Ameliorating effect of hepatocyte growth factor on inflammatory bowel disease in a murine model

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Submitted 27 September 2004; accepted in final form 11 November 2004

Oh, Koushi, Yuji Iimuro, Masaharu Takeuchi, Yasufumi Kaneda, Tsuyoshi Iwasaki, Nobuyuki Terada, Takayuki Matsumoto, Kenji Nakanishi, and Jiro Fujimoto. Ameliorating effect of hepatocyte growth factor on inflammatory bowel disease in a murine model. Am J Physiol Gastrointest Liver Physiol 288: G729–G735, 2005. First published November 18, 2004; doi:10.1152/ajpgi.00438.2004.—Hepatocyte growth factor (HGF), a multifunctional cytokine, accelerates intestinal epithelial proliferation. We studied the effects of HGF in mice with trinitrobenzene sulfonic acid-induced colitis, which shows clinical and molecular resemblance to Crohn’s disease. Mice with colitis repeatedly were transfected intramuscularly with human HGF cDNA. Weight, survival, histopathology, proinflammatory cytokine mRNAs, and leukocyte infiltration were assessed. Treatment with HGF cDNA induced tyrosine phosphorylation of intestinal c-Met/HGF receptors, inhibited apoptosis, and promoted mitosis in intestinal epithelial cells, accelerating intestinal epithelial restoration and suppressing inflammation. Transfection with HGF cDNA markedly suppressed intestinal mRNA expression of T-helper 1 cytokines such as interleukin-12 and -1B, interferon-γ, and tumor necrosis factor-α. Numbers of total and CD4-positive T cells, neutrophils, and myeloperoxidase activity in intestinal epithelium were diminished by HGF gene transfer, which also prevented weight loss, and improved survival. HGF might prove useful for controlling inflammatory bowel disease.

2,4,6-trinitrobenzene sulfonic acid; gene therapy

Etiologies of both Crohn’s disease and ulcerative colitis (UC), idiopathic chronic inflammatory disorders collectively termed inflammatory bowel diseases (IBD), remain obscure. Various experimental animal models have been devised during the last decade to clarify mechanisms underlying IBDs (5, 8, 38). Among these, rectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS) to immunocompetent mice results in transmural intestinal leukocyte infiltration accompanied by severe diarrhea and weight loss, resembling some characteristics of human Crohn’s disease (30). Inflammation in the model is characterized by dense, transmural infiltration by T cells, mainly of the CD4+ phenotype, with excessive production of T-helper cell (Th) 1-derived cytokines (31). Induction of colitis in the mice is begun by local administration of ethanol, which injures the epithelial lining of the intestine, the initial barrier against a variety of toxic agents. An imbalanced mucosal immune response then can be induced by subsequent administration of a hapten reagent, TNBS. Clinical data from patients with IBD similarly suggest the key importance of an impaired epithelial barrier and exposure of intestinal tissues to microbial toxins in initiation and progression of IBD (10). Considering this sequence, accelerating the restoration of the epithelial lining could impede expansion of intestinal inflammation caused by TNBS and also might improve the clinical status of patients with IBD.

Previous studies demonstrated participation of several growth factors and cytokines in the regulation of the intestinal epithelial restoration (6, 20, 36). Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes, has proven to be multifunctional, showing mitogenic, motogenic, morphogenic, angiogenic, and anti-apoptotic activities in a wide variety of cells and organs (3, 28), including those of the gastrointestinal tract (33). HGF has been found to act on intestinal cell populations as a positive growth regulator and to accelerate remodeling after epithelial injury (14, 32, 33). The HGF receptor, c-Met, also has been reported to appear in intestinal mucosal epithelial cells during restoration of continuity (20, 43). Thus intestinal mucosal healing appears to be stimulated by the HGF-c-Met signaling system. Indeed, expression of HGF and its receptor, c-Met, has been reported to increase in a rat model of UC and patients with UC (24, 34). Moreover, the beneficial effect of HGF administration in a rat UC model has also been reported (41). However, the effect of HGF on Crohn’s disease remains elusive.

