Hepatocyte growth factor promotes colonic epithelial regeneration via Akt signaling

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Hepatocyte growth factor (HGF) can promote the regeneration of injured organs, including HGF gene therapy by electroporation (EP) for liver injury. In this study, we investigated the effect of HGF on dextran sulfate sodium-induced colitis and tried to clarify the regenerative mechanisms of colonic epithelial cells and the signaling pathway involved. Colitis was induced by dextran sulfate sodium in mice, together with HGF gene transfer by EP. On day 10, the colitis was evaluated histologically and by Western blot analysis. The colonic epithelial cell line MCE301 was exposed to HGF protein, and its proliferation and activated signaling pathway were analyzed. In vivo, the histological score improved and the number of Ki-67-positive epithelial cells increased in the HGF-treated mice compared with the controls. In vitro, HGF stimulated the proliferation of MCE301 cells. There was enhanced phospho-Akt expression for more than 48 h after HGF stimulation, although phospho-ERK1/2 was enhanced for only 10 min. LY-294002 or Akt small interfering RNA decreased the expression of TNF-α and IFN-γ (3, 24, 26, 38). Several studies have reported that adenoviral-mediated, liposome-formulated, or naked HGF gene can be administered intrarectally, intramuscularly, or intravenously into an animal colitis model (8, 14, 20, 25). Thus HGF gene therapy might be useful for the treatment of IBD. Furthermore, in an in vitro study, HGF is effective in inducing the proliferation of intestinal epithelial cells (23). HGF exerts a protective effect on ischemia-reperfusion injury via an antiapoptotic pathway (15). These data suggest that HGF is a promising drug for the treatment of IBD.

HGF is a mesenchymal-derived pleiotropic cytokine that regulates cell proliferation, antiapoptosis, motility, morphogenesis, anti-inflammatory, and angiogenesis in a wide variety of organs, including gastrointestinal epithelial cells. These complex biological functions occur through its receptor, a transmembrane tyrosine kinase encoded by the proto-oncogene c-Met. Binding of HGF with this receptor leads to autophosphorylation on tyrosine residues in the COOH-terminal domain of c-Met, followed by the phosphorylation of downstream signaling molecules, including phosphatidylinositol 3-kinase (PI3K) and MAPK pathway proteins (41). Of these, the PI3K/Akt signaling pathway has been shown to play a role in a variety of the biological effects of HGF, depending on the system, including proliferation, antiapoptosis, and migration (5, 13, 27). The ERK1/2 signaling pathway is also involved in mediating proliferation, antiapoptosis, transformation, and differentiation (21). However, the critical signal pathway for the cellular proliferative effect of HGF is still largely unknown in colonic epithelial cells.

In this study, we examined the efficacy of HGF on acute dextran sulfate sodium (DSS) colitis using HGF gene transfer...
by electroporation. HGF gene therapy in acute DSS colitis exerted its therapeutic effects mainly by inducing epithelial cell proliferation although its anti-inflammatory effects also played a role. Furthermore, our in vitro data demonstrated that HGF induces the proliferation of colonic epithelial cells predominantly through the PI3K/Akt pathway.

MATERIALS AND METHODS

Induction of colitis. Female C57Bl/6 mice (7–8 wk old) were used throughout these experiments (Sankyo Laboratory, Osaka, Japan). Five mice were assigned to the HGF-treated group and nine mice to the control group. Mice were maintained under specific pathogen-free conditions in the animal center at University of Toyama according to animal care guidelines. Acute colitis was induced by feeding the mice 5% DSS (molecular weight 5 kDa, 15–20% sulfur content, Wako) in distilled water ad libitum, just after electroporation. On day 10 after the induction of colitis, the mice were killed and their colons were examined.

