Hepatocyte growth factor ameliorates mucosal injuries leading to inhibition of colon cancer development in mice

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Abstract. Hepatocyte growth factor (HGF), which facilitates the repair of injured mucosa, has the potential to be a new therapeutic agent for inflammatory bowel disease (IBD). However, given that the incidence of colorectal cancer increases continuously with disease duration in patients with IBD, the fact that HGF is a potent mitogen for intestinal epithelial cells may further heighten the risk of bowel cancer in this patient population. In this study, we examined the effects of recombinant HGF on colorectal cancer development in mice with or without experimentally induced colitis. Although HGF stimulated proliferation of colonic epithelial cells in normal mucosa, the development of colorectal cancer induced by repeated injection of azoxymethane (AOM) was significantly inhibited by HGF treatment. In a mouse model of colitis-associated cancer, colorectal cancer frequently developed despite only a single injection of AOM prior to three cycles of dextran sulfate sodium administration. However, HGF treatment significantly facilitated the repair of injured mucosa, leading to inhibition of colorectal cancer development in a dose-dependent manner. Thus, HGF-induced repair of injured mucosa inhibits rather than accelerates the development of colorectal cancer, and these results also suggest the importance of blocking the cycles of mucosal injury and repair to prevent colitis-associated colorectal cancer.

Introduction

Inflammatory bowel disease (IBD), such as ulcerative colitis and Crohn's disease, is an intractable disease characterized by chronic relapsing inflammation of the gastrointestinal tract. The primary therapies for IBD are anti-inflammatory and anti-immune agents, such as salazosulfapyridine, mesalazine, corticosteroids, mercaptopurine, azathioprine, methotrexate and cyclosporine (1,2). Recently, a chimeric mouse-human monoclonal antibody against tumor necrosis factor (TNF)-α was shown to be extremely effective in Crohn's disease (3,4), and another calcineurin inhibitor, tacrolimus, was effective in the treatment of IBD (5). However, side effects associated with these medications were frequently noted and colitis was often recurrent and intractable in spite of the therapy, leading not only to cessation of these agents but also to impaired quality of life.

Hepatocyte growth factor (HGF) was first purified as a potent hepatocyte mitogen from the plasma of patients with fulminant hepatic failure (6). This protein functions as a mitogen, motogen and morphogen for multiple subsets of epithelial cells, including those of the gastrointestinal tract (6-8). HGF activator and HGF activator inhibitor type-1, both HGF-associated molecules involved in the activation of HGF in injured tissues, are associated with colonic mucosal repair (9,10); HGF expression is increased in inflamed colonic mucosal tissues in patients with ulcerative colitis (11). We previously reported that systemic administration of recombinant human HGF ameliorated experimental colitis in rats (12,13). Perturbed homeostasis between luminal antigens (e.g., commensal bacteria) and mucosal immunity serves as a critical determinant in the development of gut inflammation in IBD. Therefore, treatment that enhances the remodeling and repair of injured mucosa may be essential for the treatment of IBD; this approach may ultimately prove to be more successful than either anti-inflammatory or anti-immune therapy. However, the evident mitogenic activity of HGF has the potential to increase the risk of cancer development; and in patients with IBD, particularly those with ulcerative colitis, the risk of colorectal cancer continuously increases with the duration of the disease (14,15). In fact, recent investigations have reported that the cumulative risk for colorectal cancer ranges between 10-18% in patients with a disease history of 30 or more years (16,17), and that colitis-associated colorectal cancer is responsible for 10-15% of all deaths in UC patients (18). In this context, before clinical applications of recombinant

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Key words: hepatocyte growth factor, colorectal cancer, colitis-associated cancer, mucosal injury, carcinogenesis

