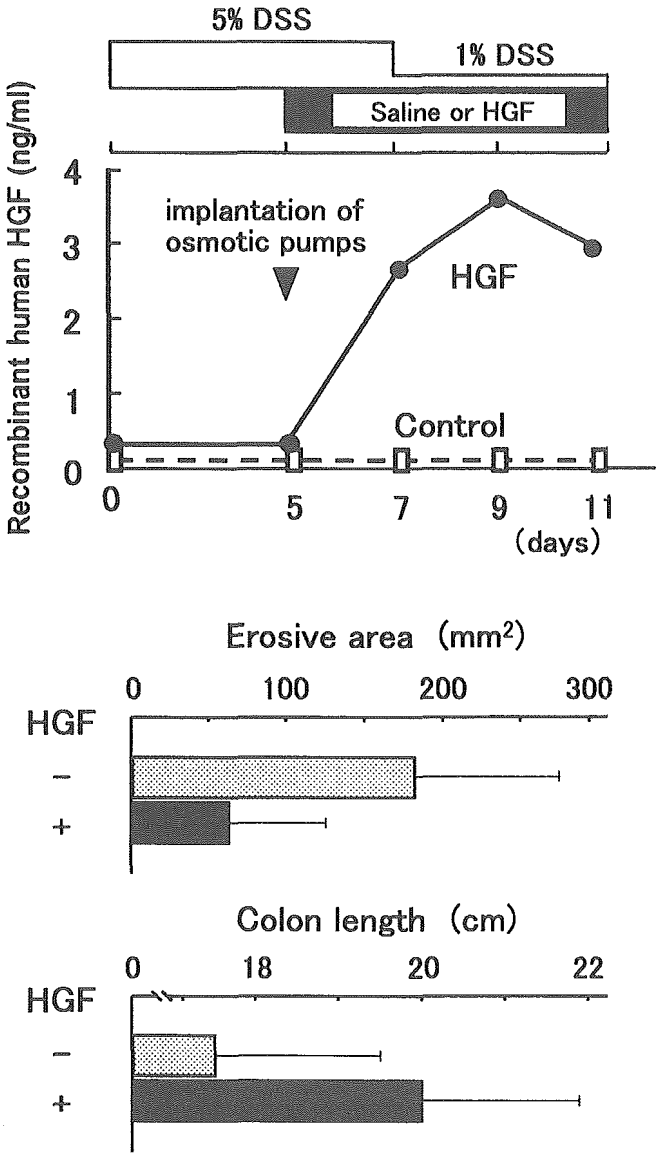


Fig. 4. HGF improves colonic erosions, and reduces large-intestinal shortening in rats with dextran sulfate sodium (*DSS*)-induced colitis. Acute colitis was induced by the administration of 5% DSS for 7 days and maintained by feeding with 1% DSS. After 5 days of 5% DSS administration, recombinant human HGF (0.2 mg/day) or saline was delivered intraperitoneally for 6 days. Colon length (from the colorectal junction to the anal verge) and the erosion area were measured on day 11. $*P < 0.05$

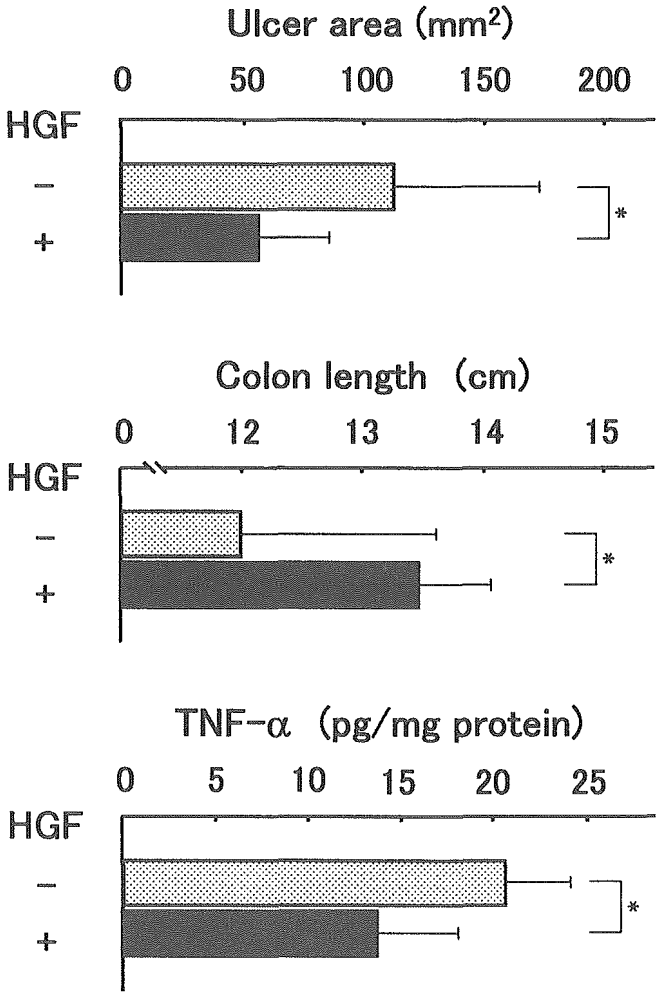


Fig. 5. HGF ameliorates large colonic ulcers in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats. Colitis was induced by a single enema of TNBS (7.5 mg; day 0). Only rats suffering from large ulcers, which occupied more than two-thirds of the luminal circumference colonoscopically on day 5, underwent intravenous injections of recombinant human HGF (1 mg/kg per day) for 5 days. Ulcer area, colon length, and tumor necrosis factor-α (*TNF-α*) levels in colon tissues were examined on day 10. $*P < 0.05$

nant human HGF promoted wound healing in animal models of colitis, including HLA-B27 transgenic rats, which exhibit phenotypic changes similar to IBD.[63–65] Ohda et al.[66] showed that the intraperitoneal administration of recombinant human HGF ameliorated DSS- and TNBS-induced colitis through the inhibition of apoptosis, rather than by stimulating the proliferation of intestinal epithelial cells. Recently, Mukoyama et al.[67] demonstrated that the rectal administration of recombinant adenovirus expressing HGF facilitated the repair of TNBS-induced mucosal injuries, via both enhanced cell proliferation and the inhibition of epithelial-cell apoptosis.

Outlook for clinical applications of recombinant human HGF

Recombinant human HGF will soon be available for administration to patients with fulminant hepatic failure. Therefore, both the clinical availability of HGF and its documented role in mucosal repair suggest that HGF has the potential to be an important new treatment

modality that promotes intestinal mucosal repair in patients with IBD. Recently, two clinical trials using recombinant growth factors for patients with IBD were performed. In a phase II study of recombinant KGF-2 for patients with active ulcerative colitis, intravenous administration of KGF-2, at a dose of 1–50μg/kg, did not effectively induce remission.[68] In that study, the dose of KGF-2 may have been too low for any therapeutic effect to be realized in IBD patients. Conversely, a placebo-controlled trial of recombinant EGF enemas in patients with active left-sided ulcerative colitis or proctitis showed that the rate of remission in patients receiving EGF enemas for 2 weeks was significantly higher than that of the placebo-treated group.[69] Given that the efficacy of growth factors in the treatment of IBD has been established, we are encouraged that HGF may be a useful treatment modality. However, several issues must be resolved before undertaking a clinical trial examining recombinant human HGF. First, because HGF is a growth factor that promotes cell proliferation, we cannot exclude the possibility that the repeated administration of recombinant human HGF might accelerate carcinogenesis. Additionally, when recombinant human HGF is administered intravenously in a bolus, only a fraction of the recombinant human HGF is distributed into colonic tissues.[70]

Increased rates of benign and malignant tumor formation occurred in the livers and mammary glands of different HGF transgenic mouse strains,[71–74] and diethylnitrosamine-induced hepatocarcinogenesis and ultraviolet radiation-induced skin carcinogenesis was accelerated in these mice.[74–76] Although continuous systemic expression of HGF did not promote intestinal tumor formation, 65%–80% of HGF transgenic mice died of intestinal pseudo-obstruction or renal failure.[77] Interestingly, transgenic mice specifically overexpressing HGF in the liver did not develop hepatocellular carcinoma, and the development of hepatic neoplasms induced by TGF-α or the c-*myc* transgene was inhibited in these mice.[78,79] These findings suggest that prolonged exposure to high doses of HGF may accelerate neoplastic development in multiple organs. Further experiments are needed to clarify the potential carcinogenicity of HGF before clinical trials can be undertaken.

The topical administration of recombinant human HGF may be a possible means to reduce the risk of HGF-induced carcinogenesis. Accordingly, we previously observed that intravenously administered recombinant human HGF was distributed mainly to the liver, with less HGF going to the colon,[70] and we also confirmed that the rectal administration of recombinant human HGF did not lead to detectable levels of human HGF in rat serum (unpublished observation). The rectal administration of the recombinant growth factors bFGF and EGF effectively treated IBD in an experimental model and a human clinical trial, respectively. Nakase et al.[80] recently reported that the rectal administration of gelatin microspheres containing IL-10 ameliorated colitis in IL-10-deficient mice. Therefore, although we need to find a suitable carrier solution, recombinant human HGF enemas may both reduce the systemic side effects of HGF administration, including carcinogenesis, as well as deliver HGF to the relevant anatomic location.

Future directions

Numerous growth factors and cytokines are associated, in a complex way, with the regeneration of injured intestinal mucosa. Recently, a prominent role for HGF (which was first purified as a potent agent stimulating liver regeneration) has been found in the repair of injured intestinal mucosa. A phase I/II study of recombinant human HGF in patients with fulminant hepatic failure will soon begin. Although additional preclinical biological studies are required, we are preparing for clinical trials to examine the safety and efficacy of rectally administered recombinant human HGF in patients with IBD. However, because the possibility of HGF-associated carcinogenicity has not been completely excluded, complete informed consent is ethically required.

References

1. Hanauer SB, Present DH. The state of the art in the management of inflammatory bowel disease. Rev Gastroenterol Disord 2003; 275:40453–62.
2. Sandborn WJ, Hanauer SB. Antitumor necrosis factor therapy for inflammatory bowel disease: a review of agents, pharmacology, clinical results, and safety. Inflamm Bowel Dis 1999;5:119–33.
3. Murphy MS. Growth factors and gastrointestinal tract. Nutrition 1998;14:771–4.
4. Beck PL, Podolsky DK. Growth factors in inflammatory bowel disease. Inflamm Bowel Dis 1999;5:44–60.
5. Dignass AU, Strum A. Peptide growth factors in the intestine. Eur J Gastroenterol Hepatol 2001;13:763–70.
6. Dignass AU. Mechanisms and modulation of intestinal epithelial repair. Inflamm Bowel Dis 2001;7:68–77.
7. Mammen JMV, Matthews JB. Mucosal repair in the gastrointestinal tract. Crit Care Med 2003;31:S532–7.
8. Okamoto R, Yajima T, Yamazaki M, Kanai T, Mukai M, Okamoto S, et al. Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. Nat Med 2002;8:1011–7.
9. Okamoto R, Watanabe M. Molecular and clinical basis for the regeneration of human gastrointestinal epithelia. J Gastroenterol 2004;39:1–6.
10. Matsumoto T, Okamoto R, Yajima T, Mori T, Okamoto S, Ikeda Y, et al. Increase of bone marrow-derived secretory lineage epithelial cells during regeneration in the human intestine. Gastroenterology 2005;128:1851–67.