Recent advances in molecular techniques have provided several gene-delivery strategies in vivo that show promise as novel therapeutic approaches using cDNAs encoding beneficial molecules. These techniques variously employ naked plasmid DNA (39), liposomes encapsulating DNA (18), and viral vectors such as adenovirus, retrovirus, and adenovirus-associated virus (9, 11, 27). Each type of delivery system has individual advantages and limitations (23). A fusigenic liposome with the hemagglutinating virus of Japan (HJV-liposome) represents an attractive in vivo gene-delivery system (22) characterized by persistent gene expression and low cytotoxicity (17). We used this system in the present study to transduce HGF in mice developing IBD, previously having achieved stable HGF expression in a rat liver cirrhosis model, a rat obstructive nephropathy model, and a mouse graft vs. host disease model (13, 26, 42). We designed the present study to assess the possible therapeutic role of HGF in TNBS-induced colitis, which resembles Crohn’s disease in humans. Repeated transfection of the human HGF gene encapsulated in HJV-liposomes induced

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phosphorylation of intestinal c-Met/HGF receptors in the mice. We found that HGF transfection inhibited apoptosis in intestinal epithelial cells and promoted constitution of the epithelium, suppressing inflammatory responses. This approach using a gene encoding a multifunctional growth factor may prove useful for the control of IBD.

MATERIALS AND METHODS

Animals. Male 12- to 14-wk-old BALB/c mice (SLC, Japan), weighing 24–28 g, were used in this study. They were maintained under specific pathogen-free conditions and fed free autoclaved chow and water ad libitum. Animal care was in accordance with the guidelines of the Hyogo College of Medicine.

Murine colitis was induced by intracoelomic administration of a hapten reagent, TNBS (Sigma Chemical, St. Louis, MO), dissolved in ethanol, as described previously (30, 31). Under ether anesthesia, mice received 1.0 mg TNBS dissolved in 100 μl of 50% ethanol via anus using an 18-gauge soft silicon catheter inserted in the proximal colon for 5 cm. They were kept in a vertical position for 1 min. Mice were checked for their body weight and general condition daily.

Preparation of HVJ-liposome. HVJ-liposome was prepared as described previously (22). Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4.8:2. Dried lipid was hydrated in 200 μl balanced salt solution (BSS: 137 mM NaCl, 5.4 mM KCl, and 10 mM Tris–HCl, pH 7.6). Plasmid DNA (20 μg) was previously incubated with nonhistone chromosomal protein (high-mobility group-I) purified from calf thymus. Liposome was prepared by shake and sonication. Purified HVJ was inactivated by ultraviolet irradiation for 3 min just before use. The liposome suspension was mixed with HVJ (10,000 hemagglutinating units) in a total volume of 1 ml BSS. The mixture was incubated at 4°C for 10 min and then for 30 min with gentle shaking at 37°C. Free HVJ was removed from the HVJ-liposomes by sucrose density centrifugation. In this study, ~20 μg pUC-SRα/HGF expression plasmid entrapped in HVJ-liposome complex was injected in gluteal muscles for every injection.

Gene transfer and experimental design. Human HGF cDNA (2.2 kb) was inserted between the Not I sites in the pUC-SRα expression plasmid vector and encapsulated in HVJ-liposome as described. Purified HGF/HVJ-liposome complexes were directly injected in gluteal muscles 3 days before and 4 days after the induction of colitis. According to our previous study, exogenous human HGF expression persisted at least for 7 days after the gene transfer using the HVJ-liposome method with a peak expression at day 4 (42). To maintain a significant level of HGF protein in plasma, we delivered the human HGF gene two times in this experiment. In the control group, they received PBS injection in the same manner. Some mice were killed 3 days after the TNBS treatment, and surviving mice were killed at day 10. Total colonic tissues were collected and used for histopathological analysis, flow cytometric analysis, enzymatic analysis of myeloperoxidase (MPO) activities, analysis of mRNA expression of inflammatory cytokines, and tyrosine phosphorylation of c-Met. In some mice, 100 mg/kg bromodeoxyuridine (BrdU) was injected intraperitoneally 1 h before death for analysis of DNA synthesis in the intestinal epithelium.

Measurement of plasma HGF level. Samples were collected 0, 4, and 10 days after the first injection of HGF-cDNA. Exogenous human and endogenous mouse HGF levels were measured in plasma by ELISA kits (Institute of Immunology, Tokyo, Japan).