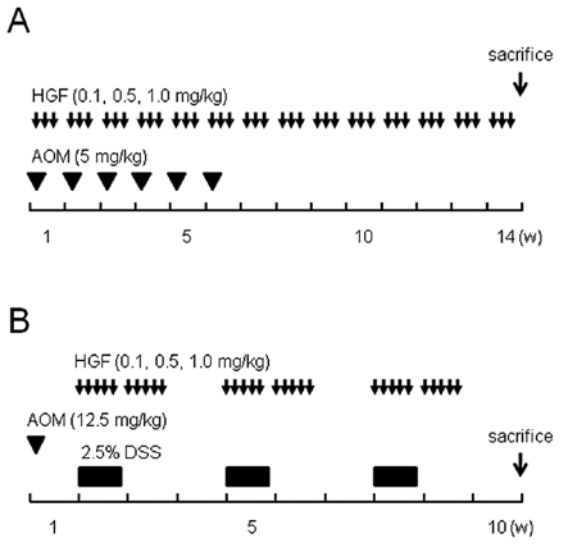

Figure 1. Animal models of colorectal cancer and HGF administration. (A) To induce AOM-induced colorectal cancer, mice received intraperitoneal injections of AOM (5 mg/kg) once a week for 6 weeks. Recombinant HGF (0.1, 0.5 or 1.0 mg/kg/day) or vehicle control were intraperitoneally administered every second day for 14 weeks. (B) Colitis-associated colorectal cancer was induced in mice by a single injection of AOM (12.5 mg) followed by three cycles of 2.5% DSS, where each cycle consisted of 5 days of DSS treatment followed by 16 days of regular water. Intraperitoneal injections of recombinant HGF or vehicle control were initiated with the DSS treatment, and continued for 2 weeks (5 days a week) in each cycle.

HGF to treat IBD patients are developed, it must first be ascertained, whether repeated doses of recombinant HGF increase the risk of accelerating the development of colorectal cancer, particularly colitis-associated colorectal cancer, and second, whether HGF-induced enhanced repair of injured mucosa leads to prevention of colitis-associated colorectal cancer. In this study, we examined the effect of repeated injections of recombinant HGF on the development of colorectal cancer in mice with or without repeated mucosal injury.

Materials and methods

Animals. Male A/J mice, 7 weeks of age, and female CBA/J mice, 5 weeks of age, were obtained from Japan SLC (Shizuoka, Japan). The animals were maintained under constant room temperature (25˚C) and given free access to water and a standard diet throughout the study. The protocol for animal studies was approved by the Ethics Committee of the Graduate School of Medicine, Kyoto University. All animal experiments were performed after a 1-week acclimation period.

Animal models of colon cancer and recombinant human (rh)-HGF administration. To assess the effects of HGF on colon tumor development, A/J mice, 8 weeks of age and weighing between 22 and 23 g, were injected intraperitoneally with azoxymethane (AOM) (5 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) once a week for 6 weeks (Fig. 1A). Intraperitoneal injections of rh-HGF (0.1, 0.5 or 1.0 mg/kg/day) or phosphate-buffered saline (PBS) were initiated with the AOM injection and were continued every second day for 14 weeks. The number, incidence and multiplicity of colon tumors were determined after the 14-week HGF regimen was complete. To induce colitis-related cancer, CBA/J mice, 7 weeks of age and weighing ~19 g, were injected with a single dose of AOM (12.5 mg/kg) followed by three cycles of 2.5% dextran sulfate sodium (DSS) adminis-tration, in which each cycle consisted of 5 days of DSS treatment followed by 16 days of regular water (Fig. 1B). Intraperitoneal injections of HGF or PBS were initiated with the DSS treatment, and were continued for 2 weeks (5 days a week) in each cycle. Two weeks after the final administration of DSS, the number, incidence and multiplicity of colon tumors were examined. To evaluate the severity of DSS-induced colitis, the disease activity index (DAI) was assessed during the first cycle of DSS and HGF administration.

Histological examination. The entire colon was excised post-mortem and fixed with 10% formalin for histological analysis. The longitudinal sections were embedded in paraffin and stained with hematoxylin and eosin (H&E). The sections were assessed according to diagnostic criteria for gastrointestinal carcinomas independently by two investigators blinded to the experimental protocol (19). Colon tumors falling under category 4, including high-grade adenoma/dysplasia, suspected carcinoma and mucosal carcinoma, were defined as colorectal cancers. The number of colorectal tumors was scored to determine the incidence (number of animals with at least one tumor) and multiplicity (number of tumors per animal) of neoplasms.