11. Gohda E, Tsubouchi H, Nakayama H, Hirono S, Takahashi K, Koura M, et al. Human hepatocyte growth factor in plasma of patients with fulminant hepatic failure. Exp Cell Res 1986;166: 139–50.
12. Gohda E, Tsubouchi H, Nakayama H, Hirono S, Sakiyama O, Takahashi K, et al. Purification and partial characterization of hepatocyte growth factor of a patient with fulminant hepatic failure. J Clin Invest 1988;81:414–9.
13. Igawa T, Kanda S, Kanetake H, Saitoh Y, Ichihara A, Tomita Y, et al. Hepatocyte growth factor is a potent mitogen for cultured rabbit renal tubular epithelial cells. Biochem Biophys Res Commun 1991;174:831–8.
14. Joplin R, Hishida T, Tsubouchi H, Daikuhara Y, Ayres R, Neuberger JM, et al. Human intrahepatic biliary epithelial cells proliferate in vitro in response to human hepatocyte growth factor. J Clin Invest 1992;90:1284–9.
15. Takahashi M, Ota S, Terano A, Yoshiura K, Matsumura M, Miwa Y, et al. Hepatocyte growth factor induces mitogenic reaction to the rabbit gastric epithelial cells in primary culture. Biochem Biophys Res Commun 1993;191:528–34.
16. Dignass AU, Lynch-Devaney K, Podolsky DK. Hepatocyte growth factor/scatter factor modulates intestinal epithelial cell proliferation and migration. Biochem Biophys Res Commun 1994;202:701–9.
17. Lacy ER. Epithelial restitution in the gastrointestinal tract. J Clin Gastroenterol 1988;10(Suppl):72–7.
18. Feil W, Wentzl E, Vattay P, Starlinger M, Sogukoglu T, Schiessel R. Repair of rabbit duodenal mucosa after acid injury in vivo and in vitro. Gastroenterology 1987;92:1973–86.
19. Moore R, Carlson S, Madara JL. Rapid barrier restitution in an in vitro model of intestinal epithelial injury. Lab Invest 1989;60:237–44.
20. McCormack SA, Viar MJ, Johnson LR. Migration of IEC-6 cells: a model for mucosal healing. Am J Physiol 1992;263G:426–35.
21. Nusrat A, Delp C, Madara J. Intestinal epithelial restitution. J Clin Invest 1992;89:1501–11.
22. Part 1. Epithelial Restitution. Gastrointestinal mucosal repair and experimental therapeutics. In: Cho CH, Wang JY, editors. Front Gastrointest Res, vol. 25. Basel: Karger; 2002.
23. Grenier A, Chollet-Martin S, Crestani B, Delarche C, El Benna J, Boutten A, et al. Presence of a mobilizable intracellular pool of hepatocyte growth factor in human polymorphonuclear neutrophils. Blood 2002;99:2997–3004.
24. Koehne P, William C, Strauss E, Schindler R, Eckardt KU, Buhrer C. Lack of hypoxic stimulation of VEGF secretion from neutrophils and platelets. Am J Physiol Heart Circ Physiol 2000; 279:H817–24.
25. Oka Y, Orth DN. Human plasma epidermal growth factor/β-urogastrone is associated with blood platelets. J Clin Invest 1983; 72:249–59.
26. Assoian RK, Sporn MB. Type β transforming growth factor in human platelets: release during platelet degradation and action on vascular smooth muscle cells. J Cell Biol 1986;102:1217–23.
27. Karey KP, Sirbasku DA. Human platelet-derived mitogens: subcellular locarilation of insulin-like growth factor I to the α-granule and release in response to thrombin. Blood 1989;74:1093–100.
28. Podolsky DK. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. Am J Physiol Gastrointest Liver Physiol 1999;277:G495–9.
29. Matsuura M, Okazaki K, Nishio A, Nakase H, Tamaki H, Uchida K, et al. Therapeutic effects of rectal administration of basic fibroblast growth factor on experimental murine colitis. Gastroenterology 2005;128:975–86.
30. Dignass AU, Tsunekawa S, Podolsky DK. Fibroblast growth factors modulate intestinal epithelial cell growth and migration. Gastroenterology 1994;106:1254–62.
31. Ciacci C, Lind SE, Podolsky DK. Transforming growth factor beta regulation of migration in wounded rat intestinal epithelial monolayers. Gastroenterology 1993;105:93–101.
32. Boros P, Miller CM. Hepatocyte growth factor: a multifunctional cytokine. Lancet 1995;345:293–5.
33. Zarnegar R, Michalopoulos GK. The many faces of hepatocyte growth factor: from hepatopoiesis to haemetopoiesis. J Cell Biol 1995;129:1177–80.
34. Naka D, Ishii T, Yoshiyama Y, Miyazawa K, Hara H, Hishida T, et al. Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. J Biol Chem 1992;267:20114–9.
35. Shimomura T, Ochiai M, Kondo J, Morimoto Y. A novel protease obtained from FBS-containing culture supernatant that processes single-chain form hepatocyte growth factor to two-chain form in serum free culture. Cytotechnology 1992;8:219–29.
36. Miyazawa K, Shimomura T, Kitamura A, Kondo J, Morimoto Y, Kitamura N. Molecular cloning and sequence analysis of the cDNA for a human serine protease responsible for activation of hepatocyte growth factor. J Biol Chem 1993;268:10024–8.
37. Itoh H, Hamasuna R, Kataoka H, Yamauchi M, Miyazawa K, Kitamura N, et al. Mouse hepatocyte growth factor activator gene: its expression not only in the liver but also in the gastrointestinal tract. Biochim Biophys Acta 2000;1491:295–301.
38. Shimomura T, Kondo J, Ochiai M, Naka D, Miyazawa K, Morimoto Y, et al. Activation of the zymogen of hepatocyte growth factor activator by thrombin. J Biol Chem 1993;268: 22927–32.
39. Miyazawa K, Shimomura T, Naka D, Kitamura N. Proteolytic activation of hepatocyte growth factor in response to tissue injury. J Biol Chem 1994;269:8966–70.
40. Miyazawa K, Shimomura T, Kitamura N. Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator. J Biol Chem 1996;271:3615–8.
41. Hori T, Ido A, Uto H, Hasuike S, Moriuchi A, Hayashi K, et al. Activation of hepatocyte growth factor in monkey stomach following gastric mucosal injury. J Gastroenterol 2004;39:133–9.
42. Shimomura T, Denda K, Kitamura A, Kawaguchi T, Kito M, Kondo J, et al. Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor. J Biol Chem 1997; 272:6370–6.
43. Itoh H, Kataoka H, Tomita M, Hamasuna R, Nawa Y, Kitamura N, et al. Upregulation of HGF activator inhibitor type 1 but not type 2 along with regeneration of intestinal mucosa. Am J Physiol Gastrointest Liver Physiol 2000;278:635–43.
44. Kataoka H, Shimomura T, Kawaguchi T, Hamasuna R, Itoh H, Kitamura N, et al. Hepatocyte growth factor activator inhibitor type 1 is a specific cell surface binding protein of hepatocyte growth factor activator (HGFA) and regulates HGFA activity in the pericellular microenvironment. J Biol Chem 2000;275:40453–62.
45. Takahashi M, Ota S, Simada T, Hamada E, Kawabe T, Okudaira T, et al. Hepatocyte growth factor is the most potent endogenous stimulant of rabbit gastric epithelial cell proliferation and migration in primary culture. J Clin Invest 1995;95:1994–2003.
46. Goke M, Podolsky DK. Regulation of the mucosal epithelial barrier. Baillieres Clin Gastroenterol 1996;10:393–405.
47. Nishimura S, Takahashi M, Ota S, Hirano M, Hiraishi H. Hepatocyte growth factor accelerates restitution of intestinal epithelial cells. J Gastroenterol 1998;33:172–8.
48. Nurstat A, Parkos CA, Bacarra AE, Godowski PJ, Delp-Archer C, Rosen EM, et al. Hepatocyte growth factor/scatter factor effects on epithelia. Regulation of intercellular junctions in transformed and nontransformed cell lines, basolateral polarization of c-met receptor in transformed and natural intestinal epithelia, and induction of rapid wound repair in a transformed model epithelium. J Clin Invest 1994;93:2056–65.
49. Kitamura S, Kondo S, Shimomura Y, Isozaki K, Kanayama S, Higashimoto Y, et al. Expression of hepatocyte growth factor and c-met in ulcerative colitis. Inflamm Res 2000;49:320–4.
50. Srivastava M, Zurakowski D, Cheifetz P, Leichtner A, Bousvaros A. Elevated serum hepatocyte growth factor in children and

