respir Cell Mol Biol*. 2003; 29:283–94. [PubMed: 12663332]
28. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol*. 2004; 4:617–29. [PubMed: 15286728]
29. Clark IM, Swingler TE, Sampieri CL, Edwards DR. The regulation of matrix metalloproteinases and their inhibitors. *Int J Biochem Cell Biol*. 2008; 40:1362–78. [PubMed: 18258475]
30. Moestrup SK, Holtet TL, Etzerodt M, Thøgersen HC, Nykjaer A, Andreasen PA, Rasmussen HH, Sottrup-Jensen L, Gliemann J. Alpha 2-macroglobulin-proteinase complexes, plasminogen activator inhibitor type-1-plasminogen activator complexes, and receptor-associated protein bind

to a region of the alpha 2-macroglobulin receptor containing a cluster of eight complement-type repeats. *J Biol Chem.* 1993; 268:13691–6. [PubMed: 7685767]

31. Hahn-Dantona E, Ruiz JF, Bornstein P, Strickland DK. The low density lipoprotein receptor-related protein modulates levels of matrix metalloproteinase 9 (MMP-9) by mediating its cellular catabolism. *J Biol Chem.* 2001; 276:15498–503. [PubMed: 11279011]
32. Nabeshima K, Inoue T, Shimao Y, Okada Y, Itoh Y, Seiki M, Kono M. Front-cell-specific expression of membrane-type 1 matrix metalloproteinase and gelatinase A during cohort migration of colon carcinoma cells induced by hepatocyte growth factor/scatter factor. *Cancer Res.* 2000; 60:3364–9. [PubMed: 10910039]
33. Kalff JC, Schraut WH, Billiar TR, Simmons RL, Bauer AJ. Role of inducible nitric oxide synthase in postoperative intestinal smooth muscle dysfunction in rodents. *Gastroenterology.* 2000; 118:316–27. [PubMed: 10648460]
34. Schwarz NT, Kalff JC, Turler A, Engel BM, Watkins SC, Billiar TR, Bauer AJ. Prostanoid production via COX-2 as a causative mechanism of rodent postoperative ileus. *Gastroenterology.* 2001; 121:1354–71. [PubMed: 11729115]
35. Ridnour LA, Windhausen AN, Isenberg JS, Yeung N, Thomas DD, Vitek MP, Roberts DD, Wink DA. Nitric oxide regulates matrix metalloproteinase-9 activity by guanylyl-cyclase-dependent and -independent pathways. *Proc Natl Acad Sci U S A.* 2007; 104:16898–903. [PubMed: 17942699]
36. Turler A, Moore BA, Pezzone MA, Overhaus M, Kalff JC, Bauer AJ. Colonic postoperative inflammatory ileus in the rat. *Ann Surg.* 2002; 236:56–66. [PubMed: 12131086]
37. Medina C, Santana A, Paz MC, Diaz-Gonzalez F, Farre E, Salas A, Radomski MW, Quintero E. Matrix metalloproteinase-9 modulates intestinal injury in rats with transmural colitis. *J Leukoc Biol.* 2006; 79:954–62. [PubMed: 16478919]
38. Santana A, Medina C, Paz-Cabrera MC, Diaz-Gonzalez F, Farre E, Salas A, Radomski MW, Quintero E. Attenuation of dextran sodium sulphate induced colitis in matrix metalloproteinase-9 deficient mice. *World J Gastroenterol.* 2006; 12:6464–72. [PubMed: 17072979]

ABBREVIATIONS

COX-2	Cyclooxygenase-2
DQ	Dye quenched
ECM	Extracellular matrix
HRP	Horse radish peroxidase
KO	Knock out
iNOS	Inducible nitric oxide synthase
mAb	monoclonal antibody
pAb	polyclonal antibody
MMP	Matrix metalloprotease
MMPi	MMP inhibitor (specifically, MMP-2/MMP-9 II)
MPO	Myeloperoxidase
NO	Nitric oxide
POI	Postoperative ileus
TIMP	Tissue inhibitor of matrix metalloprotease
WT	Wild type

Figure 1.