To evaluate the degree of DSS-induced colitis, all colonic tissues were obtained after the first cycle of HGF or PBS administration, fixed with 10% formalin and stained with H&E. Histological scoring was assessed independently by three investigators blinded to the experimental protocol, and was expressed as a combined score of inflammatory cell infiltration (0-3) and tissue damage (0-3) (20). For inflammatory cell infiltration, the presence of rare inflammatory cells in the lamina propria was scored as 0, increased number of inflammatory cells in the lamina propria as 1, confluence of inflammatory cells extending into the submucosa as 2, and transmural extension of the inflammatory cell infiltrate as 3. For epithelial damage, absence of mucosal damage was scored as 0, discrete focal lymphoepithelial lesions were counted as 1, mucosal erosion or ulceration as 2, and extensive mucosal damage and extension through deeper structures of the bowel wall as 3. The two subscores were added, and the combined histological score ranged from 0 (no change) to 6 (extensive cell infiltration and tissue damage).

Immunohistochemistry. To evaluate the proliferation of the colonic epithelium, cells undergoing proliferation were identified by immunohistochemistry for Ki-67 using anti-mouse Ki-67 monoclonal antibody (MIB-5) (DakoCytomation, Copenhagen, Denmark). Following visualization of the Ki-67 antigen, the number of Ki-67-positive cells in 10 crypts was counted under a microscope at x400 magnification in the normal mucosa and in the colon tumors independently by three investigators blinded to the experimental protocol.

Statistical analysis. Unless otherwise specified, data are expressed as the mean ± SD. Statistical parameters were ascertained with Statview J-4.5 software (Abacus Concept,

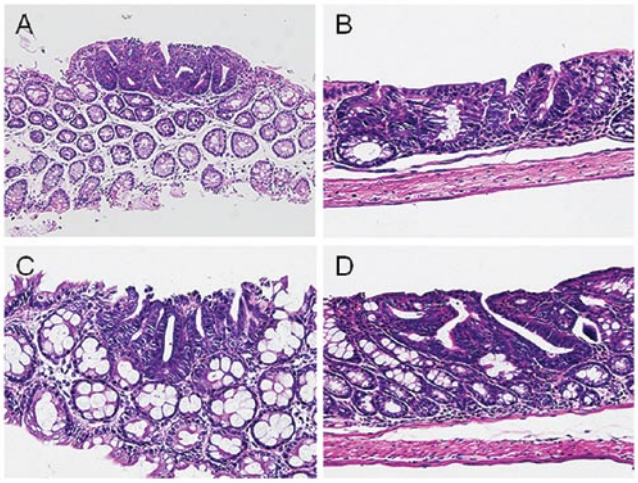

Figure 2. Representative microscopic appearance of AOM-induced colorectal cancer developing in mice treated with or without HGF. All colorectal cancers developing in AOM-treated mice were identified as intramucosal carcinomas. There was no apparent difference in the microscopic appearance of the colorectal cancers between mice treated with vehicle control (A) or HGF at a dose of 0.1 (B), 0.5 (C) or 1.0 mg/kg (D) (magnification x100).

Berkeley, CA). The differences between means were compared using the Mann-Whitney U test. Values of $p<0.05$ were considered statistically significant.

Results

Repeated intraperitoneal injection of HGF inhibits development of colorectal cancer in mice treated with AOM. First, we examined the effect of repeated doses of HGF on AOM-induced colorectal cancer. Pathologically, all colorectal cancers that developed in the AOM-treated mice were identified as intramucosal carcinomas. There was no apparent difference in the microscopic appearance of the colorectal cancers between mice treated with the vehicle control and HGF (0.1, 0.5 and 1.0 mg/kg) (Fig. 2). However, the development of colorectal cancer was significantly inhibited in mice treated with HGF at each dose (p=0.02 for each) (Table I). The incidence and multiplicity of the tumors also decreased in the mice treated with HGF at each dose, and both were significantly inhibited by 1.0 and 0.1 mg/kg of HGF administration (p=0.04 and 0.03, respectively).