young adults with inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2001;33:548–53.
51. Matsuo M, Shiota G, Umeki K, Kawasaki H, Kojo H, Miura K. Induction of plasma hepatocyte growth factor in acute colitis of mice. Inflamm Res 1997;46:166–7.
52. Ortega-Cava CF, Ishihara S, Kawashima K, Rumi MAK, Kazumori H, Adachi K, et al. Hepatocyte growth factor expression in dextran sodium sulfate-induced colitis in rats. Dig Dis Sci 2002;47:2275–85.
53. Xian CJ, Couper R, Howarth GS, Read LC, Kallincos NC. Increased expression of HGF and c-met in rat small intestine during recovery from methotrexate-induced mucositis. Br J Cancer 2000;82:945–52.
54. Itoh H, Naganuma S, Takeda N, Miyata S, Uchinokura S, Fukushima T, et al. Regeneration of injured intestinal mucosa is impaired in hepatocyte growth factor activator-deficient mice. Gastroenterology 2004;127:1423–35.
55. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel model in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 1990;98:694–702.
56. Elson CO, Sartor RB, Tennyson GS, Riddell RH. Experimental models of inflammatory bowel disease. Gastroenterology 1995; 109:1344–67.
57. Tahara Y, Ido A, Yamamoto S, Miyata Y, Uto H, Hori T, et al. Hepatocyte growth factor facilitates colonic mucosal repair in experimental ulcerative colitis in rats. J Pharmacol Exp Ther 2003;307:146–51.
58. Neurath MF, Russ I, Kelsall BL, Stuber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. J Exp Med 1995;182:1281–90.
59. Hibi T, Ogata H, Sakuraba A. Animal models of inflammatory bowel disease. J Gastroenterol 2002;37:409–17.
60. Numata M, Ido A, Moriuchi A, Kim ID, Tahara Y, Yamamoto S, et al. Hepatocyte growth factor facilitates the repair of large colonic ulcers in 2,4,6-trinitrobenzene sulfonic acid-induced colitis in rats. Inflamm Bowel Dis 2005;11:551–8.
61. Kuroiwa T, Kakishita E, Hamano T, Kataoka Y, Seto Y, Iwata N, et al. Hepatocyte growth factor ameliorates acute graft-versus-host disease and promotes hematopoietic function. J Clin Invest 2001;107:1365–73.
62. Oh K, Iimuro Y, Takeuchi M, Kaneda Y, Iwasaki T, Terada N, et al. Ameliorating effect of hepatocyte growth factor on inflammatory bowel disease in a murine model. Am J Physiol Gastrointest Liver Physiol 2005;288:G729–35.
63. Hammer RE, Makita SD, Richardson JA, Tang JP, Taurog JD. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human beta2m: an animal model of HLA-B27-associated human disorders. Cell 1990;63:1099–112.
64. Arthur LG, Kuenzler KA, Schwartz MZ. Hepatocyte growth factor ameliorates inflammatory bowel disease in a rat model. J Gastrointest Surg 2003;7;1062–8.
65. Arthur LG, Schwartz MZ, Kuenzler KA, Birbe R. Hepatocyte growth factor treatment ameliorates diarrhea and bowel inflammation in a rat model of inflammatory bowel disease. J Pediatr Surg 2004;39:139–43.
66. Ohda Y, Hori K, Tomita T, Hida N, Kosaka T, Fukuda Y, et al. Effects of hepatocyte growth factor on rat inflammatory bowel disease models. Dig DIs Sci 2005;50:914–21.
67. Mukoyama T, Kanbe T, Murai R, Murawaki Y, Shimomura T, Hashiguchi K, et al. Therapeutic effect of adenoviral-mediated hepatocyte growth factor gene administration on TNBS-induced colitis in mice. Biochem Biophys Res Commun 2005;329:1217–24.
68. Sandborn WJ, Sands BE, Wolf DC, Valentine JF, Safdi M, Katz S, et al. Repifermin (keratinocyte growth factor-2) for the treatment of active ulcerative colitis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Aliment Pharmacol Ther 2003; 17:1355–64.
69. Sinha A, Nightingale JMD, West KP, Berlanga-Acosta J, Playford RJ. Epidermal growth factor enemas with oral mesalamine for mild-to-moderate left-sided ulcerative colitis or proctitis. New Engl J Med 2003;349:350–7.
70. Ido A, Moriuchi A, Kim ID, Numata M, Nagata-Tsubouchi Y, Hasuike S, et al. Pharmacokinetic study of recombinant human hepatocyte growth factor administered in a bolus intravenously or via portal vein. Hepatol Res 2004;30:175–81.
71. Sakata H, Takayama H, Sharp R, Rubin JS, Merlino G, LaRochelle WJ. Hepatocyte growth factor/scatter factor overexpression induces growth, abnormal development, and tumor formation in transgenic mouse livers. Cell Growth Differ 1996; 7:1513–23.
72. Takayama H, LaRochelle WJ, Sharp R, Otsuka T, Kriebel P, Anver M, et al. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. Proc Natl Acad Sci USA 1997;94:701–6.
73. Gallego MI, Bierie B, Hennighausen L. Targeted expression of HGF/SF in mouse mammary epithelium leads to metastatic adenosquamous carcinomas through the activation of multiple signal transduction pathways. Oncogene 2003;22:8498–508.
74. Bell A, Chen Q, DeFrances MC, Michalopoulos GK, Zarnegar R. The five amino acid-deleted isoform of hepatocyte growth factor promotes carcinogenesis in transgenic mice. Oncogene 1999;18: 887–95.
75. Horiguchi N, Takayama H, Toyoda M, Otsuka T, Fukusato T, Merlino G, et al. Hepatocyte growth factor promotes hepatocarcinogenesis through c-Met autocrine activation and enhanced angiogenesis in transgenic mice treated with diethylnitrosamine. Oncogene 2002;21:1791–9.
76. Nooman FP, Otsuka T, Bang S, Anver MR, Merlino G. Accelerated ultraviolet radiation-induced carcinogenesis in hepatocyte growth factor/scatter factor transgenic mice. Cancer Res 2000; 60:3738–43.
77. Takayama H, LaRochelle WJ, Anver M, Bockman DE, Merlino G. Scatter factor/hepatocyte growth factor as a regulator of skeletal muscle and neural crest development. Proc Natl Acad Sci USA 1996;93:5866–71.
78. Shiota G, Kawasaki H, Nakamura T, Schmidt EV. Characterization of double transgenic mice expressing hepatocyte growth factor and transforming growth factor α. Res Commun Mol Pathol Pharmacol 1995;90:17–24.
79. Santoni-Rugiu E, Preisegger KH, Kiss A, Audolfsson T, Shiota G, Schmidt EV, et al. Inhibition of neoplastic development in the liver by hepatocyte growth factor in a transgenic mouse model. Proc Natl Acad Sci USA 1996;93:9577–82.
80. Nakase H, Okazaki K, Tabata Y, Ozeki M, Watanabe N, Ohana M, et al. New cytokine delivery system using gelatin microspheres containing interleukin-10 for experimental inflammatory bowel disease. J Pharmacol Exp Ther 2002;301:59–65.

Hepatocyte Growth Factor Facilitates the Repair of Large Colonic Ulcers in 2,4,6-Trinitrobenzene Sulfonic Acid–Induced Colitis in Rats

Masatsugu Numata, MD,† Akio Ido, MD,† Akihiro Moriuchi, MD,† Ildeok Kim, MD,† Yoshihiro Tahara, MD,* Shojiro Yamamoto, MD,* Satoru Hasuike, MD,* Kenji Nagata, MD,* Yoshifumi Miyata, MD,* Hirofumi Uto, MD,* and Hirohito Tsubouchi, MD*†*

Background: Hepatocyte growth factor (HGF) modulates intestinal epithelial cell proliferation and migration, serving as a critical regulator of intestinal wound healing. The aim of this study was to clarify the effects of administration of recombinant human HGF on colonic mucosal damage in vivo.

Methods: Rats were given 7.5 mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS) per rectum on day 0. On day 5, the degree of TNBS-induced colitis was evaluated endoscopically, and rats suffering from large ulcers (occupying more than two thirds of the luminal circumference) were treated with intravenous bolus injections of recombinant human HGF (1.0 mg/kg per day) or phosphate-buffered saline (PBS) for 5 days.

Results: Rats with TNBS-induced colitis given human HGF showed a significant reduction in colonic ulcer coverage and large intestinal shortening compared with those treated with PBS. Administration of recombinant human HGF also stimulated the proliferation of epithelial cells and reduced the inflammatory cell infiltrate. Finally, HGF treatment decreased the myeloperoxidase activity and tumor necrosis factor α levels in the TNBS-inflamed colon tissues.

Conclusions: These results indicate that intravenous injection of HGF accelerates colonic mucosal repair and reduces infiltration of inflammatory cells in rats with TNBS-induced colitis and suggest that HGF has the potential to be a new therapeutic modality to promote intestinal mucosal repair in patients with inflammatory bowel disease.

Received for publication December 6, 2004; accepted March 17, 2005.

From the *Department of Internal Medicine II, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki, Japan; and †Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan.

Supported in part by grants-in-aid from the Ministry of Science, Education, Sports, and Culture and the Ministry of Health, Labor, and Welfare of Japan.

Reprints: Hirohito Tsubouchi, Department of Internal Medicine II, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1601, Japan (e-mail: htsubo@med.miyazaki-u.ac.jp)

Key Words: 2,4,6-trinitrobenzene sulfonic acid, hepatocyte growth factor, inflammatory bowel disease, mucosal injury, mucosal repair

(*Inflamm Bowel Dis* 2005;11:551–558)

The mucosal lining of the intestinal tract is composed of a rapidly proliferating and continually renewing sheet of epithelial cells. After mucosal injury occurs, numerous growth factors and cytokines, induced in both the lumen and in submucosal locations, cooperatively stimulate epithelial mucosal repair.[1–3]

Hepatocyte growth factor (HGF) was originally purified from the plasma of patients with fulminant hepatic failure and is a major agent promoting hepatocyte proliferation.[4,5] HGF also functions as a pleiotropic factor, acting as a mitogen, morphogen, and motogen for multiple subsets of epithelial cells, including gastrointestinal epithelial cells.[4–9] HGF acts primarily by ligating the c-Met receptor at the plasma membrane.[10] Recent studies have shown that HGF expression is stimulated in inflamed colonic mucosal tissue in patients with ulcerative colitis and that plasma HGF levels are increased in animal models of acute colitis.[11–13] Additionally, 2 HGF-associated molecules involved in the activation of HGF in injured tissues, HGF activator and HGF activator inhibitor type-1, are closely associated with colonic mucosal repair.[14,15] These findings indicate an important role for HGF in intestinal mucosal wound healing.

The primary therapies for inflammatory bowel disease (IBD) are anti-inflammatory and anti-immune agents such as salazosulfapyridine, mesalazine, corticosteroids, azathioprine, 6-mercaptopurine, methotrexate, and cyclosporine.[16] A chimeric mouse-human monoclonal antibody against tumor necrosis factor α (TNFα) has also been developed and has been extremely effective in Crohn's disease.[17] The majority of patients with IBD benefit from these anti-inflammatory and/or anti-immune agents, but the disease is often recurrent and intractable. We recently reported that continuous intraperitoneal administration of recombinant human HGF, which results in detectable serum levels of human HGF, facilitated colonic mucosal repair

in experimental ulcerative colitis in rats.[18] Recombinant human HGF will soon be available for treatment of patients with severe liver disease. Therefore, in contrast to various other therapeutic agents used to suppress inflammation and immunity, HGF has the potential to be an important new modality for the promotion of intestinal mucosal repair in patients with IBDs. However, intravenously administered recombinant human HGF disappears rapidly from serum because of its short half-life,[19] and it is still not clear whether intravenous injection of recombinant HGF can facilitate wound healing of deeper and more extensive mucosal damage. In this study, we used a rat model of experimental colitis induced by a single enema of 2,4,6-trinitrobenzene sulfonic acid (TNBS) and performed colonoscopies in all rats given TNBS to confirm the radial extent of mucosal damage. Only animals suffering from large colonic ulcers were treated by intravenous bolus injections of recombinant human HGF, and we evaluated the effect of this HGF treatment on wound healing and inflammation.

MATERIALS AND METHODS

Animals

Male Wistar rats, 7 weeks of age and weighing between 140 and 150 g, 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 ethical committee of the Graduate School of Medicine, Kyoto University (Kyoto, Japan). All animal experiments were performed after a 1-week acclimation period. Colitis was induced in rats by administration of 7.5 mg of TNBS (Wako Pure Chemical Industries, Osaka, Japan) per rectum, dissolved in 0.5 mL of 50% ethanol (day 0). To evaluate the severity of colitis, the rats' body masses and disease activity index (DAI) scores were examined on days 1, 3, 5, 7, and 10.[20] Rats were killed on day 10, and we measured the length of the large intestine between the colocecal junction and the anal verge.