Real time RT-PCR time course analysis of gene expression in the mouse small bowel and colonic muscularis 3, 6, 12, and 24 hr after surgical manipulation. The induction of MMP-9 and TIMP-1 was time-dependent; reaching peak levels 12-24 hr postoperatively. Surgical manipulation resulted in a time-dependent decrease in MMP-2 gene expression in the small bowel, but had no effect in the colon. Data were calculated relative to β -actin endogenous reference gene as mean fold change relative to naïve control (NC) $\pm$ SEM, n=4 per group. One-way ANOVA with Bonferroni post testing for multiple comparisons. * $P < .05$.

AUTHOR'S NOTE: Table 3 is also provided in the text but is best presented here with Figure 2.

**Table 3. MMP-9, MMP-2, and TIMP-1 protein expression^a
(ng/g tissue wet wt)**

	Small Bowel Muscularis		Colonic Muscularis	
	Control	POI	Control	POI
MMP-9	<5	680±55*	<5	630±23*
MMP-2	88±7	150±4.8*	130±34	180±6.0
TIMP-1	13±1.2	1200±150*	28±3.8	930±110*

^a Rats were subjected to surgical manipulation and sacrificed 14 hr post-operatively. MMP-2 and TIMP-1 were assayed in tissue homogenates by ELISA, and MMP-9 was estimated by comparing Western blot band intensities to a known standard.

* Compares POI vs control, $P < 0.05$ by Student's T Test.

Figure 2.

Expression of the pro- and active forms of MMP-9 protein in the rat small bowel muscularis 14 hr after surgical manipulation. MMP-9 expression was assessed in tissue lysates by Western blot analysis. A. Tissue lysates probed with mAb L51/82 recognizing both the pro- and active forms of MMP-9. Pro-MMP-9 was abundantly expressed in tissues from surgically manipulated rats. B. Tissue lysates probed with pAb 1246 recognize the N-terminal neopeptide specific to the 82 kd active form of MMP-9. Active MMP-9 was increased after surgical manipulation. The standard in each panel was combination of 2 ng of recombinant rat pro-MMP-9 and 2 ng of rat MMP-9 converted to the active form with catalytic MMP-3.

Figure 3.

In situ zymography of rat small bowel muscularis whole mounts. A. Gelatinase activity was absent in muscularis wholemounts from non-operated controls (n=3). B. Numerous gelatinase positive immune cells were detected in muscularis harvested 14 hr postoperatively (n=3). C. Enlargement of region outlined in B. Localized regions of gelatinase activity were observed in close approximation to the cell membrane/extracellular matrix interface (arrows). D. Gelatinase activity was abolished by application of mouse anti-human MMP-9 neutralizing antibody. E. Gelatinase activity was unaffected by application of mouse anti-human MMP-2 neutralizing antibody.

Figure 4.

Real time RT-PCR analysis of the effects of iNOS gene deletion on peak MMP-9 and TIMP-1 gene expression (12 hr postoperatively). A. Time course analysis of iNOS gene expression following surgical manipulation in the wild type mouse shows significant induction in both the small bowel and colon relative to naïve control (NC). * = $P < .05$ by ANOVA, with Bonferroni post hoc testing for multiple comparisons. B. Surgically induced MMP-9 gene expression was reduced by ~70% in the small bowel muscularis and ~90% in the colonic muscularis in the iNOS KO mouse. * = $P < .05$ by unpaired Student's T-test. C. TIMP-1 gene expression was not significantly altered in the small bowel. In the colon, iNOS deletion appeared to completely prevent the surgically induced increase in TIMP-1 gene expression (small solid grey bar). However, baseline TIMP-1 copy number was markedly higher in the KO compared to WT (Table 1). When recalculated relative to the WT baseline, post-surgical relative TIMP-1 copy number is not different from WT (dotted bar; see text for

a detailed discussion). Expression levels were calculated relative to β -actin endogenous reference gene and are presented as mean fold-change relative to naïve control (NC) $\pm$ SEM, n=4 per group.

