Increased expression of midkine in the rat colon during healing of experimental colitis

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Submitted 22 August 2005; accepted in final form 29 March 2006

Yuki, Takafumi, Shunji Ishihara, M. A. K. Rumi, Cesar F. Ortega-Cava, Yasunori Kadowaki, Hideaki Kazumori, Norihisa Ishimura, Yuji Amano, Nobuyuki Moriyama, and Yoshikazu Kinoshita. Increased expression of midkine in the rat colon during healing of experimental colitis. Am J Physiol Gastrointest Liver Physiol 291: G735–G743, 2006; doi:10.1152/ajpgi.00388.2005.—Midkine (MK) is a unique growth and differentiation factor that modulates the proliferation and migration of various cells; however, little is known regarding its relationship to intestinal diseases. The aim of this study was to investigate MK expression and its role in dextran sulfate sodium (DSS)-induced colitis in rats. The expressions of MK, receptor-like protein-tyrosine phosphatase (RPTP)-β, and proinflammatory cytokines were examined in rat colonic tissues after the development of DSS-induced colitis using Northern blotting, immunohistochemistry, and laser-capture microdissection (LCM) coupled with RT-PCR. The effects of MK on the migration of intestinal epithelial cells (IEC-6) were also evaluated in vitro using an intestinal wound repair model. MK expression was significantly increased in damaged colonic mucosa, mainly from day 3 to day 5 after the end of DSS administration, with abundant MK immunoreactive signals detected in submucosal fibroblasts. Expressions of proinflammatory cytokines were most strongly induced on day 1, which preceded the augmentation of MK expression. Results of LCM coupled with RT-PCR clearly indicated RPTP-β expression in colonic epithelial cells. The migration assay showed that wound repair in the MK-treated groups was accelerated dose dependently. The present results showed for the first time that intestinal inflammation upregulates the MK-RPTP-β system, which may stimulate mucosal regeneration during the process of healing of colitis. Additional investigations regarding the role of MK may contribute to the development of new options for the treatment of inflammatory bowel diseases.

receptor-like protein-tyrosine phosphatase-β; proinflammatory cytokines; inflammatory bowel diseases; dextran sulfate sodium-induced colitis

A VARIETY OF CYTOKINES and growth factors are well-known mediators that maintain the physiological and immunological functions of the intestinal tract (12, 39). In particular, growth factors and their receptors are essential molecules to control cell-to-cell communication between epithelial and mesenchymal cells and enhance mucosal regeneration during the healing process following intestinal inflammation (4, 46). Ulcerative colitis (UC) and Crohn disease (CD) are two major forms of human inflammatory bowel diseases (IBD), which are characterized by chronic intestinal immune-mediated disorders of unknown etiology. In IBD, several kinds of growth factors, including heparocyte growth factor (HGF) (20, 35, 45), epidermal growth factor (EGF) (1, 25), and transforming growth factor (TGF)-α (8, 11), have been shown to be increased in the intestinal inflammatory site, suggesting a possible link between those growth factors and mucosal regeneration by epithelial cell proliferation and migration. In recent years, a number of basic and clinical studies have focused on growth factors and their role in IBD with the objective of developing a novel therapy (31, 33, 34, 42).

Midkine (MK) has been identified as the product of a retinoic acid-responsive gene and is a member of a newly described family of heparin-binding growth and differentiation factors (16, 17, 26). MK was recently demonstrated to bind to receptor-like protein-tyrosine phosphatase (RPTP)-β, a chondroitin sulfate proteoglycan that is expressed abundantly in the brain (21). In the central nervous system, the MK-RPTP-β system is physiologically involved in neuronal organogenesis, as it influences cellular proliferation, differentiation, migration, and adhesion (21, 22). This system also plays an important regulatory role at sites of epithelial-mesenchymal interactions during the process of inflammation in several organs and is transiently upregulated in damaged tissues after injury, where it enhances tissue reconstruction during the repair process (14, 28). We recently reported that the MK-RPTP-β system was upregulated in rat stomachs with experimental mucosal lesions induced by indomethacin and acetic acid, suggesting that MK may stimulate mucosal regeneration during the healing process following gastric mucosal damage (23, 50).