Colonoscopy

Colonoscopic examinations were performed using a BF3C40 bronchofiberscope (Olympus Co., Tokyo, Japan), on days 5 and 10. The endoscope was inserted through the anus of rats with TNBS-induced colitis under the inhalation anesthesia of diethyl ether. When the endoscope was fully inserted (~8 cm), it was possible to observe at least two thirds of the entire colon.

Administration of Recombinant Human HGF

Recombinant human HGF was kindly provided by Mitsubishi Pharma Co. (Tokyo, Japan). After endoscopic examination on day 5, either recombinant human HGF (1.0 mg/kg) in phosphate-buffered saline (PBS) or PBS alone was intravenously injected in a bolus to TNBS-induced colitis rats for 5 days (from days 5 to 9).

Measurement of the Area of Colonic Erosions

The large intestines of treated rats were obtained on day 10 and opened longitudinally. After measurement of colon lengths from the colocecal junction to the anal verge, large intestines were fixed with 10% formalin for 3 days. The areas of colonic ulcers were measured using a VM-30 micrometer (Olympus Co.).

Histologic Examination

As described above, the entire colon was excised postmortem and fixed with 10% formalin for histologic analysis. The longitudinal sections were embedded in paraffin and stained with hematoxylin and eosin (H&E). Histologic scoring was assessed independently by 2 investigators blinded to the previous protocols, using the following histopathological grading system: grade 0, normal findings; grade 1, mild mucosal and/or submucosal inflammatory infiltrate (admixture of neutrophils) and edema, punctuate mucosal erosions associated with capillary proliferation, but muscularis mucosa intact; grade 2, grade 1 changes involving 50% of the specimen; grade 3, prominent inflammatory infiltrate and edema (neutrophils usually predominating), frequently with deeper areas of ulceration extending through the muscularis mucosae into the submucosa, and rare inflammatory cells invading the muscularis propriae but without muscle necrosis; grade 4, grade 3 changes involving 50% of the specimen; grade 5, extensive ulceration with coagulative necrosis bordered inferiorly by numerous neutrophils and lesser numbers of mononuclear cells, and necrosis that extends deeply into the muscularis propria; grade 6, grade 5 changes involving 50% of the specimen.[21,22]

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity in the colon tissues, which is directly related to the number and activity of infiltrating myeloid cells, was measured as described previously.[23,24] Colonic MPO activity is expressed in units per gram of wet tissue.

Immunohistochemistry

To evaluate proliferation of the colonic epithelium, 50 mg/kg of 5-bromo-2′-deoxyuridine (BrdU; Wako Pure Chemical Industries) was injected intraperitoneally 1 hour before death, and cells synthesizing DNA were identified by immunohistochemistry using a BrdU In-Situ Detection Kit (BD Bioscience, San Diego, Calif.) according to the manufacturer's instructions. We then counted the BrdU-positive

cells in 10 crypts of the normal mucosa and in the mucosa-bordering ulcers (5 mm from the ulcer edges).

Measurement of TNFα and Interferon γ in Colon Tissues

Colon tissues were homogenized in cold PBS and centrifuged at 20,000*g* for 15 minutes at 4 °C. We determined the total protein in the supernatants with a Protein Assay Kit (Bio-Rad, Hercules, Calif.), and TNFα and interferon γ (IFNγ) levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit (DIACLONE SAS, Besancon, France) according to the manufacturer's instructions.

Statistical Analysis

Unless otherwise specified, data are expressed as mean ± SD. Statistical parameters were ascertained with Statview J-4.5 software (Abacus Concept, Berkeley, Calif.). The differences between means were compared by the unpaired Student *t* test. Values of $P < 0.05$ were considered significant.

RESULTS

Administration of Recombinant Human HGF Reduced DAI Scores in Rats with Large Colonic Ulcers Induced by TNBS

To evaluate the degree of TNBS-induced colitis before HGF administration, we performed colonoscopic examinations on day 5. Although rectal administration of TNBS induced ulcers in the large intestines of all rats, approximately 80% of the animals exhibited large ulcers occupying more than two thirds of the luminal circumference (Fig. 1A). Only rats suffering from these larger colonic ulcers underwent treatment with intravenous recombinant human HGF.

We evaluated body weight changes and DAI scores of TNBS-induced colitis rats treated with PBS or HGF (Fig. 2). Body weights decreased until 5 days after TNBS administration and then gradually increased. Treatment with HGF did not affect sequential changes in body weight (Fig. 2A). However, in TNBS colitis rats given human HGF, the DAI scores were significantly reduced (3.7 ± 0.7) compared with those given only PBS (5.1 ± 0.4) by day 10 ($P = 0.0001$; Fig. 2B).

HGF Administration Facilitated the Repair of Colonic Ulcers and Reduced Colitis-induced Shortening of the Large Intestine

Because experimental colitis induced by a single enema of 7.5 mg TNBS spontaneously disappeared within 12 to 14 days, the colonic ulcers and the lengths of the large intestine were examined in rats treated with either PBS or HGF on day 10. Before death, we evaluated colonoscopic appearance in PBS- or HGF-treated rats (Fig. 1B). In PBS-treated rats, the depth of colonic ulcers was slightly reduced compared with

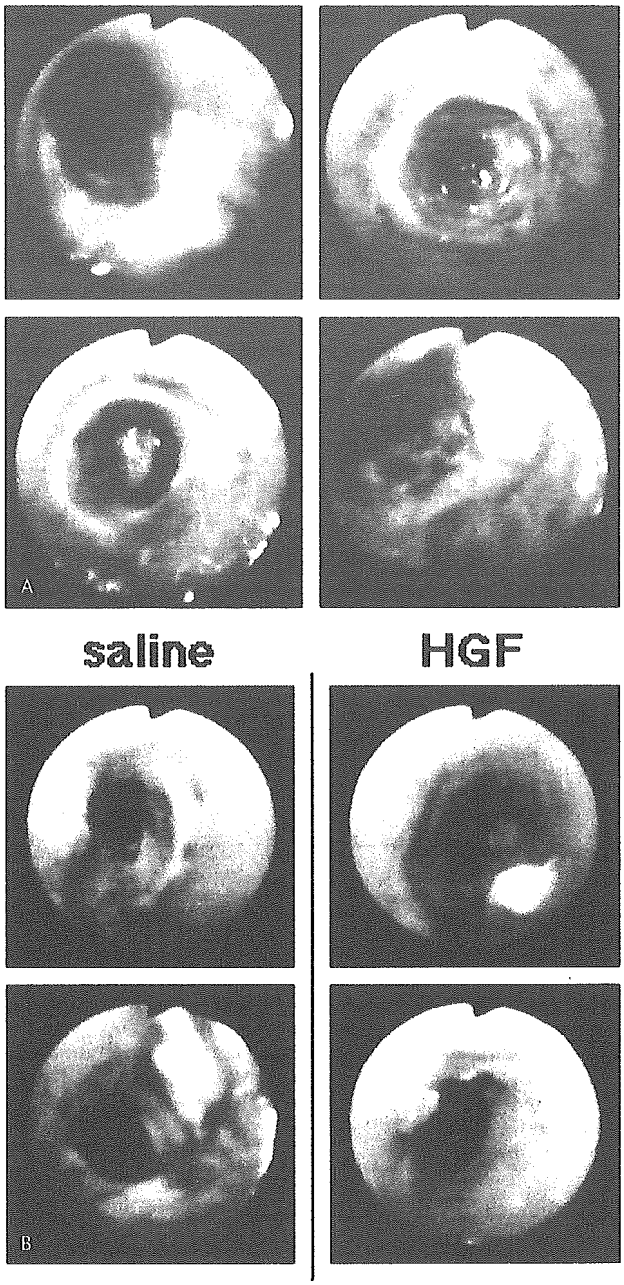


FIGURE 1. Representative colonoscopic appearances of large colonic ulcers in TNBS-administered rats. A, colonoscopies were performed 5 days after a single enema of TNBS (7.5 mg/rectum). Administration of TNBS induced large and deep colonic ulcers that occupied more than two thirds of the luminal circumference in 80% of rats. B, on day 10, rats treated with PBS or recombinant human HGF were subjected to colonoscopies. Rats treated with PBS exhibited large colonic ulcers that bled readily from the bordering mucosa, whereas those treated with HGF exhibited reduced ulcer size and mild contraction of the mucosa surrounding healing ulcers.

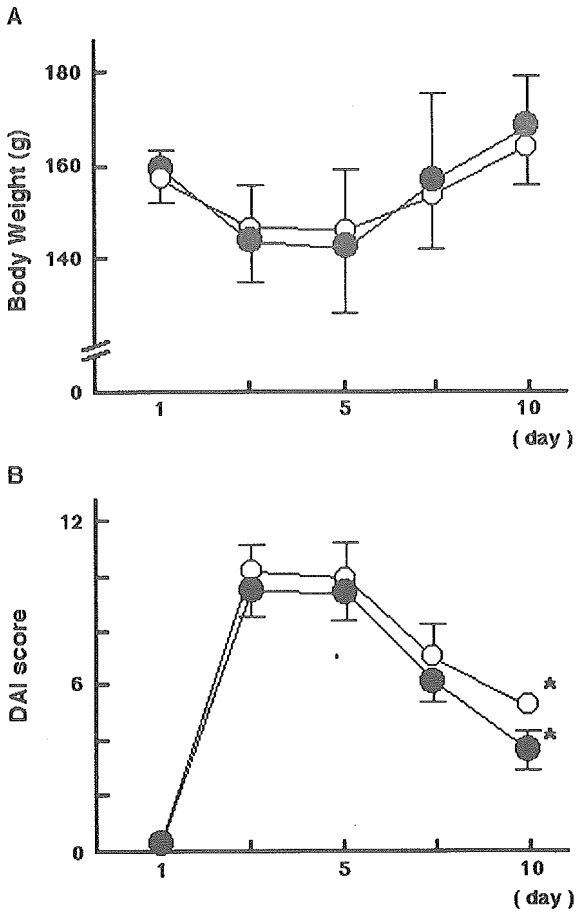


FIGURE 2. Intravenous HGF administration reduces disease activity but does not affect changes in body weight in rats with TNBS-induced colitis. After rectal TNBS administration, sequential changes in body weight (A) and DAI (B) were examined in PBS- or HGF-treated rats (○, n = 8; ●, n = 9). Although the intravenous injections of recombinant human HGF for 5 days did not affect the changes in body weight, the disease activity in rats treated with HGF was significantly reduced on day 10 (*, $P = 0.0001$).

those developed by day 5 after a single enema of TNBS (Fig. 1A), but the large colonic ulcers still remained, and bleeding from the bordering mucosa occurred readily. In contrast, HGF-treated rats exhibited remarkable amelioration of colonic ulcers, with mild contraction of the surrounding mucosa (Fig. 1B). After death, we measured the areas of colonic ulcers and the colon lengths (Fig. 3). In TNBS-induced colitis rats treated with recombinant human HGF, the areas covered by ulcers within the large intestine were significantly reduced in size (54.4 ± 32.3 mm^2) compared with those treated with PBS (112.8 ± 61.2 mm^2; $P = 0.037$; Fig. 3A), and the colons were significantly longer (13.3 ± 0.7 cm) than those in PBS-treated rats (11.8 ± 1.7 cm; $P = 0.043$; Fig. 3B).