HGF gene transfer by electroporation. Plasmid pKSCX-HGF was constructed by inserting the full-length cDNA for rat HGF into plasmid as described previously (42, 43). Briefly, 50 μg of plasmid DNA (HGF-treated group) or only TE (0.01 M Tris-HCl, 1 mM EDTA/Na2) buffer (control group) were injected into the tibialis anterior muscle while the muscle was held by an electrode, and electric pulses were delivered to the muscle by an electric pulse generator (CUY 21; Tokiwa Science, Tokyo, Japan). The pulse was a square wave at 25 V throughout its duration. Three pulses of the indicated voltage, followed by three more pulses of opposite polarity, were administered at each injection site at a rate of one pulse/s, with a duration of 100 ms for each pulse.

Histopathological analysis. The entire colon was carefully opened longitudinally and fixed in 10% formalin. Paraffin sections cut in transverse, and 3 μm-thick slices were prepared and stained by hematoxylin and eosin. We analyzed three different regions of the distal colon at 5, 10, or 15 mm from the anal verge.

Grading of histological changes. To evaluate the histological changes, we followed the grading score as previously described (4, 9, 17, 28). The degree of crypt disruption and inflammation was graded semiquantitatively from 0 to 4 as follows. For the crypt score, the grading was 0 = normal morphology; 1 = loss of one-third of crypt; 2 = loss of two-thirds of crypt; 3 = loss of entire crypt with surface epithelium remaining intact; 4 = loss of entire crypt and surface epithelium. For the inflammation score, the grading was 0 = no infiltrate; 1 = infiltrate around the base of the crypt; 2 = infiltrate reaching to the Lamina muscularis mucosa; 3 = extensive infiltration reaching to the L. muscularis mucosa and thickening of the mucosa with abundant edema; 4 = infiltration of the L. muscularis submucosa. Samples were evaluated independently by two investigators blinded to the mice examined.

Immunostaining of phospho-c-Met and Ki-67. In brief, deparaffinized sections were immersed in a citrate-based solution in a micro-

*Fig. 1. Histological findings of hematoxylin and eosin staining in acute dextran sodium sulfate (DSS)-induced colitis. A and B: control group, higher magnification image from A (magnification ×100) is presented in B (magnification ×200). C and D: hepatocyte growth factor (HGF)-treated group, higher magnification image from C (magnification ×100) is presented in D (magnification ×200). The HGF-treated group had enhanced development of regenerative epithelia and reduced inflammatory cell infiltrates. Histological score was assessed at 10 days in DSS-induced colitis (see MATERIALS AND METHODS). E: crypt score was 2.48 ± 1.42 in the control group and 1.13 ± 1.19 in the HGF-treated group. EP, electroporation. F: inflammation score was 3.59 ± 0.84 in control group and 2.67 ± 1.40 in the HGF-treated group. The crypt score and inflammation score of the control group were significantly higher than those of the HGF-treated group (E, **P < 0.005; F, *P < 0.05) (control group, n = 9; HGF-treated group, n = 5).*
wave oven for 15 min. After blocking the endogenous peroxidase with hydrogen peroxide, the sections were incubated with polyclonal anti-phospho-c-Met (pYpYpY1230/1234/1235) antibody (Biosource 1:50) overnight at 4°C or polyclonal Ki-67 antibody (Ylem) for 60 min at room temperature. After being washed with PBS, the section was treated with EnVision Peroxidase, Rabbit (Dako) for 30 min. The peroxidase activity was visualized with a diaminobenzidine solution. For quantitative analysis, the number of Ki-67-positive cells per 1,000 epithelial cells was counted by analyzing four mice from each group.

TUNEL staining. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) was performed using the ApopTag Plus peroxidase in situ apoptosis detection kit (CHEMICON) according to the manufacturer’s instructions. In brief, paraffin sections were digested with 20 µg/ml of proteinase K (TaKaRa, Shiga, Japan) for 10–15 min at room temperature, and reacted with terminal deoxynucleotidyl transferase enzyme for 60 min at 37°C. The sections were then incubated with anti-digoxigenin conjugate at room temperature for 30 min, followed by incubation with diaminobenzidine solution. The number of TUNEL-positive epithelial cells per crypt was determined by analyzing five mice from each group.