Although MK has been suggested to be a key molecule in the repair of gastrointestinal mucosal injury, nothing is known regarding its role in the pathogenesis of IBD. In the present study, we investigated the in vivo expression of MK and RPTP-β in rat large intestines after inducing colitis with dextran sulfate sodium (DSS). DSS-induced colitis is widely used as a model of human UC, whose possible pathogenesis includes reproducible and well-characterized mucosal injury with cellular infiltration, as well as interference with the normal interactions among epithelial and mesenchymal cells (5, 24, 30). In addition, we examined the effects of MK on cell restitution in vitro using an intestinal wound repair model developed with cultured epithelial monolayer sheets (29). The present results revealed for the first time that intestinal inflammation-dependent upregulation of the MK-RPTP-β system accelerates mucosal regeneration during experimental colitis, which may contribute to the development of a new therapy for IBD.
MATERIALS AND METHODS

Animals and experimental colitis. Seven-week-old male Sprague-Dawley (SD) rats were obtained (Nihon Clea, Tokyo, Japan) and then cared for and handled in accordance with guidelines of the National Institutes of Health and the Institute for Animal Experimentation of Shimane University. Our experiment and protocols were submitted to and approved by the Institute for Animal Experimentation of Shimane University. The animals were housed under constant environmental conditions with circadian light-dark cycles and given an initial adaptation period of 1 wk. In the experimental groups, colitis was induced by DSS (5 kDa; Wako Pure Chemical Industries, Osaka, Japan), which was dissolved in drinking water to a concentration of 5% (wt/vol). The solution was changed each day and administered for 7 days. The control group was given water lacking DSS. The animals were killed by an overdose of diethyl ether, after which the colons were extracted and divided into proximal, medial, and distal portions. The proximal colon was the cecum, whereas the remaining portion was divided into two segments of equal length. For this study, only the distal colon was examined, since it is considered to be the most vulnerable to DSS-induced colitis. Furthermore, a segment of each distal colon was dissected to investigate mRNA expression or for histological examinations.

RNA extraction and RT-PCR analysis. Total RNA was extracted from frozen tissues, and cells were cultured using a single-step guanidinium thiocyanate-phenol-chloroform method (Isogen; Nippon Gene, Tokyo, Japan). Each RNA sample was subjected to RT using a first-strand synthesis kit (Stratagene Toyobo, Tokyo, Japan) to prepare cDNA. The resulting first-strand cDNA was amplified by a PCR method. When total RNA was extracted from laser-captured samples, RT-PCR amplification of the samples was performed as described previously (50). The primer sequences for RT-PCR were designed by using the Primer 3.0 software (41). The primers used for cDNA amplification were: MK (479 bp), 5'-AGGGCTCTTGGCTGTCACT-3' and 5'-GGTGTCTTGAGCTGCCAGGCA-3'; RPTP-β cDNA (354 bp), 5'-GTGAAGCCGACCTGCTGTA-3' and 5'-TGGGACCCCAAAGTCTCTGATAG-3'; cytokertatin-20 cDNA (305 bp), 5'-CACATCATCCGGGACTACAGTCTT-3' and 5'-CTTCGACTCTTCTTGGATCC-3'; CD45 cDNA (588 bp), 5'-CACCCACCGGAGCTCAACACTTCTGAACTCAGTATAGGCA-3' and 5'-CTTCGACTTCTTCTTGGATCC-3'; CD45 (588 bp), 5'-CGAGCAAGGGGATCTCTCAGGTCTGAGTATAGGCA-3' and 5'-GTGAAGCCGACCTGCTGTA-3'; and β-actin cDNA (550 bp), 5'-TGGGTATGGGTGCTAGAGAGGAC-3' and 5'-GCATCTCTTCTGCTGAGTCT-3'. RPTP-β has three alternative splicing isoforms; however, since it is not known which is expressed in the colon, the RPTP-β primers were set for the common region. After an initial hot start at 95°C for 10 min, PCR was carried out in a DNA thermal cycler (PerkinElmer, Foster City, CA) for 35 cycles, with denaturing at 95°C for 45 s; annealing at 60°C for 45 s (MK and RPTP-β), 55°C for 30 s (cytokeratin-20 and CD45), 68°C for 30 s (β-actin); and extension at 72°C for 90 s (MK and RPTP-β), 40 s (cytokeratin-20 and CD45), or 30 s (β-actin). A final elongation step was carried out for 7 min at 72°C. The PCR products were then sequenced directly, and RNA extracted from the rat brains was used as a positive control.