Western blot and immunoprecipitation analysis. Total colonic tissues were removed at day 10, and total protein was extracted from homogenized tissues. The protein samples were adjusted to the same concentration for Western blotting. For immunoprecipitation of c-Met-protein, lysates were incubated with the protein A-agarose beads (Pierce, Rockford, IL) pretreated with an anti-mouse c-met antibody. Each sample containing 20 μg total protein was separated by SDS-PAGE on 7% acrylamide slab gels. After electrophoresis, the gel was transferred to a polyvinylidene difluoride sheet and then probed with the anti-mouse c-met antibody (sc-8057; Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-phosphotyrosine antibody (PY-20; ICN, Transduction Laboratories, Lexington, KY).

Histopathological analysis. At death, the total colon was removed and carefully opened longitudinally on the opposite side to the mesentery. The colonic tissues were washed with PBS and fixed in 10% formalin in PBS overnight at room temperature. After being embedded in paraffin, 4-μm sections were prepared and stained with hematoxylin and eosin (H&E). Histological changes of the colon were evaluated in a blind fashion according to a semiquantitative grading method previously reported elsewhere (30). Moreover, existence of epithelial restoration, namely appearance of growing epithelial cells over the disrupted mucosal layer, was evaluated in the H&E staining in each experimental group.

To assess DNA synthesis in the intestinal epithelium, BrdU incorporation in the epithelial cells was determined in mice treated with BrdU 1 h before death. The paraffin-embedded sections were incubated in 4 N HCl for 20 min at 37°C and washed several times with dH2O and 1× PBS. After blocking of endogenous peroxidase with peroxidase blocking agent, sections were incubated with mouse monoclonal anti-BrdU antibody (1:200, clone Bu20a 1:30; DAKO, Kyoto, Japan) for 10 min at 25°C. After being washed, the sections were incubated for 60 min with biotin-conjugated anti-mouse antibody, labeled with peroxidase streptavidin, and examined after being incubated with diamobenzidine-H2O2 substrate.

Detection of apoptotic cells in large intestine. To detect apoptotic cells in large intestine, the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) method, which detects in situ DNA fragmentation, was applied using the Apo Tag in situ Apoptosis Detection System according to the standard protocol (Oncor, Gaithersburg, MD).

Measurement of MPO activities. MPO activity is an index of neutrophils, monocytes, and macrophages infiltrating into the inflamed colonic tissue. A modified method of MPO activity measurement, which was previously described, was used (25).

Analysis of cytokine mRNA expression by RNase protection assay. Purified total RNA was extracted from total colonic tissue at day 10 using a standard acid-guanidium-phenol-chloroform method (1). Cytokine mRNAs were detected using a RiboQuant MultiProbe RNase protection assay system (PharMingen, San Diego, CA) by standard protocols (37). Briefly, multiprobes were 32P labeled and synthesized by T7 RNA polymerase. Purified riboprobes were then hybridized overnight with 10 μg of the RNA samples. The hybridized RNA was treated with RNase, and single-strand RNA was digested. Remaining dRNA samples were then electrophoresed in 6% polyacrylamide-Tris-borate-EDTA-urea minigels (Novex, San Diego, CA). The gels were dried, exposed, and quantitated in a PhosphorImager (BAS 2000; Fuji, Mountain View, CA).

Flow cytometric analysis. Mouse colonic mucosal mononuclear cells were prepared as described previously (15), with minor modification. Briefly, total colonic tissues were removed, and the content was washed with PBS at day 10. The mucosa was dissected from the underlying muscularis using iris scissors, and cells were passed through the mesh and suspended in PBS. Cell suspensions were prepared in PBS containing 1% FCS and 0.1% sodium azide. The cells were incubated with anti-Fe receptor mAb (2.4G2) for 10 min at 4°C and then incubated with FITC-conjugated mAb and PE-conjugated mAb for 30 min. The stained cells were washed two times, resuspended, and analyzed using FACScan (Becton-Dickinson, Mountain View, CA).