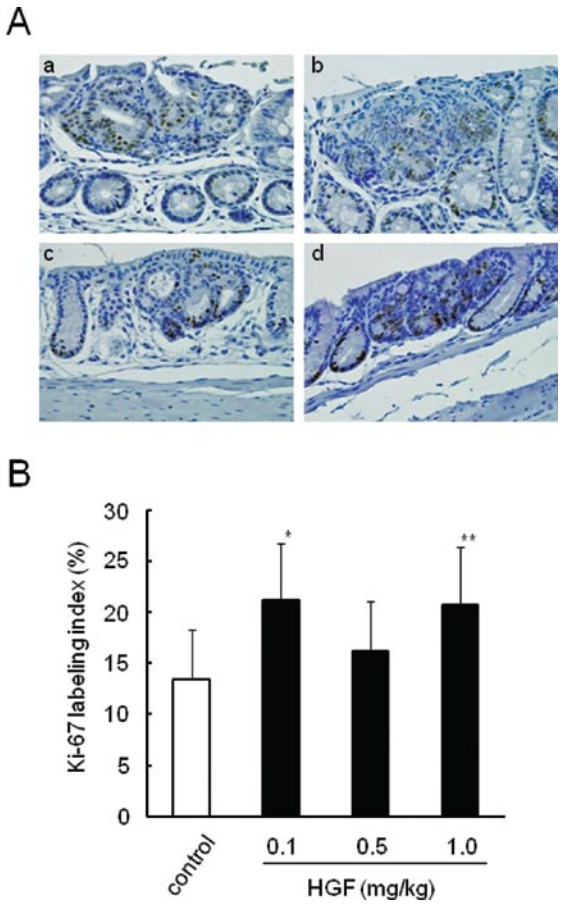

Figure 3. Repeated intraperitoneal injections of HGF stimulates the proliferation of colonic epithelial cells in normal mucosa. The proliferation of colonic epithelial cells was examined by immunohistochemistry using anti-mouse Ki-67 monoclonal antibody. (A) Ki-67-positive cells were observed in both cancerous and non-cancerous colon tissues in AOM-treated mice receiving vehicle control (a) or HGF at a dose of 0.1 (b), 0.5 (c) or 1.0 mg/kg (d). (B) The Ki-67 labeling index (the number of Ki-67-positive epithelial cells in 10 crypts) in normal, non-cancerous colonic mucosa increased in mice treated with HGF; repeated administration of HGF at a dose of 0.1 or 1.0 mg/kg significantly stimulated the proliferation of colonic epithelial cells. $^{*}p=0.001$ and $^{**}p=0.002$ vs. vehicle control.

HGF administration stimulates the proliferation of epithelial cells in normal colonic mucosa. Since HGF is a potent mitogen for intestinal epithelial cells, we determine whether HGF stimulated cell proliferation of colonic mucosa using immuno-

Table I. Repeated doses of HGF inhibit development of AOM-induced colon cancer.

	No. of tumors			
	Total	Mean ± SD	Tumor incidence	Tumor multiplicity
Vehicle control (n=20)	40	2.00±2.22	0.65	3.08
HGF				
0.1 mg/kg (n=14)	6	0.43±0.65[a]	0.36	1.20[c]
0.5 mg/kg (n=13)	5	0.38±0.65[a]	0.31	1.25
1.0 mg/kg (n=14)	6	0.43±0.76[a]	0.29[b]	1.50

Tumor incidence was compared by Fisher's exact test, and other parameters were compared by the Mann-Whitney U test. [a]p=0.02, [b]p=0.04 and [c]p=0.03, compared with vehicle control.

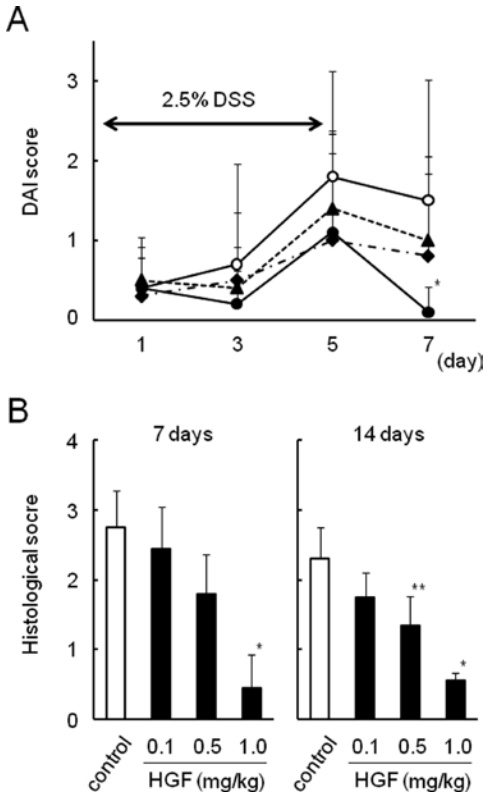

Figure 4. Repeated doses of HGF ameliorated DSS-induced colitis. (A) Mice were treated with vehicle control (○) or HGF at a dose of 0.1 (▲), 0.5 (◆), or 1.0 mg/kg (●) for seven days. Treatment with HGF at a dose of 1.0 mg/kg significantly decreased DAI scores in comparison with mice treated with vehicle control (*p=0.02 vs. vehicle control). (B) Repeated HGF doses of 0.5 and 1.0 mg/kg significantly improved the histological score (*p=0.03 and **p=0.045 vs. vehicle control).