Northern blot analysis. Total RNA was extracted as previously described (50), after which 20 µg were electrophoresed on a 1% agarose gel containing 0.66 mmol/l of formaldehyde, then transferred to a supported nitrocellulose membrane (Hybond-c Extra; Amersham International, Little Chalfont, UK) and UV linked. Probes were prepared by PCR amplification of rat first-strand cDNA using primers for MK and β-actin. Other probes used for Northern blot analysis were a 0.6-kb cDNA of rat interleukin (IL)-1β, a 0.5-kb cDNA of rat TNF-α, and a 0.3-kb cDNA of rat cytokine-induced neutrophil chemoattractant (CINC)2-β. All probes were 32P labeled. Hybridization was carried out at 42°C, then the membranes were washed first for 5 min and then for 10 min at 37°C with 2× SSC containing 0.2% SDS and finally for 10 min at 50°C in 0.1× SSC containing 0.2% SDS. A bio-image analyzer (BAS 2000; Fujix, Tokyo, Japan) was used to detect signals from the hybridized radiolabeled DNA probes, and the intensity of each was normalized using the intensity of the β-actin mRNA signals as a reference.

Immunohistochemistry. For immunostaining, the distal colon was rinsed immediately after being opened in segments and then immersed in embedding medium (Tissue-Tek OCT compound; Sakura Finetecnical, Tokyo, Japan), frozen on dry ice, and stored at −70°C. Frozen samples of 6-µm-thick sections were prepared and fixed in cold acetone for 10 min. After washing, endogenous peroxidase activity was blocked with 3% H2O2 in water for 10 min at room temperature, followed by incubation with normal blocking serum for 30 min. Subsequently, sections were incubated for 2 h at room temperature with anti-MK primary antibody (Ab) (sc-1398; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:50 dilution, and then the staining was processed using a commercial immunoperoxidase staining kit (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Sections were counterstained with hematoxylin. Negative controls were produced by omitting the primary Ab. Because previous studies have shown that MK is abundantly expressed in mesenchymal cells, including fibroblasts in a variety of tissues (14, 21, 22, 28), we performed two-color immunofluorescence stainings for MK and vimentin, a marker of fibroblasts, in the histological section. The frozen sections were fixed in acetone, then labeled with anti-MK (1:50, Santa Cruz) and anti-vimentin (1:100, Dako). Binding of the primary Ab was detected by FITC-conjugated anti-goat immunoglobulin (Dako) and rhodamine-conjugated anti-mouse immunoglobulin (Dako).

Laser-capture microdissection. Fresh frozen colon tissues were cut into 5-µm-thick sections using a cryostat, then mounted on noncoated glass slides, fixed immediately with 70% ethanol for 30 s, and washed with diethylpyrocarbonate (DEPC)-treated water for 30 s. Next, the sections were stained rapidly with hematoxylin for 30 s, dehydrated by passage through an ethanolic gradient, counterstained with eosin Y for 1 min, dehydrated again with an ethanol gradient, cleared with xylene, and air-dried. The sections were laser microdissected using the laser-capture microdissection (LCM) system LM200 (Olympus, Tokyo, Japan), as reported previously (18). In brief, tissue sections on slides were placed on the stage of a microscope, and an area of tissue was selected and covered with LCM transfer film. The focus of the microscope was adjusted so that the field of view of the required specific portion of tissue was the same size as that of the desired target. Under direct microscopic observation, the targeted cells were subjected to brief laser pulses directly from above using a laser beam 30 µm in diameter and a laser power of 60 mW. Samples captured by 5,000 shots on each transfer film cap were immersed in RNA extraction solution. To exclude the possibility that contaminating cells within the colonic epithelial preparation may be responsible for the detection of RPTP-β expression, we performed RT-PCR to detect the cytokeratin-20 (an epithelial maker) and CD45 (pan-leukocyte maker) mRNA simultaneously in LCM samples, as reported previously (13, 18, 36).