Intravenous Injection of Human HGF Enhances the Regeneration of the Colonic Epithelium and Decreases the Inflammatory Cell Infiltrate in Rats with TNBS Colitis

We also evaluated the effect of HGF on TNBS-induced colonic ulcers in rats by histologic analysis. In TNBS-induced colitis rats treated with PBS, extensive mucosal damage, inflammatory cell infiltrates, and edema were observed on day 10 (Fig. 4A). After treatment with recombinant human HGF for 5 days, rats with TNBS colitis had an enhanced regenerative epithelium and a decrease in inflammatory cell infiltrates (Fig. 4A). Consequently, HGF administration significantly reduced the histologic score to 4.3 ± 0.9 compared with 5.4 ± 0.9 for PBS-treated rats ($P = 0.030$; Fig. 4B). Next, we measured MPO activity in normal or TNBS-inflamed colon tissues with or without HGF treatment to evaluate the degree of inflammation. Although the MPO activity was significantly increased in TNBS-inflamed colon tissues (12.29 ± 0.98 U/g wet tissue on day 10 compared with 0.75 ± 0.44 for normal colon tissues; $P = 0.0009$), administration of recombinant human HGF significantly reduced the accumulation of MPO in TNBS-inflamed colons by approximately 50% (6.41 ± 1.94 U/g wet tissue; $P = 0.0013$).

We examined the BrdU labeling index to evaluate the proliferation of the colonic epithelium (Fig. 5). In TNBS colitis rats treated with PBS, BrdU-positive epithelial cells at the edges of ulcers increased to 12.2 ± 7.9/crypt compared with those in normal mucosa (1.7 ± 2.1/crypt). When the rats were treated with recombinant human HGF, we observed a significant increase in BrdU-positive cells within both the mucosal epithelium surrounding colonic ulcers (30.4 ± 2.4/crypt) and the normal mucosal epithelium (11.3 ± 1.5/crypt; $P = 0.047$ and 0.004 in comparison with PBS-treated rats, respectively; Figs. 5, A and B).

TNBS-induced Colitis Rats Treated with Recombinant Human HGF Exhibited Reductions in TNFα and IFNγ in Colon Tissues

Because rats treated with human HGF exhibited a reduction in inflammatory cell infiltrates, we examined colonic tissue levels of TNFα and IFNγ (Fig. 6). After administration of TNBS, tissue levels of TNFα were 20.8 ± 3.5 and 20.2 ± 2.9 (pg/mg protein) on days 5 and 10, respectively (Fig. 6A). Colonic TNFα levels were 13.6 ± 4.8 pg/mg protein on day 10 in rats treated with recombinant human HGF ($P = 0.028$). Levels of IFNγ in colon tissues on day 5 (23.8 ± 3.9 pg/mg) were similar to those in normal rats (day 0; 19.5 ± 10.0 pg/mg; Fig. 6B). TNBS-induced colitis rats given PBS exhibited a significant increase in colon tissue IFNγ on day 10 (51.1 ± 13.7 pg/mg) compared with those on day 5 ($P = 0.003$). In rats treated with recombinant human HGF, the IFNγ level in colon

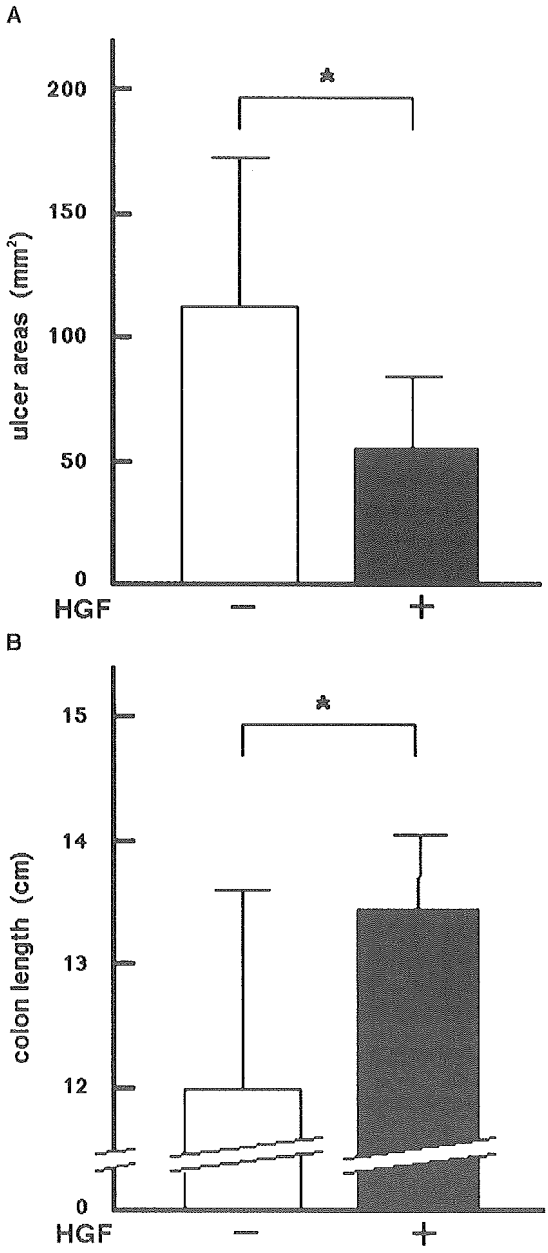


FIGURE 3. Administration of HGF reduces colonic ulcers and prevents shortening of the large intestine in rats with TNBS-induced colitis. The areas of the colonic ulcers and the lengths of the large intestines in PBS- or HGF-treated rats with TNBS-induced colitis (n = 8 or 9, respectively) were determined on day 10. A, the areas of colonic ulcers in rats treated with recombinant human HGF were significantly reduced compared with ulcers in PBS-treated rats (*, $P = 0.037$). B, the large intestines of rats treated with HGF were significantly longer than those treated with PBS (*, $P = 0.043$).

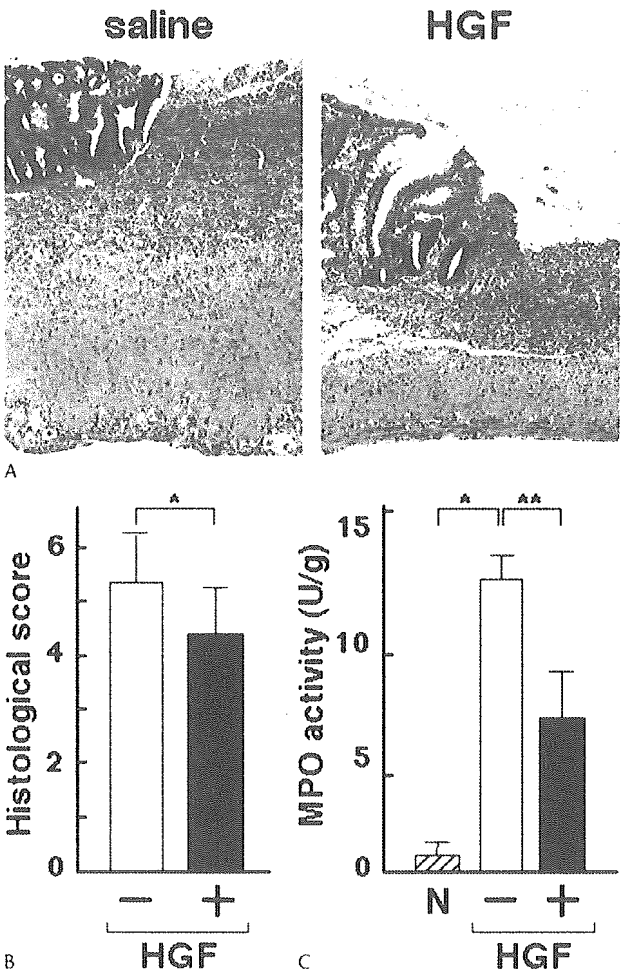
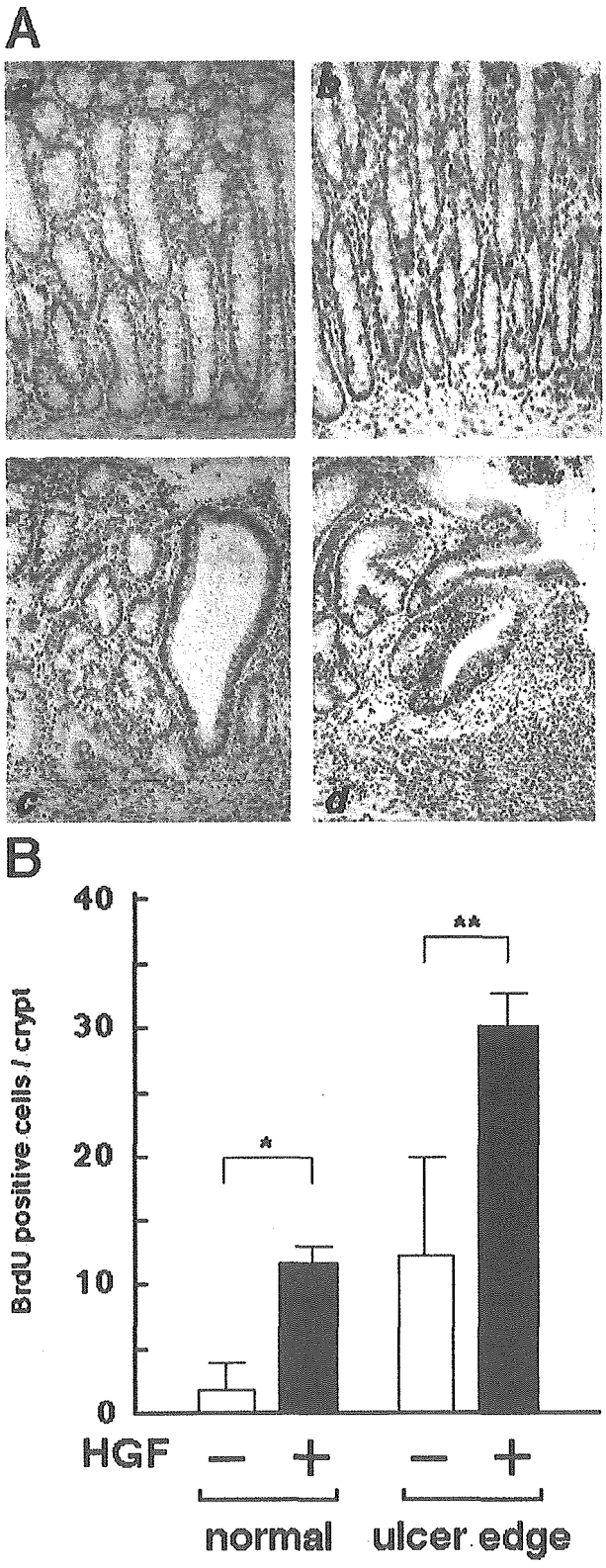


FIGURE 4. Intravenous injection of recombinant HGF enhances colonic epithelium regeneration and decreases the inflammatory infiltrate in rats with TNBS-induced colitis. Large intestines, which were obtained on day 10 from colitis rats treated with PBS or HGF (n = 8 or 9, respectively), were opened longitudinally and stained with H&E. A, representative microscopic appearance of colonic ulcers in PBS- and HGF-treated rats. In TNBS-induced colitis rats treated with PBS, extensive mucosal damage and marked inflammatory cell infiltration were observed on day 10. Treatment with recombinant HGF enhanced the development of regenerative epithelium and reduced inflammatory cell infiltrates and edema (magnifications, ×100). B, blind histologic scoring was performed. Rats treated with PBS had a higher histologic score than those treated with recombinant human HGF (*, $P = 0.030$). C, MPO activity in the normal (N) or TNBS-inflamed colon tissues (n = 3 or 4, respectively) was measured. Colonic MPO activity observed on day 10 in rats with TNBS-induced colitis was significantly higher than in normal rats (*, $P = 0.0009$). HGF treatment significantly reduced the MPO activity in rats with TNBS-induced colitis (**, $P = 0.0013$).



tissues was reduced to 36.8 ± 9.7 pg/mg; however, the difference was not significant ($P = 0.067$).