Preparation of tissue homogenate. A 2-cm-long section of distal colon tissue from the anal verge was homogenized in 0.01 M TNE buffer (10 mmol/l Tris, pH 7.8, 150 mmol/l NaCl, 1% NP-40, 1 mmol/l EDTA, pH 8.0) (1 ml/2 cm length of tissue) containing 5% of a protease inhibitor cocktail (Sigma, St. Louis, MO), followed by centrifugation at top speed for 30 min. The protein concentrations were assayed by using Bio-Rad protein assay solution (Bio-Rad, Hercules, CA).

Western blot analysis. Samples were separated by electrophoresis on 7.5–15% polyacrylamide gels and transferred to Immobilon-PVDF (Millipore, Bedford, MA). The membrane was exposed to the following specific polyclonal antibodies: phospho-ERK1/2 antibody (Thr202/Tyr204), phospho-Akt antibody (Ser473), phospho-JNK antibody (Thr183/Tyr185), phospho-p38 antibody (Thr180/Tyr182), Akt antibody (New England Biolabs, Beverly, MA; 1:1,000), GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200), c-Met antibody (Santa Cruz Biotechnology; 1:100), and phospho-c-Met (pYpYpY1230/1234/1235) antibody (Biosource; 1:1,000). The membrane was washed and exposed to alkaline phosphatase-conjugated secondary antibodies and visualized by incubation in CDP star assay buffer (New England Biolabs) according to the protocol provided by the company.

Immunoprecipitations. For detection of phosphorylated c-Met, immunoprecipitation of c-Met was performed by the Catch and Release immunoprecipitation system (Upstate Biotechnology, Lake Placid, NY), using 500 µg protein of tissue lysates. The eluted proteins were then studied by Western blot analysis with anti-phospho-c-Met antibody.

Characteristics of MCE301 cells and cell culture. A mouse colonic epithelial cell line MCE301, which was established from transgenic mice harboring a temperature-sensitive simian virus 40 large

![Fig. 2. Anti-phospho-c-Met immunohistochemistry in DSS-induced colitis (A, control group; B, HGF-treated group; C, negative control). A: very few cells were positive in the cytoplasm of the control group (arrow) (magnification ×400). B: the cytoplasm of the upper epithelia was diffusely stained in the HGF-treated group. However, a few cells (arrows) are positive in the basal crypt. (magnification ×400). C: a representative section from the HGF-treated group was incubated with only the secondary antibody, without the primary antibody. No staining was detected. (magnification ×400)](http://ajpgi.physiology.org/)

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T-antigen, was used (37). These cells preserve the normal characteristics of colon epithelial cells at 33°C (36). The cells were cultured in DMEM/Ham’s F-12 (1:1) (GIBCO-BRL, Grand Island, NY), supplemented with 20 ng/ml insulin, 20 ng/ml transferrin, 1.22 ng/ml ethanolamine, 0.0914 ng/ml sodium selenite, 10 ng/ml EGF, and 2% fetal bovine serum on a type I collagen-coated dish (Asahi Techno Glass, Tokyo, Japan). The cell growth, which was arrested with serum-free medium for 24 h before the stimulation of HGF or EGF, was assessed by Western blot analysis and proliferation assay.

Fig. 3. Ki-67 immunohistochemistry in DSS-induced colitis (A, control group; B, HGF-treated group, magnification ×200). A: Ki-67-positive cells are only seen at the base of the crypts. B: Ki-67-positive cells extend to almost half of the basal crypts. C: the number of positive epithelial cells per 1,000 epithelial cells from 10 sections of each group was calculated. The number of positive cells in the HGF-treated group was increased compared with the control group (*P < 0.0001). Control group, n = 4; HGF-treated group, n = 4.