Effects of proinflammatory cytokines on MK expression in 3Y1 cells. To study the influence of proinflammatory cytokines toward MK expression in vitro, the rat fibroblast cell line 3Y1 was obtained from JBRB Cell Bank (Health Science Research Resources Bank, Osaka, Japan). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 0.1% glucose and 10% heat-inactivated fetal bovine serum (FBS), then four groups of cultured cells were inoculated onto collagen type I-coated plastic culture dishes. After changing the DMEM with 2% FBS, the first group was left alone to serve as a control, whereas the other three were incubated with 10 ng/ml of rat recombinant IL-1β (Genzyme-Technie, Minneapolis, MN), 50 ng/ml of rat recombinant tumor necrosis factor (TNF-α) (Peprotech EC, London, UK), or 40 ng/ml of rat recombinant CINC2-β (Peprotech EC). In rats, CINC2-β is the human IL-8 homolog, which is produced by the cells in inflamed sites in various tissues and acts as a potent chemotactic agent and mediator of inflammation (9, 48). Each group
of cells was incubated for 3, 6, 12, 24, or 48 h before total RNA was extracted.

**Assay for cell migration of IEC-6 cells after stimulation with MK.** To study the effects of MK on cell migration, the rat small intestine epithelial cell line IEC-6 from Riken Cell Bank (Tsukuba, Japan) was obtained. The cells were grown in DMEM with 4 μg/ml of insulin and 5% heat-inactivated FBS, and four groups of cultured cells were inoculated onto collagen type I-coated plastic culture dishes. After changing the DMEM with 1% FBS without insulin, the first group was left alone to serve as a control, whereas the others were incubated with 0.1, 1.0, and 5.0 nM of human MK (Peptide Institute, Osaka, Japan). After making a round wound in the three experimental groups, they were incubated for an additional 48 h. The process of restoration was monitored using an inverted phase-contrast microscope at 0, 12, 24, 36, and 48 h after induction of the artificial wounds. Changes in cell-free areas were analyzed quantitatively with an IBAS image analyzer (Carl Zeiss, Oberkochen, Germany), as described previously (29).

In addition, to quantify the migration effects of MK on cultured IEC-6, the different migration assay was performed on 24-well Boyden chambers as described by the manufacturer (Chemical International). Briefly, IEC-6 cells were incubated in serum-free DMEM medium for 18–24 h prior to assay. After incubation, cells were harvested using trypsin/EDTA and redissolved in serum-free media so that the cell number became 0.5–1.0 × 10^6 cells/ml. Approximately 300 μl of prepared cell suspension was added into the insert, and the
The lower chamber was filled with 500 μl of serum-free media with different doses of recombinant MK (1.0 nM and 5.0 nM). After 24 h of incubation, the inserts were removed and stained. After several washings with distilled water, the cells were extracted, and optical density, corresponding to the number of cells per well, was taken at 560 nm.

**Assay for effect of MK on growth of IEC-6 cells.** Assessment of the MK-induced growth effect was performed by an assay of cellular DNA synthesis, which was assessed by [3H]thymidine incorporation. IEC-6 cells were seeded into 24-well plates for an initial 24-h culture. After changing the medium to DMEM with 1% FBS but without insulin, MK at a concentration of 0.1, 1.0, or 5.0 nM was added, and incubation continued for 48 h. Then, 1.0 Ci of [methyl-3H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added to each well during the last 4 h of MK treatment. The assay protocol was then applied as described earlier.