DISCUSSION

In this animal model of TNBS-induced colitis, granulomas with inflammatory cell infiltrates in all layers were visible in the intestine.[25] Isolated macrophages produce large amount of interleukin (IL)-12, and lymphocytes produce large amounts of IFNγ and IL-2, indicating that the colitis in this model is induced by a T-helper type 1 response.[26] Thus, animals with TNBS-induced colitis are considered an appropriate model for Crohn's disease. Susceptibility to TNBS varies in each animal, however, resulting in different colitis levels after a single enema of TNBS. In this study, we performed colonoscopic examinations 5 days after the TNBS enema, and only animals with large colonic ulcers—80% of TNBS-treated rats—were given HGF. Using colonoscopy in living animals, we were able to evaluate not only the severity of colitis, but also the healing process of the injured mucosa. Colonoscopic examination of rodents has been recently reported to be feasible and enables continuous observation of dextran sulfate sodium (DSS)-induced colitis without the need for death.[27,28] Therefore, colonoscopy is advantageous to evaluate the response of injured colonic mucosa to experimental therapeutics.

We have previously reported that the continuous intraperitoneal delivery of recombinant human HGF allows detection of the human HGF in rat serum and successfully stimulates colonic mucosal repair in rats with DSS-induced colitis.[18] Recombinant human HGF circulating in the blood is thought to interact with c-Met, a specific receptor for HGF expressed on the basolateral membranes of intestinal epithelial cells,[29] and this interaction leads to the stimulation of cell migration and proliferation. In this study, daily intravenous bolus injections of recombinant human HGF stimulated the proliferation of colonic epithelial cells and facilitated the repair of the deep and large TNBS-induced colonic ulcers. The healing of deeper, penetrating injuries requires reparative mechanisms involving not only epithelial cells, but also nonepithelial cells, angiogenesis, and scarring responses in the submucosal and

FIGURE 5. Proliferation of colonic epithelial cells is stimulated by intravenous administration of recombinant human HGF. A, representative photographs of BrdU immunohistochemistry in normal mucosa (a and b) and that bordering ulcers (c and d) in rats with TNBS-induced colitis, which were administered PBS (a and c) or recombinant HGF (b and d; magnifications, ×400). B, BrdU-positive cells were counted, and the number of positive cells per crypt was calculated. In rats treated with PBS alone (n = 8), BrdU-positive cells in the mucosa-bordering ulcers were increased in comparison with those in the normal mucosa. HGF administration, however, stimulated the proliferation of epithelial cells in both normal and border mucosa (* and **, $P = 0.004$ and 0.047, respectively; n = 9).

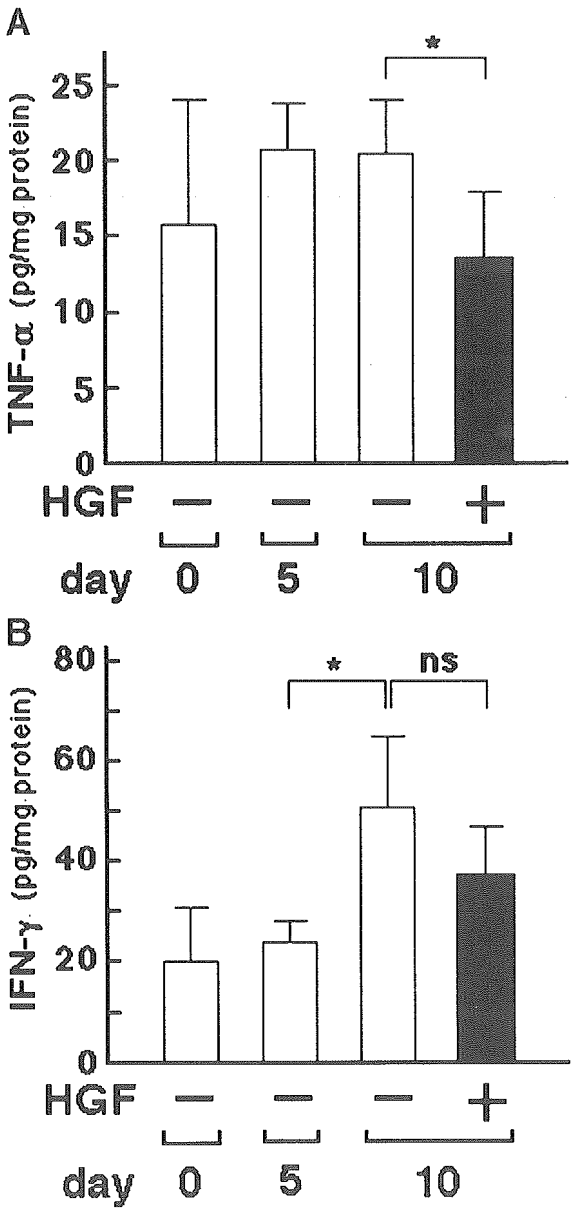


FIGURE 6. HGF administration reduces TNFα and IFNγ in colon tissues of rats with TNBS-induced colitis. The levels of TNFα and IFNγ in colon tissues treated with PBS or HGF were determined by ELISA on days 0 (normal rat), 5, and 10 of TNBS administration (n = 4). A, intravenous HGF administration significantly reduced TNFα in colon tissues compared with those in PBS-treated rats on day 10 (*, $P = 0.028$). B, rats given TNBS exhibited a significant increase in IFNγ levels in colon tissues by day 10 (*, $P = 0.003$ compared with those on day 5). The IFNγ levels in colon tissues of HGF-treated rats decreased by day 10. However, these differences were not statistically significant ($P = 0.067$) compared with those of PBS-treated rats.

serosal layers of the intestines. In such a response, angiogenesis is initiated and fibroblasts participate in depositing extracellular matrix components and in shaping the resulting granulated tissue.[30] HGF is known to act not only on epithelial cells, but also on nonepithelial cells (including vascular endothelial cells), and also functions as an angiogenic factor.[31] Therefore, despite the short half-life of the recombinant protein (~2.5 min),[19] intravenous bolus injections of human HGF may effectively ameliorate TNBS-induced colonic ulcers. Additionally, because HGF also acts as an antifibrogenic and angiogenic factor, its administration may potentially suppress development of fibrosis, which causes intestinal stenosis in patients with Crohn's disease.

In injured intestinal tissues, myofibroblasts beneath the epithelial lesion are known to secrete HGF.[3,32,33] Inflammatory cells, such as neutrophils, also produce HGF along with proinflammatory cytokines.[34] Thus, HGF plays a pivotal role in tissue repair. Recently, several groups have reported that HGF induces lymphocyte function-associated antigen-1–mediated adhesion of neutrophils to endothelial cells and also stimulates transmigration of inflammatory cells, including neutrophils and lymphocytes.[35–37] These findings suggest that HGF participates in the activation of the nonspecific cellular inflammatory response in the microenvironment of injured tissues. In this study, although TNBS-induced colitis rats treated with PBS exhibited remarkable infiltration of inflammatory cells, expanding deeply into the large intestinal wall, the inflammatory cell infiltrate and the corresponding increase in proinflammatory cytokines were reduced by treatment with recombinant human HGF. The effect of exogenous HGF on the immune and inflammatory cells in the submucosa remains obscure. However, it is possible that HGF-induced enhancement of mucosal repair allows for a more rapid recovery of epithelial barrier function, leading to a reduced exposure to various luminal agents that contribute to persistent colitis. Therefore, the observed reduction of inflammation in HGF-treated rats may result from a decrease in exposure to luminal stimuli rather than from the direct influence of HGF on immune and inflammatory cells.

We have recently reported that recombinant human HGF, injected intravenously in a bolus, primarily distributes to the liver and that the HGF content in colon tissues is much smaller than in liver, spleen, adrenal glands, and kidneys.[19] We show here that daily intravenous bolus injections of recombinant HGF for 5 days ameliorated large colonic ulcers, despite the small amount of recombinant protein that is supposedly delivered to colonic tissues. c-Met is expressed on basolateral membranes of intestinal epithelial cells,[29] and an increase in recombinant HGF circulating in the blood is therefore considered to stimulate mucosal repair. However, once intestinal mucosal injury occurs, intracellular junctions between epithelial cells are loosened, and the separation, spreading, and migration of epithelial cells are facilitated by the reparative process. This suggests that, compared with

normal mucosa, the distribution of c-Met in epithelial cells is altered. Therefore, luminal administration of recombinant human HGF also has the potential to ameliorate intestinal tissue injury with less adverse side effects, such as renal toxicity.

In conclusion, we performed colonoscopies in living animals to study the various susceptibilities to TNBS in each animal, and we showed that repeated intravenous injections of recombinant human HGF facilitates the repair of large, deep colonic ulcers in a rat model of TNBS-induced colitis. Clarification of the carcinogenic risk will require further study, as will the development of an efficient drug delivery system. However, in contrast to anti-inflammatory or anti-immune agents, recombinant human HGF may be a new modality to eradicate uncontrolled and perpetuated inflammation through enhanced mucosal wound healing in patients with IBD. Additionally, because HGF induces a rapid recovery of the epithelial barrier, HGF treatment may also be more physiological than treatments with anti-inflammatory or anti-immune agents.

ACKNOWLEDGMENTS

The authors thank Sayoko Ohara, Kana Ohara, and Yuko Nakamura for technical assistance..