Fig. 4. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) staining in DSS-induced colitis (A, control group; B, HGF-treated group, magnification ×200). A: TUNEL-positive cells (arrows) are seen at the apex of the crypts in the control group. B: TUNEL-positive cells (arrow) are seldom seen in the HGF-treated group. C: the number of positive epithelial cells per crypt from 7 sections in the HGF-treated group and 6 sections in the control group was calculated. The number of positive epithelial cells was 1.32 ± 0.56/crypt and 0.83 ± 0.24/crypt in the control and HGF-treated groups, respectively. The differences were not statistically significant (ns) (P = 0.0619). Control group, n = 5; HGF-treated group, n = 5.
Proliferation assay. MCE301 proliferation was assessed by the incorporation of 5-bromo-2′-deoxyuridine (BrdU) (Cell Proliferation ELISA kit, Roche Diagnostics, Mannheim, Germany). Briefly, cells (2 × 10³ cells/well) were preincubated in 96-well plates with serum-free medium for 24 h. After exposure to HGF or EGF for 24 h, 10 μM BrdU was added to the medium and the cells were incubated for another 3–18 h. BrdU incorporation was determined with a peroxidase-conjugated anti-BrdU antibody, and the absorbance was measured. HGF (R & D Systems, Minneapolis, MN) or EGF (BD Bioscience, San Diego, CA) was added at concentrations ranging from 0 to 100 ng/ml. The cell proliferation index was calculated as the increase in the absorbance compared with the untreated cells.

Cell signaling pathway. To investigate the cell signaling pathway involved in proliferation, the specific inhibitors LY-294002 (a PI3K/Akt pathway inhibitor; Calbiochem, San Diego, CA), PD-98059 (an ERK1/2 pathway inhibitor; Calbiochem), and SB-203580 (a p38 pathway inhibitor; Calbiochem) were used. Cells were pretreated with the inhibitors, which were dissolved in DMSO for 30 min before HGF exposure. The proliferative effect was assessed by BrdU incorporation. The control cells were treated only with DMSO, in which inhibitors were dissolved. The concentrations of these inhibitors were as follows: 10 μM LY-294002, 10 μM PD-98059, 5 μM SB-203580.

Akt siRNA transfection. MCE301 cells (1 × 10⁴ cells/well) were seeded on a 96-well plate with serum-free media. Transient transfections of Akt-small interfering RNA (siRNA) (Akt1/2/3 Kinases ShortCut siRNA mix, New England Biolabs) or C-siRNA (control siRNA, Lit28i Poly linker ShortCut siRNA mix, New England Biolabs) were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, the siRNA complex (final concentration, 30 nM) was gently overlaid onto the cells and cultured for 18 h. The siRNA-transfected cells were treated with or without 50 ng/ml HGF for 24 h. After incubation with BrdU for another 3 h, the proliferative effect was assessed by BrdU incorporation.

Statistical analysis. All calculations were carried out with StatView 5.0J software (Abacus Concepts, Berkeley, CA). Values are expressed as means ± SD. Differences between groups were evaluated by Student’s t-test. A P value <0.05 was considered to be statistically significant.

RESULTS

HGF gene therapy attenuated acute DSS-induced colitis. As described previously, plasmid HGF concentrations, which were 0.52 ng/ml before electroporation, began to increase on day 4, peaked on days 6–9 at 2.1 ng/ml, and then decreased to essentially normal concentrations by 3 wk (43). On the base of these data, the time course of the experimental schedules was planned.

Fig. 5. Expression of phospho-c-Met and phospho-Akt in DSS-induced colitis. A: similar c-Met expression was detected in both the control and HGF-treated groups. The expression of phospho-c-Met protein (immunoprecipitated with anti-c-Met antibody) was enhanced in the HGF-treated group compared with the control group (n = 3) compared with the control group (n = 3), although similar amounts of Akt protein were expressed in both groups. On the other hand, phosphorylated ERK1/2 was variably detected in both groups.