Statistical analysis. Results for the group were compared by Mann-Whitney’s U-test and X-test. Survival data were plotted by the Kaplan-Meier method and analyzed by Long-Rank test. A P value <0.05 was considered significant.
**RESULTS**

Expression of HGF and its biological activity in colonic epithelium. We previously reported that repeated transfection of human HGF gene in skeletal muscle resulted in a sustained high serum concentration of human and endogenous rat or mouse HGF (13, 26, 42). HGF expression initially was transient, but repeated HGF gene injections showed a booster effect, and stable HGF production was obtained. Human and mouse HGF both were detected by ELISA in sera from mice transfected with the human HGF gene in this TNBS-induced colitis model. The total concentration of HGF (human and mouse) in serum was 0.20 ng/ml before injection, 1.70 ng/ml at 4 days after the first injection of HGF cDNA, and 1.63 ng/ml at 10 days. Amounts of c-Met/HGF receptor protein in the intestine increased in TNBS-treated control mice compared with untreated normal mice, whereas expression was relatively low in HGF-treated mice exposed to TNBS (Fig. 1A). The receptor contains an intracellular tyrosine kinase domain, and biological activities of HGF are produced by tyrosine residue phosphorylation in c-Met. Tyrosine phosphorylation of c-Met was barely detectable in intestine from PBS-treated control mice given TNBS, although strong tyrosine phosphorylation of c-Met was observed in intestine from mice given the HGF gene and TNBS (Fig. 1A). These results indicate that introduction of the HGF gene produced biologically effective amounts of HGF protein and activated the c-Met receptor in the intestine. Thus HGF could exert its biological effect in the intestines of mice with TNBS-induced colitis.

HGF gene-mediated amelioration of colitis and weight loss. Consistent with previous reports, TNBS-induced colitis in our mice was evident as severe inflammation by 2–3 days after TNBS administration and persisted for 3 wk (8). In control mice given PBS rather than the transgene, massive infiltration of lymphocytes in the submucosal layer accompanied by disruption of the mucosal layer was observed in the intestine at day 10 (Fig. 1, D and E). In contrast, HGF gene induction resulted in a significant decrease in inflammation and also attenuated the disruption of the mucosal layer (Fig. 1, F and G). The effect of HGF on epithelial injury was assessed using a grading system described in MATERIALS AND METHODS (30). As shown in Fig. 1H, transfection with the HGF gene significantly improved scores at 10 days after TNBS administration. Some mice were killed 3 days after TNBS administration to examine whether ethanol/TNBS caused the same degree of early intestinal epithelial injury in PBS-treated and HGF-treated mice, which proved to be true. Both groups showed disruption of the epithelial layer and transmural infiltration of inflammatory cells. Mean scores at day 3 were 3.5 ± 0.5 in PBS-treated and 3.6 ± 0.5 in HGF gene-treated mice (no significant difference).

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**Fig. 1.** A: expression of c-Met, the hepatocyte growth factor (HGF) receptor, and its phosphorylation (Western blot) are shown. The c-Met protein was overexpressed in the colonic tissues in the PBS-treated group, whereas the expression was relatively low in HGF gene-treated animals. However, strong tyrosine phosphorylation of c-Met was observed only in the HGF gene-treated group, suggesting significant biological activity of HGF in this group. B-G: histological analysis of the large intestine after the induction of colitis was performed at day 10 [hematoxylin and eosin (H&E), original magnification ×100 (B, D, and F) and ×400 (C, E, and G)]. The large intestines from untreated normal mice (B and C), mice treated with PBS/2,4,6-trinitrobenzene sulfonic acid (TNBS) (D and E), and mice treated with HGF/TNBS (F and G) are shown. Severe transmural infiltration by inflammatory cells and thick intestinal walls accompanied by disruption of the mucosal layer were observed in the PBS/TNBS group (D and E). B–G are representative of 35–37 individual animals among the TNBS-treated mice. H: histological grading scores, which are semiquantitatively calculated at day 10, are presented. PY, phosphotyrosine.
Reconstruction of colonic epithelium often was observed in specimens from HGF gene-treated mice at day 10, with epithelium extending over previously disrupted regions (Fig. 2, A and B). In PBS control mice given TNBS, restoration of epithelium was found in 14 of 25 mice (56%), whereas restoration was evident in 17 of 20 mice treated with HGF (85%, P = 0.036, Fig. 2G). Because HGF is a potent mitogen for epithelial cells, we assessed BrdU incorporation in the colonic epithelium. The number of BrdU-positive cells in the intestine was much greater in mice treated with HGF than in PBS-treated control mice (Fig. 2, C and D). Apoptosis of intestinal epithelium has been reported in the TNBS-induced colitis model (12). When apoptosis was investigated with the TUNEL method for detecting DNA fragmentation in situ, a number of apoptotic cells were observed in the epithelial crypts of PBS-treated control mice (Fig. 2E), whereas HGF gene treatment inhibited this apoptosis (Fig. 2F). Thus HGF gene transfection appeared to improve recovery from intestinal damage through its anti-apoptotic effect and through accelerated restoration of epithelial continuity after injury.