**Western blot analysis for MK-induced ERK1/ERK2 activation in IEC-6 cells.** MK-induced MAP kinase ERK1/ERK2 activation in IEC-6 cells was investigated using Western blot analysis of the phosphorylated ERK1/ERK2 protein. A constitutive high level of ERK1/ERK2 phosphorylation in IEC-6 cells was inhibited by depriving serum and insulin 24 h before MK treatment. Then, cells were treated with 5.0 nM of MK for 15, 30, or 60 min, and total cellular proteins were extracted in SDS sample buffer in the presence of protease inhibitors (aprotinin, leupeptin, and PMSF) and phosphatase inhibitors (Na3VO4 and NaF). Equal amounts of proteins (~20 μg) from the cells with or without MK treatment were separated on SDS-PAGE, then electroblotted onto PVDF membranes and processed for Western analysis. Phosphorylated ERK1/ERK2 was detected using a mouse MAb (E-11, Sc-7383; Santa Cruz Biotechnology) in a 1:750 dilution. The phosphatase inhibitor NaF was used in all the buffers for both the incubation and washing procedures. After phosphorylated ERK1/ERK2 was detected, the membranes were stripped, and total ERK1/ERK2 was detected using the same blots. Total ERK1/ERK2 was detected using a rabbit polyclonal Ab (Calbiochem-Novabiochem) at a concentration of 0.5 μg/ml (1:1,000 dilution). Blotting steps were then performed as described earlier.

**Statistical analysis.** Data are means ± SE. ANOVA and the Student’s t-test were used for statistical determinations. Multiple comparisons were performed with an LSD test following ANOVA. P values of <0.05 were considered statistically significant.

**RESULTS**

**Expression of MK in rat distal colon with DSS-induced mucosal lesions.** Following the induction of DSS colitis, the expression of MK mRNA was investigated in the rat distal colons using Northern blot analysis. The gene expression of

**Fig. 3.** A: time-course changes of IL-1β, TNF-α, and CINC-2β gene expression during DSS-induced colitis. Total RNA (20 μg) was extracted at the indicated times after the end of DSS administration, and Northern blotting was performed using IL-1β, TNF-α, and CINC-2β cDNA probes. B and C: relative signal intensities of IL-1β, TNF-α, and CINC-2β mRNA levels were normalized with that of β-actin mRNA, then quantified using an image analyzer. Values are means ± SE obtained from 3 independent experiments. *P < 0.05 vs. control.

**Fig. 4.** A: time-course changes of RPTP-β gene expression during DSS-induced colitis. Total RNA was extracted from colonic tissues at the indicated times after the end of DSS administration, and the gene expression of RPTP-β was examined by RT-PCR. B: relative signal intensities of RPTP-β mRNA levels were normalized with that of β-actin mRNA levels, then quantified using an image analyzer. Values are means ± SE obtained from 3 independent experiments. *P < 0.05 vs. control.
MK was significantly increased during DSS-induced colitis and peaked 3 days after the end of DSS administration (Fig. 1). After Northern blot analysis, immunostaining was performed to determine localization of the MK protein in the histological sections. Abundant MK immunoreactive signals were detected after treatment with DSS, which were mainly identified in the mucosal and submucosal layers of the distal colon (Fig. 2, A and B). The morphological characteristics of the MK-positive cells were quite similar to those of fibroblasts (Fig. 2C). To verify whether fibroblasts are the cells expressing MK, we performed two-color immunofluorescence stainings for MK and vimentin in the frozen sections, 5 days after the induction of DSS-dependent colitis. A representative image (submucosal layer) of the immunofluorescence is shown in Fig. 2D. Double immunoreactive signals for MK (green) and vimentin (red) were detected in the cells present in the submucosal layer of the colon, showing that MK is expressed in vimentin-positive mesenchymal cells including fibroblasts in the colonic tissues.