Fig. 6. Protein activation by HGF in MCE301 cells. A: c-Met protein activation by HGF in MCE301 cells. MCE301 cells were either treated with 50 ng/ml of recombinant HGF for 30, 60, or 120 min or left untreated after serum starvation for 24 h. Cell extracts of 100 μg protein were subjected to Western blot analysis with anti-phospho-c-Met antibody (top) and anti-c-Met antibody (bottom). c-Met expression was constant, but phosphorylation of c-Met peaked at 30 min after HGF treatment. B: phosphorylation of both Akt and ERK1/2 induced by HGF. Activation of the signal transduction pathway after HGF stimulation was examined in MCE301 cells by Western blot analysis. The MCE301 cells were serum starved for 24 h and then stimulated with 100 ng/ml HGF for 10, 30, 60, and 120 min and 24 and 48 h. Cell extracts of 35 μg protein were subjected to Western blot analysis. Phospho-Akt was detected at 10 min and was sustained for up to 48 h. On the other hand, phospho-ERK1/2 was detected only at 10 min.
In this experimental model, anal bleeding was observed on day 3 in both groups, which continued afterward. Histologically, the DSS-induced colitis in the control group was composed of crypt disruption, epithelial destruction, and marked infiltration by inflammatory cells (Fig. 1, A and B). On the other hand, the HGF-treated group had milder changes and attenuated acute colitis (Fig. 1, C and D). The crypt score was 2.48 ± 1.42 in the control group and only 1.13 ± 1.19 in the HGF-treated group (Fig. 1E, P < 0.005). The inflammation score in the HGF-treated group was 2.67 ± 1.40, compared with 3.59 ± 0.84 in the control group (Fig. 1F, P < 0.05). Thus HGF gene transfer decreased the crypt and epithelium destruction as well as the inflammatory infiltrate in DSS-induced colitis.

**HGF gene therapy activates c-Met.** Activation of the c-Met receptor was assayed by immunohistochemistry using anti-phospho-c-Met antibody in DSS-induced colitis (Fig. 2). In the control group, very few cells were positive in the cytoplasm of the upper epithelia, and none were detected in the basal crypt (Fig. 2A). However, in the HGF-treated group, the cytoplasm of the upper epithelia was intensively stained (Fig. 2B), and a few cells were positive in the basal crypt. HGF treatment induced the phosphorylation of the c-Met receptor in colonic epithelia and activated HGF/c-Met signaling. The mesenchymal cells were also positive in both groups.

**HGF gene therapy promoted proliferation of colonic mucosa.** The proliferation of the colonic epithelium was assayed by Ki-67 immunohistochemistry (Fig. 3, A and B). Ki-67 positive epithelial cells were found at the basal area of the crypts in both groups. In the HGF-treated group, Ki-67-positive cells were markedly increased to 446.8 ± 70.9/1,000 epithelial cells compared with 243.4 ± 72.5 in the control group (P < 0.0001, Fig. 3C).

**HGF gene therapy did not affect apoptosis of colonic mucosa.** Apoptosis of the colonic epithelium was assayed by TUNEL immunohistochemistry (Fig. 4, A and B). In the HGF-treated group, TUNEL-positive epithelial cells were decreased to 0.83 ± 0.24/crypt compared with 1.32 ± 0.56/crypt in the control group (P = 0.0619, Fig. 4C). However, the difference between the two groups was not statistically significant.

**Enhanced expression of phospho-c-Met and phospho-Akt in the colon with HGF.** Since phosphorylation at tyrosine residues of the c-Met protein is a hallmark of activation of the receptor, we examined the phosphorylation of the c-Met receptor. In the HGF-treated group, expression of phospho-c-Met protein immunoprecipitated with anti-c-Met antibody was higher than in the control group (Fig. 5A), which was consistent with the immunohistochemical study. Thus HGF/c-Met signaling was effectively activated in this model.