Figure 5. MMP-9 KO mice are resistant to postoperative ileus (POI). A. Representative digital photomicrographs of small bowel muscularis whole mount harvested 24 hr postoperatively. Myeloperoxidase (MPO) positive leukocytes were absent in non-operated control animals, but were present in large numbers within the muscularis of surgically manipulated animals with POI (arrows). The numbers of leukocytes were dramatically reduced in tissue from MMP-9 KO mice. B. Histogram summarizing MPO-positive cell counts among experimental groups for statistical comparison. Data are mean $\pm$ SEM, n=6 per group. Statistical analysis was by one-way ANOVA with Bonferroni post hoc testing for multiple comparisons. *Compares surgically manipulated WT with WT control, $P < 0.0001$; # compares surgically manipulated KO with WT, $P < .001$. C. Histogram summarizing gastrointestinal transit (Geometric Center; GC) among experimental groups. GC was reduced in the WT mouse 24 hr after surgical manipulation indicating delayed transit. The MMP-9 KO mouse was partially protected. Data are mean $\pm$ SEM, n=6 per group. * Compares surgically manipulated WT with WT control, $P < .01$ relative to control; # compares KO with WT, $P < .05$. One-way ANOVA with Tukey post hoc testing for multiple comparisons

Figure 6.

Inhibition of MMP-9 catalytic activity protects wild type mice from intestinal smooth muscle dysmotility. A. Treatment with MMP-2/MMP-9 II (MMPi; 10 mg/kg) inhibited the influx of myeloperoxidase (MPO)-positive leukocytes into the small bowel and colonic muscularis determined 24 hr after colonic manipulation (CM). Data are mean $\pm$ SEM, $n=4$ per group. * Compares CM with control, $P < .0001$; # compares MMPi treated with vehicle treated, $P < .0001$. B. Representative traces showing colonic circular smooth muscle contractile responses to 100 μ M bethanechol. In control tissues, bethanechol induces a tonic contraction overlain by a series of phasic contractions. Surgical manipulation resulted in a reduction in magnitude of the tonic contraction and a loss of the phasic response. Contractile responses were improved in animals treated with MMPi. C. Integrated colonic smooth muscle contractile responses to increasing concentrations of bethanechol. Treatment with MMPi prevented the surgically induced reduction in the magnitude of the integrated contractility response. Data are mean $\pm$ SEM, $n=4$ per group. Histogram summarizes peak integrated contractile responses to 100 μ M bethanechol in small bowel and colonic smooth

muscle. Contractility is inhibited after surgical manipulation and improved after treatment with MMPi. Data are mean $\pm$ SEM, n=4 per group. * Compares vehicle treated with control, $P < 0.001$; # compares MMPi treated with vehicle treated, $P < 0.01$. D. Histogram summarizing Geometric Center as a measure of gastrointestinal transit. The surgically induced delay in transit is attenuated with MMPi treatment. Data are mean $\pm$ SEM, n=8 per group. * Compares untreated and vehicle treated with control, $P < .001$; # compares MMPi treated with untreated and vehicle treated, $P < .001$. One-way ANOVA with Tukey post hoc testing for multiple comparisons.

Table 1MMP-9, MMP-2, and TIMP-1 protein expression^a (ng/g tissue wet wt)

Gene	Small Bowel Muscularis		Colonic Muscularis	
	Control	POI	Control	POI
MMP-9	<5	680 ± 55 *	<5	630 ± 23 *
MMP-2	88 ± 7.0	150 ± 4.8 *	130 ± 34	180 ± 6.0
TIMP-1	13 ± 1.2	1200 ± 150 *	28 ± 3.8	930 ± 110 *

^aRats were subjected to surgical manipulation and sacrificed 14 hr post-operatively. MMP-2 and TIMP-1 were assayed in tissue homogenates by ELISA, and MMP-9 was estimated by comparing Western blot band intensities to a known standard.

* Compares POI vs control, $P < 0.05$ by Student's T-Test