Proinflammatory cytokine gene expression in rat distal colons with DSS-induced mucosal lesions. Gene expression of the proinflammatory cytokines IL-1β, TNF-α, and CINC2-β was investigated in rat distal colons with DSS-induced mucosal lesions by Northern blot analysis. The gene expression of all cytokines was increased and peaked 1 day after the end of DSS administration (Fig. 3). In particular, IL-1β and CINC2-β mRNA was markedly increased.

Gene expression of RPTP-β in rat distal colons with DSS-induced mucosal lesions. RPTP-β has been recognized as a receptor of MK, thus the expression of the RPTP-β gene in distal colon tissues was also examined by RT-PCR. Gene expression was decreased 1 day after the end of DSS administration, although it gradually returned to a normal level on day 7 after the induction of DSS-induced colitis (Fig. 4, A and B). Next, we investigated whether the RPTP-β gene was expressed in epithelial cells from a normal distal colon using LCM coupled with RT-PCR. Frozen histological sections were used for LCM, and colonic epithelial cells were carefully captured. Colonic mucosa samples before (Fig. 5A) and after (Fig. 5B) removal of the epithelial cells and LCM-captured epithelial cells are shown in Fig. 5C. To detect possible contamination of the captured epithelial cells by leukocytes, CD45 gene expression in the LCM samples was examined by RT-PCR. Then CD45-negative and cytokeratin-20-positive LCM samples were tested for evaluation of RPTP-β gene expression in the colonic epithelial cells. The RT-PCR results are shown in Fig. 5D. RPTP-β gene expression was detected clearly in the CD45-negative, cytokeratin-20-positive LCM samples, suggesting that RPTP-β is essentially expressed in colonic epithelial cells.

Effects of inflammatory cytokines on MK expression in 3Y1 cells. Since the increased expression of MK was preceded by that of proinflammatory cytokines in the distal colon during
DSS-induced colitis, the direct effects of those proinflammatory cytokines on MK gene expression were investigated using a rat fibroblast cell line, 3Y1. The expression of the MK gene in 3Y1 cells after stimulation with IL-1β, TNF-α, and CINC2-β was examined by Northern blot analyses, with time-course changes shown in Fig. 6, A and B. Stimulation by IL-1β and TNF-α, although not by CINC2-β, time-dependently induced MK expression in the 3Y1 cells.

Effects of MK on cellular proliferation and migration of the IEC-6 cell line. Based on our in vivo results, we speculated that MK produced by fibroblasts may influence the proliferation or migration of colonic epithelial cells during DSS-induced colitis. Since the IEC-6 cells, taken from a rat intestinal epithelial cell line, expressed the MK receptor RPTP-β (Fig. 7A), they were used in an in vitro study to evaluate the effects of MK on epithelial cell proliferation and migration. Initially, we examined the effects of MK on cell restitution using an intestinal wound repair model developed with cultured epithelial monolayer sheets. Although each cell-free area was repaired within 48 h after wounding, the wound repair in MK-treated groups was accelerated dose dependently compared with the control (Fig. 7B and C). In addition, to quantify the migration effects of MK on cultured IEC-6, a different migration assay was performed on 24-well Boyden chambers. This assay also showed significant effects of MK on the migration of IEC-6 cells (Fig. 7D). To assess the effects of MK on epithelial cell proliferation, we performed a [3H]thymidine incorporation assay. Epithelial cell proliferation was not stimulated by any of the concentrations of MK used (Fig. 8A). Furthermore, since MAP kinase ERK1/ERK2 activity has been reported to be one of the major intracellular signals of MK-dependent cell proliferation (37, 38), we examined MK-induced MAP kinase activity.

Fig. 6. A: time-course changes of MK gene expression in 3Y1 cells following stimulation with IL-1β (10 ng/ml), TNF-α (50 ng/ml), and CINC2-β (40 ng/ml). Total RNA (20 μg) was extracted at the indicated times after the addition of proinflammatory cytokines, and Northern blotting was performed using each cDNA probe. B: relative signal intensities of MK mRNA levels were normalized with that of β-actin mRNA, then quantified using an image analyzer. Values are means ± SE obtained from 3 independent experiments.