The main outward signs of TNBS-induced colitis in this experiment were severe diarrhea and loss of body weight; some mice died of extensive wasting. Characteristic signs of potentially lethal colitis persisted up to 3 wk. Survival rates with such involvement are shown in Fig. 2H. More than 50% of mice treated with PBS died within 2 wk, whereas mice treated with the HGF gene exhibited a significantly higher survival rate (Fig. 2H; 50 vs. 75%, P < 0.05). PBS-treated control animals gradually lost weight (Fig. 2I), whereas mice receiving the HGF gene began to gain weight at day 3 and failed to develop wasting, appearing healthy from that time (Fig. 2I).

**Downregulation of the inflammatory response by HGF.** Intrarectal administration of TNBS induces chronic active inflammation, evident as dense infiltration of the colon by inflammatory cells, among which CD4+ T cells are considered to predominate (30). The population of mucosal mononuclear cells isolated from the lamina propria of the large intestine was analyzed using FACS (Fig. 3A). HGF gene treatment significantly decreased the total number of T cells (CD3+) in the lamina propria, from $6.1 \times 10^5$ to $2.1 \times 10^5$, at 10 days after induction of colitis. More than 50% of mice treated with PBS/TNBS died within 2 wk. In contrast, mice treated with HGF gene transfer exhibited a significantly higher survival rate (*P < 0.05). I: changes in body weight of mice after TNBS treatment. Each point represents pooled average body weight with an error bar (SE; n = 10–32). Mice that received HGF gene transfer began to gain body weight at day 3 and failed to develop wasting.
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TNBS administration. HGF gene therapy also significantly decreased the number of CD4^+ T cells from 4.7 × 10^5 to 1.3 × 10^5. Numbers of CD8^+ T cells and neutrophils also were decreased significantly by HGF treatment. As a quantitative assessment of neutrophil infiltration in the colon, we assayed MPO activity in colonic extracts. In TNBS-treated mice, MPO activity was seven times that in control mice without colitis, whereas HGF treatment resulted in a significant reduction in MPO activity (P < 0.05, Fig. 3B).

Previous studies of cytokine production in TNBS-induced colitis demonstrated that Th1 cytokines were dominant in this model (7, 12, 30). First, we tried to measure serum concentrations of Th1 cytokines using ELISA kits, but failed to detect the expression accurately at day 10. Next, we analyzed expression of mRNAs encoding cytokines, including TNF-α, IL-12, IFN-γ, and IL-1β, in the large intestine using RNase protection assays (Fig. 3C). TNBS treatment enhanced expression of TNF-α, IL-12, IFN-γ, and IL-1β mRNA beyond that in untreated control mice. HGF treatment strongly suppressed colonic mRNA expression for these cytokines after TNBS treatment.

DISCUSSION

Recently, various anti-cytokine strategies against TNBS-induced colitis, a murine model of human IBD, have been reported to reduce colitis (2, 12, 19, 30, 31). HGF, originally identified as a potent mitogen for hepatocytes, has a variety of effects (3, 28, 29), including anti-apoptotic activities in a wide variety of organs, including those of the gastrointestinal tract (26, 33, 40). In the present study, we demonstrated a beneficial effect of HGF gene transfer in mice with TNBS-induced colitis, which resembles human Crohn’s disease.