histochemistry for Ki-67. Colonic epithelial cells positive for Ki-67 were observed in both cancerous and non-cancerous colon tissues (Fig. 3A). Repeated doses of HGF markedly inhibited the development of AOM-induced colorectal cancer (Table I), and the resulting small number of tumors in the HGF-treated mice prevented us from being able to quantify the Ki-67-positive cells in colorectal cancer. However, the Ki-67 labeling index in normal, non-cancerous mucosa significantly increased in mice treated with 0.1 and 1.0 mg/kg of HGF (p=0.001 and 0.002, respectively) (Fig. 3B).

Repeated doses of HGF inhibits development of AOM-induced colorectal cancer in mice with experimental colitis. Before investigating the effects of HGF on the development of colitis-associated colorectal cancer, the degree of DSS-induced colitis in mice treated with or without HGF was evaluated. Mice administered 2.5% DSS for 5 days, which were treated with vehicle control, exhibited only mild mucosal injury with low DAI (<2) and histological scores (<3) (Fig. 4). Repeated doses of HGF ameliorated DSS-induced colitis in a dose-dependent manner. HGF administration at a dose of 1.0 mg/kg for 7 days significantly reduced both DAI and histological scores (p=0.02 and 0.03, respectively), and 0.5 and 1.0 mg/kg of HGF administered for 14 days also significantly improved histological scores (p=0.045 and 0.03, respectively) (Fig. 4).

To examine the effect of repeated doses of HGF on colitis-associated colorectal cancer, mice were administered a single injection of AOM, followed by three cycles of 1-week 2.5% DSS dosing and 2-week cessation and simultaneous treatment with HGF (0.1, 0.5 and 1.0 mg/kg) or vehicle control for 2 weeks (Fig. 1B). Repeated doses of HGF suppressed the number, incidence and multiplicity of colorectal cancers in a dose-dependent manner; treatment with 1.0 mg/kg of HGF significantly reduced the number and multiplicity of colitis-associated colorectal cancers (p=0.01 and 0.02, respectively) (Table II). Pathologically, all colorectal cancers were identified as intramucosal carcinomas, and there was no difference in microscopic appearance between mice treated with vehicle control and HGF (Fig. 5). We evaluated the epithelial cell proliferation in colitis-associated colorectal cancers and normal, non-cancerous, mucosas 7 days after the third HGF treatment. Although the Ki-67 labeling index in colorectal cancers was significantly increased in comparison with normal, non-cancerous mucosa (p=0.03), there was no significant difference in cell proliferation in both the cancerous and non-cancerous colon tissues between AOM/DSS-treated mice receiving vehicle control and HGF (Fig. 6).

Discussion

HGF is a multifunctional polypeptide produced by mesenchymal cells and functions as a mitogen, morphogen and/or motogen for multiple subsets of epithelial cells. HGF has the potential to be a novel therapeutic agent for intractable diseases of various organs, including the liver (21,22), nervous system (23), lung

Table II. Repeated doses of HGF inhibit development of colitis-associated colon cancer.

	No. of tumors			
	Total	Mean ± SD	Tumor incidence	Tumor multiplicity
Vehicle control (n=10)	66	6.60±5.27	0.90	7.33
HGF				
0.1 mg/kg (n=10)	66	6.60±4.99	1.00	6.60
0.5 mg/kg (n=10)	37	3.70±1.95	0.90	4.11
1.0 mg/kg (n=10)	16	1.60±1.51[a]	0.60	2.67[b]

Tumor incidence was compared by the Fisher's exact test, and other parameters were compared by the Mann-Whitney U test. [a]p=0.01 and [b]p=0.02, compared with vehicle control.