*P < 0.05 vs. control.

Fig. 7. A: representative RT-PCR results of RPTP-β gene expression in intestinal epithelial cells (IEC-6). B and C: time-course changes of restoration of epithelial sheet around an artificial wound in control and MK-treated cells. The MK-treated denuded areas recovered faster in a dose-dependent manner. D: quantifying the migration effects of MK (1 nM and 5 nM) on cultured IEC-6; this assay was performed on 24-well Boyden chambers. Values are means ± SE obtained from 3 independent experiments. *P < 0.05 vs. control.
ERK1/ERK2 activation in IEC-6 cells using Western blot analysis for the phosphorylated ERK1/ERK2 protein. However, the immunoreactive signals of phosphorylated ERK1/ERK2 in the MK-treated group were not different from those in the control group (Fig. 8B).

DISCUSSION

Various growth factors have been reported to stimulate the proliferation and migration of intestinal epithelial cells, although little is known regarding the role of MK in intestinal inflammation. In the present study, we focused on MK expression in experimental colitis in rats and investigated its function using an in vitro intestinal wound repair model. MK expression was upregulated in rat large intestines during the DSS-induced colitis. Our findings also demonstrated that proinflammatory cytokines induced the upregulation of MK, which may ultimately enhance mucosal restitution during the process of repair of colitis.

MK is expressed in human fetal brain tissue and a number of other organs and is also known to modulate epithelial-mesenchymal interactions during fetal development and organogenesis (32). Its expression is gradually downregulated with age and shows a restricted pattern with lower levels in the colon, stomach, lungs, and kidneys in adults (47, 49). MK expression has been reported to be induced in areas after a variety of tissue injuries, including cerebral and myocardial infarction, as well as arterial endothelial injury, suggesting that the protein may play an important role in tissue repair and remodeling in adult organs (6, 10, 14). In the present study, MK expression was found to be significantly increased in damaged colonic mucosa from rats, and abundant immunoreactive MK signals were detected in submucosal fibroblasts but not in mucosal epithelial cells, mainly from 3 to 5 days after the end of DSS administration. On the other hand, the gene expressions of proinflammatory cytokines were most strongly induced on day 1, which preceded the augmentation of MK expression. In the present DSS-induced colitis rat model, the most severe mucosal injury was generally observed just after stopping DSS drinking (day 1), and it gradually subsided thereafter over a short period. Thus the time-course changes of MK expression observed in our study suggest that MK may have some influence on mucosal repair following DSS-induced colitis.

RPTP-β has been identified as an MK receptor, as well as a key molecule that modulates MK-dependent cell-to-cell communication between epithelial and mesenchymal cells (22). We speculated that MK expressed by submucosal fibroblasts may exert regulatory effects on epithelial cell functions through RPTP-β during intestinal inflammation. Our results of LCM coupled with RT-PCR showed that the rat colonic epithelial cells expressed RPTP-β in a constitutive manner. Although the expression of RPTP-β in colonic tissues was transiently decreased after the induction of colitis, it gradually returned to the basal level within 7 days. In DSS-induced colitis, a large number of epithelial cells are lost from the mucosa due to mucosal injury, which may have an influence on the transient decrease of RPTP-β expression in the injured colonic tissues. During subsequent periods of colonic mucosal regeneration, RPTP-β expression was found to parallel MK expression; therefore, it is reasonable to assume that the MK-RPTP-β system stimulates reepithelialization during the repair process. On the other hand, a recent study has experimentally shown that MK enhances early stages of collagen gel construction, which suggests that MK may be a modulator of cell-matrix interactions (fibroblast-collagen interactions) (44). We have also shown previously that MK plays an important role on the formation of granulation tissues during the healing of experimental chronic gastric ulcer model (23). Together, these findings suggest that the MK-RPTP-β system may stimulate not only epithelial cells but also fibroblasts and generally regulate wound healing during DSS-induced colitis.