The first goal of this experiment was to obtain a stable, persistent HGF concentration in plasma. HVJ- and liposome-mediated gene delivery has been characterized as providing persistent gene expression with low cytotoxicity (17). Based on our previous study (26, 42), expression of exogenous human HGF in plasma was obtained by injection of the human HGF gene, encapsulated in the HVJ-liposome, in the gluteal muscles of the mice. The receptor for HGF is a tyrosine kinase receptor encoded by c-met, and the membrane-spanning β-chain of the c-Met receptor contains an intracellular tyrosine kinase domain (4). Although c-Met protein was expressed more strongly in inflamed colonic tissue after TNBS treatment (Fig. 1A), the biological activity of HGF, which was demonstrated by tyrosine residue phosphorylation in c-Met, was very low. In contrast, in HGF gene-treated mice, expression of c-Met was relatively low, but the degree of tyrosine phosphorylation of c-Met was very high, indicating functional effectiveness of HGF gene transfer. We had some difficulty in explaining the lower expression of c-Met protein in HGF-treated mice. Considering that control mice without colitis also had relatively low expression of c-Met, lower expression in HGF-treated mice may have indirectly reflected nearly complete epithelial reconstitution at the time point examined. Alternatively, down-regulation of c-Met protein expression after stimulation by HGF possibly may be involved (16).

The mechanism of TNBS-induced colitis is believed to involve disruption of the protective epithelial barrier, followed by imbalance of the mucosal immune response to TNBS. No difference in inflammatory scores was seen between the PBS/TNBS-treated and the HGF gene/TNBS-treated mice at day 3, suggesting that cytoprotective and anti-inflammatory effects of HGF against ethanol/TNBS injury may not be the most important factors in this model. Instead, when HGF gene transfer later was seen to significantly attenuate transmural inflammation at day 10, this was accompanied by enhanced repair of the epithelial lining. Transmural infiltration of leukocytes, includ-
ing the lamina propria, and elevated MPO activity in the inflamed colonic tissue after the TNBS treatment instead were greatly attenuated by HGF gene transfer (Fig. 1, D–H, and Fig. 3B). Flow cytometric analysis in PBS/TNBS mice demonstrated a large number of T cells, especially CD4+ T cells, infiltrating in colonic tissues after TNBS treatment, resembling findings in human Crohn’s disease (7, 35). Infiltration of CD4+ and CD8+ T cells, as well as neutrophils, was reduced significantly by HGF gene transfer (Fig. 3A). More detailed histological analysis demonstrated that HGF accelerated proliferation of the intestinal epithelium by day 10 (Fig. 2, A and B), which was confirmed by comparing BrDU incorporation in proliferating epithelial cells (Fig. 2D). Importantly, numbers of apoptotic cells in the intestinal epithelium after TNBS administration clearly were reduced by HGF gene transfer (Fig. 2F). These results suggest that HGF stimulates proliferation and retards apoptosis in the disrupted intestinal epithelial barrier, thus restoring continuity of epithelial cells and in turn lessening subsequent inflammation caused by TNBS and intestinal bacterial toxins.

In most experimental models of chronic intestinal inflammation, including TNBS-induced colitis, various inflammatory cytokines, such as IL-12, INF-γ, and TNF-α, were shown to be overexpressed in inflamed intestinal tissue. In the present analysis, elevated mRNA expression for Th1 cytokines, such as IL-12 and INF-γ, was detected after TNBS exposure. Furthermore, mRNA expression of proinflammatory cytokines, such as TNF-α and IL-1β, was upregulated in this experimental colitis, although we failed to detect serum expression of these cytokines by ELISA at day 10. HGF gene transfer markedly suppressed mRNA expression of these cytokines (Fig. 3C). An anti-inflammatory effect of HGF has been reported previously (21). However, a direct suppressive effect of HGF on inflammatory cytokine production is still obscure. We speculate that, instead, the ameliorating effect of HGF observed in this study reflected mitogenic, morphogenic, and anti-apoptotic effects on epithelial cells rather than direct anti-inflammatory activity. Early restoration of the injured mucosal barrier could prevent prolonged exposure of the submucosal layer to bacterial toxins, leading to reduction in infiltration by inflammatory cells and in production of inflammatory cytokines.

In summary, stable, persistent HGF expression in mice was obtained by repeated intramuscular injections of a vector containing the human HGF gene, ameliorating potentially lethal colitis induced by TNBS. Although the mechanisms underlying this beneficial effect of HGF have not yet been firmly established, treatment with HGF may represent a promising new therapeutic approach to human Crohn’s disease.

REFERENCES