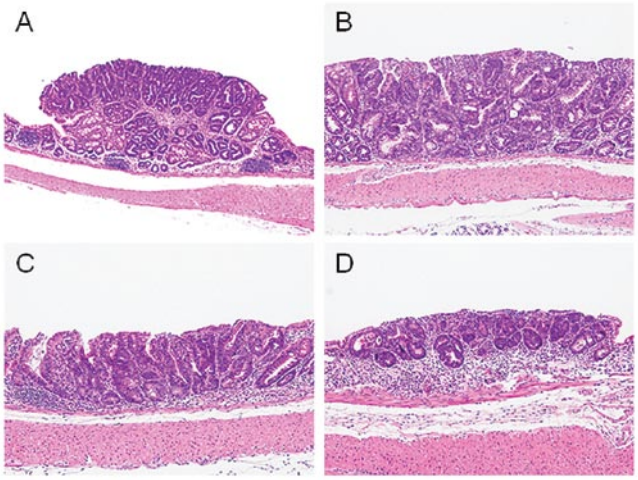

Figure 5. Representative microscopic appearance of colitis-associated colorectal cancer developing in AOM/DSS-treated mice. All colitis-associated colorectal cancers were identified as intramucosal carcinomas. There was no apparent difference in the microscopic appearance of colorectal cancers between AOM/DSS-treated mice receiving vehicle control (A) or HGF at a dose of 0.1 (B), 0.5 (C) or 1.0 mg/kg (D) (magnification x100).

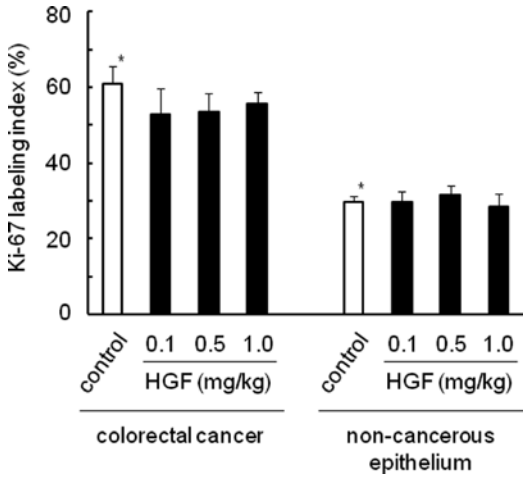

Figure 6. The proliferation of colonic epithelial cells was not persistently stimulated by repeated doses of recombinant HGF in mice with AOM/DSS-induced colorectal cancer. The Ki-67 labeling index in colitis-associated colorectal cancer and normal mucosa was examined in mice treated with AOM/DSS 7 days after the third HGF treatment. Although cell proliferation was significantly stimulated in colorectal cancer in comparison with non-cancerous mucosa ($^{*}p=0.03$), there was no significant difference in the Ki-67 labeling index in cancerous and non-cancerous tissues between mice treated with vehicle control or HGF.

(24), heart (25) and kidney (26). We therefore developed translational medicine protocols for recombinant human HGF, including preparation of a GMP-grade compound, various preclinical safety tests, and a phase I/II clinical trial to evaluate the safety, pharmacokinetics and clinical efficacy of recombinant human HGF in patients with fulminant hepatitis (27). Since HGF is a potent mitogen for hepatocytes, we confirmed before initiating a human trial that repeated doses of intravenous HGF did not accelerate the development of hepatocellular carcinoma in rats fed a choline-deficient, L-amino acid-defined diet (28). We recently reported that treatment with recombinant HGF stimulates the repair of injured intestinal mucosa, leading to amelioration of experimental colitis (12,13). However, repeated doses of HGF may exhibit oncogenic potential; therefore the carcinogenic potential of HGF should be evaluated as precisely as possible before clinical applications of this agent.