The intracellular signaling pathways were examined next. Expression of phospho-Akt protein in the HGF-treated group was higher than in the control group, while total Akt expression was not changed. On the other hand, expression of phospho-ERK1/2 was variably detected in each sample (Fig. 5B). Moreover, phosphorylation of p38 and JNK was not clearly detected by Western blot analysis in either group (data not shown).

Therefore, the in vivo results indicated that HGF gene therapy promoted epithelial cell proliferation and induced the PI3K/Akt pathway rather than the ERK1/2 pathway.

**HGF receptor expression and activation by HGF in MCE301 cells.** Western blot analysis with an anti-c-Met antibody revealed that the c-Met receptor is expressed in MCE301 cells. As shown in Fig. 6A, tyrosine phosphorylation of the c-Met protein was enhanced after HGF treatment in MCE301 cells, peaking at 30 min.

**HGF increased phosphorylation of both Akt and ERK1/2.** Signal transduction pathways controlled by kinases modulate critical cellular functions such as cell growth, apoptosis, and differentiation. To determine which signal transduction pathway was activated by HGF stimulation in MCE301 cells, the phosphorylation of Akt or MAPK was analyzed by Western blotting. As shown in Fig. 6B, Akt was phosphorylated within 10 min after HGF stimulation, and the increased phosphorylation was maintained for 48 h. Phospho-ERK1/2 was transiently detected at 10 min after HGF stimulation. Moreover, the phosphorylation of p38 and JNK was examined, but was not clearly detected by Western blot analysis (data not shown).

**HGF promotes cell proliferation of MCE301 cells.** MCE301 cells were exposed to HGF (10, 50, 100 ng/ml) or EGF (100 ng/ml) protein for 24 h, and the proliferation was evaluated by BrdU incorporation (Fig. 7). BrdU incorporation demonstrated that HGF promoted cell proliferation in a concentration-dependent fashion, although 100 ng/ml of HGF did not cause any significant increase in proliferation over 50 ng/ml. In addition, 100 ng/ml of HGF did not cause any significant difference in proliferation from 100 ng/ml of EGF.

**HGF exerts its proliferative effects via the PI3K/Akt signaling pathway.** To determine which signaling pathway is responsible for the proliferative action of HGF, the cells were pretreated with three inhibitors, LY-294002, PD-98059, or SB-203580, followed by stimulation with HGF. The activation of
Akt induced by HGF was inhibited by LY-294002 and the activation of ERK1/2 was inhibited by PD-98059 (Fig. 8A).

Next, the signaling pathway responsible for the proliferative action exerted by HGF in MCE301 cells was evaluated. Using PI3K/Akt and ERK1/2 inhibitors before the HGF treatment, the proliferative action was assayed by BrdU incorporation. As demonstrated in Fig. 8B, BrdU incorporation which was enhanced by HGF treatment for 24 h, was inhibited to nearly the basal level with LY-294002, whereas it was reduced to 83% of the HGF-induced value with PD-98059 and not changed with SB-203580. When the cells were treated with each inhibitor alone, BrdU incorporation was not affected (data not shown), indicating that these effects were not secondary to the toxicity of the inhibitors. The proliferative effect of HGF is mainly exerted via the PI3K/Akt signaling pathway in MCE301 cells.

Akt-siRNA inhibited proliferation in MCE301 cells. To demonstrate the direct effect of Akt on cell proliferation in colonic epithelial cells, Akt was downregulated with siRNA, and cell proliferation was determined by BrdU incorporation. As shown in Fig. 9, BrdU incorporation which was enhanced by HGF treatment of the control cells (C-siRNA), was blocked by Akt-siRNA. These experiments demonstrated the involvement of Akt signaling in the proliferation of colonic epithelial cells following HGF stimulation.