Elucidation of the regulation of MK expression is important for understanding the precise mechanism of intestinal regeneration. It has been reported that proinflammatory cytokines upregulate several growth factors in inflammatory conditions (12, 39). For example, in water immersion stress-induced damaged gastric mucosa, we previously found that the proinflammatory cytokine CINC2-β stimulates expression of the regenerating gene (Reg), a growth factor (19). In the present study, since the augmentation of MK expression was observed following an increased expression of proinflammatory cytokines, this indicates that MK expressed on the cell surface of submucosal fibroblasts may play an important role in tissue repair and remodeling in adult organs (6, 10, 14). In the present study, MK expression was found to be significantly increased in damaged colonic mucosa from rats, and abundant immunoreactive MK signals were detected in submucosal fibroblasts but not in mucosal epithelial cells, mainly from 3 to 5 days after the end of DSS administration. On the other hand, the gene expressions of proinflammatory cytokines were most strongly induced on day 1, which preceded the augmentation of MK expression. In the present DSS-induced colitis rat model, the most severe mucosal injury was generally observed just after stopping DSS drinking (day 1), and it gradually subsided thereafter over a short period. Thus the time-course changes of MK expression observed in our study suggest that MK may have some influence on mucosal repair following DSS-induced colitis.
those findings, no significant effect on epithelial cell proliferation was observed. Since we did not detect MK-dependent ERK1/ERK2 activation, a major intracellular signaling pathway in MK-dependent cell proliferation (31, 32), we concluded that the proliferating effect of MK toward IEC-6 cells was lower than that on other cells with a different tissue origin. Our previous study also demonstrated that MK does not stimulate the proliferation of RGM1, a normal rat gastric epithelial cell line. Together, these results suggest that MK is an essential stimulator of IEC migration and may contribute to the restitution process during recovery from colitis. Although MK exhibited a significant effect on migration of the rat small intestinal epithelial cell line IEC-6, such effects were not observed on human colorectal carcinoma cell line Caco-2 (data not shown). This may be related to the differential effect of MK on small and large IECs. However, as Caco-2 is a malignant cell line with possible defect in cell adhesion structures, to evaluate the effects of MK on the migration of colonic epithelial cells precisely, further studies using a nonmalignant colonic cell line will be necessary.

Previous reports have shown that several growth factors and cytokines modulate epithelial cell restitution through the effect of TGF-β in the IEC-6 model of intestinal wound healing (2, 3). In addition, the phenomenon that we observed, MK-induced migration of IEC-6 cells, while there was no effect on cell proliferation, is often observed in TGF-β-mediated repair (43). These findings suggest that MK-mediated production of TGF-β may stimulate cell migration in the IEC-6 monolayer model of wound healing.

A variety of therapeutic drugs, including aminosalicylates, corticosteroids, and immune modulators, have been used for the treatment of IBD, though they are unable to completely cure or control the disease. Several growth factors have been reported to be effective for tissue repair following experimentally induced colitis (1, 8, 11, 20, 25, 35, 45). Recently, Matsuura et al. (27) reported the therapeutic effects of a rectal murine murine colitis. Although the growth factor did not have a strong anti-inflammatory effect, it was able to stimulate mucosal regeneration during the healing process. The present study clearly showed that MK stimulates intestinal epithelial restitution, suggesting its possible use as a therapeutic option for the treatment of IBD. However, further studies addressing in vivo effects of MK should be carefully performed before it can be assessed for its potential as a therapeutic, because MK is one of the growth factors and has several mitogenic or oncogenic potentials including cell growth, inhibiting apoptosis, and angiogenesis (7, 15, 40).

In summary, we investigated the colonic expression of MK and RPTP-β after development of DSS-induced colitis in rats and evaluated the role of MK with intestinal epithelial functions in vitro. The present results showed for the first time that intestinal inflammation upregulates the MK-RPTP-β system, which may stimulate mucosal restitution during the process of healing of colitis. Further investigations regarding the role of MK may contribute to the development of a new therapy for IBD.

REFERENCES