In the present study, we first examined the effects of repeated HGF dosing on AOM-induced colorectal cancer in mice without colitis, and showed that HGF treatment significantly inhibited the development of colorectal cancer in normal mucosa despite stimulation of epithelial cell proliferation. Recent investigations found that HGF prevented intestinal epithelial cells from apoptosis (29,30). HGF is thought to interact with c-Met, a specific receptor for HGF that is expressed on the basolateral membranes of epithelial cells (31); recombinant HGF administered intraperitoneally stimulated the proliferation of epithelial cells in colonic mucosa through an increase in serum HGF concentration (12). HGF/c-Met signaling has been reported to have both positive and negative effects on carcinogenesis. In cell culture studies, HGF can either increase cell survival (32-34) or inhibit cell growth and induce apoptosis in a variety of cancer cell lines (35-37). In transgenic mice expressing HGF, various types of tumors developed, including liver, breast, skin and muscle (38-40). In contrast, transgenic mice expressing HGF under control of the albumin promoter did not form liver tumors (41), and transgenic coexpression with HGF suppressed both c-Myc- (42) and transforming growth factor (TGF)-α-driven (43) hepatocarcinogenesis. Conversely, increased MET signaling in early-stage colorectal cancer is a common occurrence, and elevated MET expression or amplification in advanced disease is linked to metastatic progression (44,45). However, cancer cells with increased MET expression lack autocrine HGF production (46), suggesting that the receptor exists in a ligand-independent active conformation. Additionally, the loss of c-Met signaling in hepatocytes enhanced rather than suppressed the early-stage chemical hepatocarcinogenesis (47,48). Therefore, the net outcome of HGF/c-Met activation in normal intestinal epithelial cells is phenotypically different from the effect on colorectal cancer cells, and does not necessarily lead to the enhanced development of colorectal cancer.

In AOM/DSS-treated mice, although mice administered 2.5% DSS exhibited mild colonic mucosal injury, enhanced development of colorectal cancer was observed in comparison with mice treated with a repeated injection of AOM alone (Tables I and II). Changes in TGF-β signaling or activation of the intrinsic tyrosine kinase of the EGF receptor while stimulating the synthesis of TGF-α have been reported to contribute to tumor development in mice treated with AOM (49-51). However, despite a single injection of AOM before DSS administration, repeated mucosal injury and repair facilitated the development of colorectal cancer, demonstrating the strong association between chronic mucosal inflammation and colorectal cancer. Although the molecular nature of this connection is largely unknown, the importance of inflammation is highlighted by the dependence of tumor growth and progression on the canonical activation of nuclear factor (NF)-κB by inhibitor of the NF-κB kinase-dependent pathway, which is crucial for tumor growth and progression (52). Additionally, recent investigations suggest that mechanisms include chronic formation of reactive oxygen species (53) and

tumorigenesis induced by chronic epithelial exposure or inflammatory stimuli, such as interleukin-6 and tumor necrosis factor-α (54-57). Thus, the close link between chronic inflammation and colorectal cancer also suggests that therapeutic modalities blocking the cycles of mucosal injury and repair possibly contribute to prevention of colitis-associated colorectal cancer. We showed here that repeated doses of HGF significantly facilitated the repair of injured mucosa in AOM/DSS-treated mice (Fig. 4), and consequently inhibited the development of colitis-associated colorectal cancer (Table II). Although HGF ameliorates mucosal injury through its mitogenic and anti-apoptotic effects on colonic epithelial cells (12,13,30), improvement of the microenvironment, which means the reduction of chronic epithelial exposure to proinflammatory cytokines, growth factors and reactive oxygen species throughout repeated cycles of mucosal injury and repair, is considered to be a primary contributor to the prevention of colitis-associated colorectal cancer.

The effectiveness and impact of HGF on various intractable diseases have been extensively investigated primarily using *in vivo* HGF gene transfer or HGF transgenic mice that led to persistent exposure to HGF (23-25). We showed here that there was no significant difference in epithelial cell proliferation in the colonic mucosa of AOM/DSS-treated mice after repeated HGF administration (Fig. 6). These findings seem reasonable due to the short half-life of recombinant HGF (58). Therefore, from the standpoint of carcinogenic risk, treatment with recombinant HGF can provide a higher level of safety than HGF gene therapy.

This study demonstrated that repeated doses of recombinant HGF significantly inhibited AOM-induced colorectal cancer despite its stimulatory effect on colonic epithelial cell proliferation, and efficiently attenuated colonic mucosal injury, leading to prevention of colitis-associated colorectal cancer. HGF/c-Met activation has been reported to result in both activation and inhibition of carcinogenesis, and therefore this issue should be addressed more extensively. However, the inhibitory effect of recombinant HGF on the development of colorectal cancer, particularly in mice with repeated mucosal injury, offers a significant contribution towards a novel therapeutic approach that facilitates the repair of injured mucosa, which differs from existing anti-inflammatory and anti-immune therapies.

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