REFERENCES

1. Murphy MS. Growth factors and the gastrointestinal tract. *Nutrition.* 1998;14:771–774.
2. Beck PL, Podolsky DK. Growth factors in inflammatory bowel disease. *Inflamm Bowel Dis.* 1999;5:44–60.
3. Dignass AU, Sturm A. Peptide growth factors in the intestine. *Eur J Gastroenterol Hepatol.* 2001;13:763–770.
4. Gohda E, Tsubouchi H, Nakayama H, et al. Human hepatocyte growth factor in plasma from patients with fulminant hepatic failure. *Exp Cell Res.* 1986;166:139–150.
5. Gohda E, Tsubouchi H, Nakayama H, et al. Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. *J Clin Invest.* 1988;81:414–419.
6. Igawa T, Kanda S, Kanetake H, et al. Hepatocyte growth factor is a potent mitogen for cultured rabbit renal tubular epithelial cells. *Biochem Biophys Res Commun.* 1991;174:831–838.
7. Joplin R, Hishida T, Tsubouchi H, et al. Human intrahepatic biliary epithelial cells proliferate in vitro in response to human hepatocyte growth factor. *J Clin Invest.* 1992;90:1284–1289.
8. Takahashi M, Ota S, Terano A, et al. Hepatocyte growth factor induces mitogenic reaction to the rabbit gastric epithelial cells in primary culture. *Biochem Biophys Res Commun.* 1993;19:528–534.
9. Dignass AU, Lynch-Devaney K, Podolsky K. Hepatocyte growth factor/scatter factor modulates intestinal epithelial cell proliferation and migration. *Biochem Biophys Res Commun.* 1994;202:701–709.
10. Weidner KM, Sachs M, Birchmeier W. The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. *J Cell Biol.* 1993;121: 145–154.
11. Kitamura S, Kondo S, Shimomura Y, et al. Expression of hepatocyte growth factor and c-met in ulcerative colitis. *Inflamm Res.* 2000;49:320–324.
12. Matsuo M, Shiota G, Umeki K, et al. Induction of plasma hepatocyte growth factor in acute colitis of mice. *Inflamm Res.* 1997;46:166–167.
13. Ortega-Cava CF, Ishihara S, Kawashima K, et al. Hepatocyte growth factor expression in dextran sodium sulfate-induced colitis in rats. *Dig Dis Sci.* 2002;47:2275–2285.
14. Itoh H, Kataoka H, Tomita M, et al. Upregulation of HGF activator inhibitor type 1 but not type 2 along with regeneration of intestinal mucosa. *Am J Physiol Gastrointest Liver Physiol.* 2000;278:G635–G643.
15. Kataoka H, Shimomura T, Kawaguchi T, et al. Hepatocyte growth factor activator inhibitor type 1 is a specific cell surface binding protein of hepatocyte growth factor activator (HGFA) and regulates HGFA activity in the pericellular microenvironment. *J Biol Chem.* 2000;275: 40453–40462.
16. Hanauer SB, Present DH. The state of the art in the management of inflammatory bowel disease. *Rev Gastroenterol Disord.* 2003;3:81–92.
17. Sandborn WJ, Hanauer SB. Antitumor necrosis factor therapy for inflammatory bowel disease: a review of agents, pharmacology, clinical results, and safety. *Inflamm Bowel Dis.* 1999;5:119–133.
18. Tahara Y, Ido A, Yamamoto S, et al. Hepatocyte growth factor facilitates colonic mucosal repair in experimental ulcerative colitis in rats. *J Pharm Exp Therap.* 2003;307:146–151.
19. Ido A, Moriuchi A, Kim IL, et al. Pharmacokinetic study of recombinant human hepatocyte growth factor administered in a bolus intravenously or via portal vein. *Hepatol Res.* 2004;30:175–181.
20. Stucchi AF, Shofer S, Leeman S, et al. NK-1 antagonist reduces colonic inflammation and oxidative stress in dextran sulfate-induced colitis in rats. *Am J Physiol Gastrointest Liver Physiol.* 2000;279:G1298–G1306.
21. Macpherson BR, Pfeiffer CJ. Experimental production of diffuse colitis in rats. *Digestion.* 1978;17:135–150.
22. Tsune I, Ikejima K, Hirose M, et al. Dietary glycine prevents chemical-induced experimental colitis in the rat. *Gastroenterology.* 2003;125:775–785.
23. Bradley PP, Priebat DA, Christensen RD, et al. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol.* 1982;78:206–209.
24. Moreels TG, Nieuwendijk RJ, De Man JG, et al. Concurrent infection with Schistosoma mansoni attenuates inflammation induced changes in colonic morphology, cytokine levels, and smooth muscle contractility of trinitrobenzene sulphonic acid induced colitis in rats. *Gut.* 2004;53:99–107.
25. Hibi T, Ogata H, Sakuraba A. Animal models of inflammatory bowel disease. *J Gastroenterol.* 2002;37:409–417.
26. Neurath MF, Fuss I, Kelsall BL, et al. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med.* 1995;182: 1281–1290.
27. Ahn BK, Ko KH, Oh TY, et al. Efficacy of use of colonoscopy in dextran sulfate sodium induced ulcerative colitis in rats: the evaluation of the effects of antioxidant by colonoscopy. *Int J Colorectal Dis.* 2001;16:174–181.
28. Huang EH, Carter JJ, Whelan RL, et al. Colonoscopy in mice. *Surg Endosc.* 2002;16:22–24.
29. Nusrat A, Parkos CA, Bacarra AE, et al. Hepatocyte growth factor/scatter factor effects on epithelia. Regulation of intercellular junctions in transformed and nontransformed cell lines, basolateral polarization of c-met receptor in transformed and natural intestinal epithelia, and induction of rapid wound repair in a transformed model epithelium. *J Clin Invest.* 1994; 93:2056–2065.
30. Mammen JMV, Matthews JB. Mucosal repair in the gastrointestinal tract. *Crit Care Med.* 2003;31(Suppl):S532–S537.
31. Grant DS, Kleinman HK, Goldberg ID, et al. Scatter factor induces blood vessel formation in vivo. *Proc Natl Acad Sci USA.* 1993;90:1937–1941.
32. Goke M, Kanai M, Podolsky DK. Intestinal fibroblast regulate intestinal epithelial cell proliferation via hepatocyte growth factor. *Am J Physiol Gastrointest Liver Physiol.* 1998;274:G809–G818.
33. Hori T, Ido A, Uto H, et al. Activation of hepatocyte growth factor in monkey stomach following gastric mucosal injury. *J Gastroenterol.* 2004;39: 133–139.
34. Grenier A, Chollet-Martin S, Crestani B, et al. Presence of a mobilizable intracellular pool of hepatocyte growth factor in human polymorphonuclear neutrophils. *Blood.* 2002;99:2997–3004.
35. Adams DH, Harvath L, Bottaro DP, et al. Hepatocyte growth factor and macrophage inflammatory protein 1b: Structurally distinct cytokines that induce rapid cytoskeletal changes and subset-preferential migration in T cells. *Proc Natl Acad Sci USA.* 1994;91:7144–7148.
36. Mine S, Tanaka Y, Suematu M, et al. Hepatocyte growth factor is a potent trigger of neutrophil adhesion through rapid activation of lymphocyte function-associated antigen-1. *Lab Invest.* 1998;78:1395–1404.
37. Beilmann M, Vande Woude GF, Dienes HP, et al. Hepatocyte growth factor-stimulated invasiveness of monocytes. *Blood.* 2000;95: 3964–3969.

Review TRENDS in Microbiology Vol.12 No.8 August 2004

The role of Paneth cells and their antimicrobial peptides in innate host defense

Tokiyoshi Ayabe[1], Toshifumi Ashida[1], Yutaka Kohgo[1] and Toru Kono[2]

[1]Department of Internal Medicine III, Asahikawa Medical College, 2-1-1-1 Midorigaoka-Higashi, Asahikawa 078-8510, Japan
[2]Department of Surgery II, Asahikawa Medical College, 2-1-1-1 Midorigaoka-Higashi, Asahikawa 078-8510, Japan

The intestinal epithelium is the largest surface area that is exposed to various pathogens in the environment, however, in contrast to the colon the number of bacteria that colonize the small intestine is extremely low. Paneth cells, one of four major epithelial cell lineages in the small intestine, reside at the base of the crypts and have apically oriented secretory granules. These granules contain high levels of antimicrobial peptides that belong to the α-defensin family. Paneth cells secrete these microbicidal granules that contain α-defensins when exposed *ex vivo* to bacteria or their antigens, and recent evidence reveals that antimicrobial peptides, particularly α-defensins, that are present in Paneth cells contribute to intestinal innate host defense.

Multicellular organisms use various strategies to protect themselves against microbes. In mammals, the immune system is the host's defense against possible pathogens and consists of innate and adaptive immune mechanisms. Adaptive immunity, which is only found in vertebrates, is well characterized. By contrast, innate immunity is not considered as a vital defense system because it is thought to consist of only passive, non-specific reactions. It is known that non-vertebrates, such as insects and plants, produce antimicrobial peptides (AMPs) and that these are the effectors of the innate immune response [1–3]. Vertebrates, ranging from fish and frogs to humans, also produce these endogenous AMPs [4–7].

A continuous monolayer of gastrointestinal epithelial cells functions as the primary physical barrier against microbial invasion. There is much evidence to support the hypothesis that a variety of epithelia are actively involved in the innate host defense [8]. In intestinal epithelia, Paneth cells are the producers of AMPs [9–11]. Recent analyses of the molecular mechanisms that AMPs and pattern recognition molecules use in innate immunity have illustrated that this type of immune response is a vital, evolutionarily conserved and complex system for responding to microbial infection [12–14]. AMPs, especially defensins, are summarized in Box 1 (see also Table 1). In this review, recent advances in understanding the roles of Paneth cells and their AMPs in innate host defense will be discussed and summarized.

Corresponding author: Tokiyoshi Ayabe (ayabe@asahikawa-med.ac.jp).

Innate immunity in intestinal mucosa

The mammalian intestinal epithelium is the largest host surface area that is exposed to pathogens. It is well known that the number of bacteria that colonize the small intestine is extremely low relative to that found in the colon. Factors that result in the low bacterial numbers of the small intestinal lumen include barriers such as intestinal motility, digestive juice, mucus or the immune cell response (Box 2). The role of adaptive immunity in small intestinal responses to infection has been vigorously studied, particularly with respect to the contribution of gut-associated lymphoid tissue, γδ-T cells or cytokines, such as interleukin (IL)-2, IL-7 and IL-18 [15–17]. By contrast, the concept that innate immunity is a key component of the small intestinal barrier had not received extensive consideration until recently. Phagocytes that migrate from circulation and reside in the lamina propria probably have a role in the removal of invasive microbes. In addition, the small intestine is known to contain AMPs

Box 1. Defensins as antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are small peptides, usually consisting of less than 40 amino acids, which have a broad spectrum of microbicidal activities against Gram-negative and Gram-positive bacteria, fungi, protozoa and even enveloped viruses [13]. More than 500 AMPs have been discovered, and because of their structural diversity they have been classified on the basis of their secondary structure: magainins and numerous cathelicidins contain an α-helical structure, defensins have a β-sheet that contains three disulphide bonds, and a cathelicidine, known as PR-39, has a linear non-α-helical structure [46–50].