**DISCUSSION**

Serum HGF level is elevated in patients with UC (35), and HGF and c-Met expression is increased in the inflamed mucosa of UC (16). These observations suggest HGF/c-Met system is involved in the repair process of the inflamed mucosa of UC. Recently, it has been reported that the administration of HGF facilitates colonic mucosal repair in an animal colitis model, and HGF protein can act as a potent anti-inflammatory cytokine by decreasing the expression of TNF-α and IFN-γ. Several studies demonstrated that its anti-inflammatory effects are a major function of HGF in colitis (24, 25). Our study also found decreased inflammation scores in HGF-treated mice compared with the controls. In kidney studies, HGF ameliorated acute renal inflammation in part by downregulating E-selectin-mediated macrophage adhesion to the inflamed endothelium (7). Since the mechanism for its anti-inflammatory effects has not been elucidated in colitis, further studies are needed to answer this question.

Several studies have focused on HGF as a proliferative and antiapoptotic drug, as well as an anti-inflammatory one. Ohda et al. (26) showed that the intraperitoneal administration of recombinant human HGF ameliorated 2,4,6-trinitrobenzene sulfonic acid (TNBS)- and DSS-induced colitis through antiapoptosis rather than by stimulating epithelial cell proliferation, except for anti-inflammation. Other reports have suggested that the intrarectal administration of recombinant adenovirus expressing HGF (20) or the naked HGF gene (8, 14) facilitates the repair of TNBS- and DSS-induced mucosal injuries, via both enhanced cell proliferation and the inhibition of epithelial cell apoptosis. This study showed that naked gene therapy by electroporation ameliorated DSS-induced colitis mainly...
tered naked gene therapy was consistently higher than that in phospho-Akt in HGF-treated mice by intrarectally adminis-
trated c-Met, prolonged the immunoreactivity of gastrointesti-
nal mucosa.

In colorectal cancers, c-Met is overexpressed in the vast majority of adenomas, invasive carcinomas, metastases, and through epithelial cell proliferation. This discrepancy between studies may be due to differences in the experimental models such as the reagent, its dosage, and the duration of treatment.

Since this study showed greater cell proliferation than apoptosis with HGF, we focused on the function of HGF on epithelial cell proliferation in vitro. The MCE301 cells used in this study are accepted as a normal colon epithelium (36, 37). Exposure to HGF increased the proliferation of MCE301 cells in a dose-dependent manner, and the activity of 100 ng/ml HGF was almost the same as that of EGF, which is regarded as a strong mitogenic factor of colonic epithelium. First, we confirmed that c-Met was phosphorylated transiently following HGF treatment and that Akt phosphorylation was sustained for 48 h, although phospho-ERK expression was transient. This suggests that HGF activity continues via the Akt signal pathway for 48 h. We also performed these studies using specific signaling pathway inhibitors and demonstrated that the PI3K/Akt signaling pathway mainly contributed to the proliferation of colonic epithelial cells. For colonic epithelial cells, the cell signaling pathways activated by HGF have not yet been clarified. This is the first study to provide evidence that the proliferation of normal colonic epithelial cells induced by HGF is mediated via the PI3K/Akt signaling pathway. This was further confirmed by using siRNA for Akt. Our in vitro results, indicating that cell proliferation stimulated by HGF was exerted via the PI3K/Akt pathway rather than the ERK1/2 pathway, were consistent with our in vivo study.

Both Akt and ERK1/2 are the major downstream molecules of HGF signals which regulate the proliferation and apoptosis of cells (41). Kanbe et al. (14) demonstrated that the level of phospho-Akt in HGF-treated mice by intrarectally adminis-
tered naked gene therapy was consistently higher than that in the control mice with DSS-induced colitis, which is in agreement with our results. On the other hand, another study demonstrated that ERK1/2 was increased in mice that were treated with intrarectally administered recombinant adenovirus expressing HGF following TNBS-induced colitis (20). Thus the signaling molecules activated by HGF stimulation may depend on the animal model and conditions.

In both normal intestinal epithelial cells and colorectal cancer cells, PI3K activity has been reported to be essential for proliferation, and their proliferation is induced by EGF, which is consistent with our study (12, 33). It was also reported that the activation of c-Met enhances X-linked inhibitor of apoptosis protein through the Akt pathway and is correlated with colorectal cancer tumor progression (40). In the stomach, HGF contributes to gastric mucosal repair and c-Met is activated in gastric carcinoma tissue (11). Although ERK1/2 protein phosphorylation is speculated to be involved in HGF-induced proliferation in gastric cancer cells (29), the critical signal pathway for the proliferative effect of HGF on normal gastric mucosa is still largely unknown. To our knowledge, this is the first study to determine the proliferative signal pathway of HGF in normal gastrointestinal epithelial cells. Moreover, we examined the antiapoptotic effect of HGF in MCE301 cells. HGF prevented cisplatin or TNF-α/cycloheximide-induced apoptosis and protected cells from apoptosis through the ERK1/2 pathway in the presence of inhibitors (data not shown).

Gene therapy by adenoviral-mediated, liposome-formulated, or naked gene is now moving from experimental studies into clinical applications. Viral vector gene transfer has proved efficient but does not allow repetitive injection. Although the administration of liposome-formulated DNA or naked plasmid DNA is feasible, the levels of induced protein are therapeutically unstable (2). Recently, DNA electroporation has greatly improved the efficiency of nonviral gene transfer. Electroporation in vivo shows consistently high levels of gene expression for many genes (1, 42, 43).

We previously reported that HGF gene electrotransfer is effective for acute liver injury and hepatectomy on liver cirrhosis (42, 43). The plasma concentration of HGF after electroporation increased four times the baseline amount by day 6 and remained elevated for nearly 3 wk. We examined the effects of HGF gene transfer by a single electroporation against acute DSS-induced colitis and showed that this type of gene transfer ameliorated acute DSS-induced colitis. Thus HGF gene transfer by electroporation may be effective therapy.

Phospho-c-Met immunohistochemistry showed that mainly the upper epithelial cells were stained in the HGF-treated group, while only a few basal epithelial cells were positive. Although there was wider localization of Ki-67-positive cells than phospho-c-Met-positive cells in the basal layer, phospho-c-Met-positive cells were still presented in the proliferative zone, which is important for mucosal repair. A recent immunohistochemical study showed that the cell proliferative zone appears to be independent of phospho-c-Met staining in an injured gastric model, which may indicate that the gastric mucosa is repaired in a rapid fashion (22). It also may suggest that the antibody we used, which recognizes autophosphory-
lated c-Met, prolonged the immunoreactivity of gastrointestinal mucosa.

In colorectal cancers, c-Met is overexpressed in the vast majority of adenomas, invasive carcinomas, metastases, and...
UC-associated colorectal cancers (6). Despite those observations, HGF/c-Met activation in the development of colorectal carcinoma remains poorly understood. It has been reported that overexpressed HGF/c-Met in transgenic mice can promote tumorigenicity, and HGF/c-Met has also been implicated in the invasion and metastasis of tumor cells because of its profound effects on cell motility (32, 39). Recently c-Met and β-catenin pathways are reported to be mutually activated and generated a self-amplifying positive feedback loop in colorectal carcinomas (31).

Short-term treatment by HGF might be feasible and beneficial for colitis, although its use for this needs to be carefully considered. Actually, HGF is now under clinical trial in Japan for fulminating hepatitis. Moreover, further studies using a safer growth factor reconstituted of HGF, such as metron factor-1 (19), are warranted.

In conclusion, we examined the effects of HGF on acute DSS-induced colitis. HGF gene therapy by electroporation exhibited beneficial therapeutic effects through epithelial cell proliferation. Furthermore, our in vitro data demonstrated that HGF induced the proliferation of colorectal epithelial cells via the PI3K/Akt pathway.

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