Defensins consist of cationic AMP families: α-defensins, β-defensins and θ-defensins. Defensin peptides function in a non-oxidative microbicidal manner against a variety of microbes. The α-defensins were among the first AMP families to be characterized, having been defined as major constituents of the primary granules of mammalian phagocytic leukocytes [51]. In addition, α-defensins are abundant in cytosolic granules of Paneth cells in human and mouse small intestine [9,10]. Amino acid sequences of some mouse and human α-defensins are shown in Table 1 in the main text. β-defensins are more widely expressed, being detected in neutrophils, airway epithelium, kidney, pancreas, salivary gland, skin and on every mucosal surface that has been investigated [51–53]. The first circular peptide to be discovered in the animal kingdom was the θ-defensin that is found in primate neutrophils; this defensin is a potent salt-insensitive microbicidal molecule [54].

Table 1. Amino acid sequences of α-defensins in mouse and human

In mouse Paneth cells	
Cryptdin-1	LRDLVCYCRSRGCKGRERMNGTCRKGHLLYTLCCR
Cryptdin-2	LRDLVCYCRTRGCKRRERMNGTCRKGHLMYTLCCR
Cryptdin-3	LRDLVCYCRKRGCKRRERMNGTCRKGHLMYTLCCR
Cryptdin-4	GLLCYCRKGHCKRGERVRGTC-G-IRFLYCCPRR
Cryptdin-5	LSKKLICYCRIRGCKRRERVFGTCRNLFLTFVFCCS
Cryptdin-6	LRDLVCYCRARGCKGRERMNGTCRKGHLLYMLCCR
In human neutrophils (HNP-1 ~ HNP-4) and Paneth cells (HD5 and HD6)	
HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-2	CYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-3	DCYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-4	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRVD
HD5	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR
HD6	AFTCHCRRS-CYSTEYSYGTCTVMGINHRFCCL

that might contribute to the innate immunity against microbial colonization.

The intestinal epithelium is continually renewed throughout mammalian life, as the progeny of stem cells migrate from their site of origin in crypts upward onto villi, during which time they differentiate into columnar cells, enteroendocrine cells or goblet cells. In contrast to these three epithelial lineages, Paneth cells [18] migrate downward from the stem cell zone toward the base of the crypt. Evidence from many lines of investigation support the hypothesis that the release of AMPs by epithelial cells contributes to innate mucosal immunity. In the small intestine, only Paneth cells appear to contain and release AMPs. M cells are known to exist in gut-associated lymphoid tissue, and pathogens or allergens are taken via M cells and presented to lymphocytes. This is an example of crosstalk between innate and adaptive immunity in the gut.

The role of Paneth cells in host defense

Paneth cells at the base of crypts in the small intestine contain lysozyme, secretory phospholipase A_2 and α-defensins in secretory granules, all of which have *in vitro* antimicrobial activities [19,20]. In contrast to villus enterocytes that have a lifetime of 2–3 days, Paneth cells live for more than 20 days and are renewed at a slower rate. In certain abnormal inflammatory conditions, Paneth cell-like cells can be found ectopically, such as in the colon during inflammatory bowel disease or in the pancreas during chronic pancreatitis [21]. The roles of tumor necrosis factor (TNF)-α, NOD2, CD95 ligand and zinc-binding proteins in Paneth cells are not understood, but their presence suggests that Paneth cell secretions might influence many different activities in the microenvironment of the crypt [22–24]. Recently, NOD2 expression in Paneth cells of both normal and Crohn's disease patients was reported [25].

Box 2. Factors involved in barrier functions for gastrointestinal tract

- Low pH of gastric juice
- Mucus
- Intestinal motility
- Tight junctions
- Regeneration of epithelial cells
- Antimicrobial peptides (e.g. defensins)
- Antimicrobial proteins (e.g. lysozyme and lactoferrin)
- Paneth cells
- Phagocytic cells
- Lymphocytes
- Antibodies
- Gut-associated lymphoid tissues
- M cells
- Normal flora

Paneth cell α-defensins, cryptdins in mice, and HD5 and HD6 in humans are antimicrobial constituents of apically oriented granules that have been shown to exhibit potent antimicrobial activities against microorganisms (including Gram-positive and Gram-negative bacteria, fungi and protozoa) in assays *in vitro* [26]. They have been recovered from intact tissue and from the lumen of the small intestine. As typical α-defensin family peptides, cryptdins are cationic peptides ranging from 3 to 4 kilodaltons in size. Six cryptdin peptides, cryptdin-1 to cryptdin-6, have been isolated and characterized in mice.

Satoh *et al.* [27,28] concluded from microscopic observations that *in vivo* fecal administration to the ileum induced morphological changes in Paneth cells in rat small intestine. When small intestinal crypts were stimulated *in vitro* by a cholinergic secretagogue, the Ca^{2+} dynamics were detected exclusively in Paneth cells [29]. Garabedian *et al.* [30] have shown that mice transgenic for attenuated diphtheria toxin A, which is expressed under the direction of the mouse cryptdin-2 gene promoter (CR2-tox), were deficient in mature Paneth cells. Because CR2-tox mice exhibited no phenotype in a conventional specific pathogen-free environment, the authors suggested that antimicrobial factors produced by Paneth cells are not required to prevent microbial colonization in a laboratory setting. However, because of the transient Paneth cell deficiency phenotype in CR2-tox mice, the function of Paneth cells and the role of their AMPs remain to be defined.

Proteolytic activation is required for precursors of α-defensin peptides to have microbicidal activity. In mouse small intestine, matrix metalloproteinase-7 (MMP-7, also known as matrilysin) colocalizes with cryptdins in Paneth cell granules and is the processing enzyme for procryptdins. Wilson *et al.* [31] showed that MMP-7-deficient mice, which lack mature cryptdins, exhibited decreased antimicrobial activity against orally administered bacteria *in vivo*, and were more susceptible to oral *Salmonella* infection than wild-type mice [31]. This was the first evidence that AMPs derived from Paneth cells contribute to the innate enteric host defense *in vivo*. In mice, the activation of cryptdin peptides by MMP-7 primarily occurs before secretion [32]. In human Paneth cells, a trypsin expressed in Paneth cell granules processes HD5. However, activation of HD5 occurs at secretion or shortly thereafter, because mature HD5 is not detected in Paneth cells [33].

In contrast to mice, human Paneth cells lack MMP-7. Human Paneth cells release the α-defensin HD5 precursor, $proHD5_{(20-94)}$, into the small intestinal lumen, which is then processed rapidly after secretion by anionic and/or meso isoforms of trypsin [34,35]. The trypsin cleavage site

in proHD5$_{(20-94)}$ lies between residues R62 and A63 and gives rise to the major form of the mature peptide found in washes of the small intestinal lumen [33]. The differences between mouse and human α-defensin processing suggest that the capacity for releasing mature microbicidal α-defensins is conserved, but that differing mechanisms have evolved to ensure the delivery of functional peptides into the lumen.

Microbicidal secretions by Paneth cells in response to bacteria

Paneth cells secrete α-defensins and other granule constituents in response to carbamyl choline, bacteria or their antigens, such as lipopolysaccharide (LPS) and muramyl dipeptide [35]. In mice, cryptdins constitute the majority of antimicrobial activity in Paneth cell secretions. Although the mechanisms are not known, the observation that bacteria induce Paneth cell secretion selectively suggests that a regulated pathway links bacterial recognition to the exocytotic apparatus. As illustrated in Figure 1, these recent studies have defined the role of the Paneth cell in innate enteric immunity and have established a new role of mucosal immunity at the intestinal epithelial surface. The Ca^{2+}-activated potassium channel, *mIKCa1*, is Paneth cell-specific in mouse small intestinal epithelium and functions in the secretory response of Paneth cells to bacteria [32]. For example, *IKCa1* channel-specific blockers inhibited LPS-induced release of cryptdins by Paneth cells in a dose-dependent manner [36]. Studies using mice transgenic for HD5, which express human Paneth cell α-defensin, showed that HD5 in mouse Paneth cells is highly protective against an orally administered lethal dose of virulent *Salmonella* [37]. These findings further confirm that Paneth cell α-defensins contribute to innate enteric immunity. To date, there is no evidence to suggest how Paneth cells recognize individual bacteria. Although Paneth cells might not selectively recognize differences between commensal or virulent bacteria, they do recognize bacterial factors [35]. Paneth cells are responsible for maintaining the homeostasis of the villus–crypt microenvironment by controlling the portal of microbes.

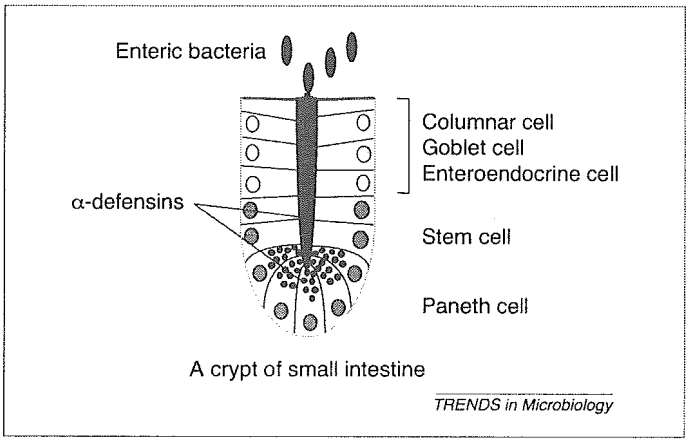


Figure 1. Secretion of microbicidal α-defensins by Paneth cells in response to bacteria. Small intestinal Paneth cells contribute to mucosal innate host defense by sensing bacteria or bacterial antigens, such as lipopolysaccharide, and secreting microbicidal peptides, particularly α-defensins [35].

The contribution of Paneth cell α-defensins to enteric mucosal immunity is most evident from the phenotype of mice transgenic for the human Paneth cell α-defensin HD5 (tg-HD5) [37]. These tg-HD5 mice specifically express the human minigene in Paneth cells and are completely immune to oral infection by *Salmonella enterica* serovar Typhimurium, even when challenged orally with an inoculum that is 1×10^4 times the LD_{50} (lethal dose 50). Recently, recombinant HD5 and mouse cryptdins have been found to have similar bactericidal activities against wild-type *S. typhimurium* (H. Tanabe, A.J. Ouellette and C.L. Bevans, unpublished), dispelling the view that immunity to *Salmonella* results from the introduction of a peptide with superior bactericidal activity. Perhaps the presence of HD5 in mouse Paneth cell secretions modifies the composition of the microflora by unknown selective means, conferring greater resistance to *S. typhimurium*.

A recent report has presented evidence that oral inoculation of mice with wild-type *S. typhimurium* decreased levels of cryptdin mRNAs and peptides [38]. Interestingly, inoculation with attenuated *Salmonella* serovar Typhimurium strains and strains lacking the SPI1 type III secretion system, or *Listeria monocytogenes*, did not modulate cryptdin levels. The authors speculated that the observed effects could have resulted from direct interactions between *Salmonella* and Paneth cells or from the effects of *Salmonella* on villous enterocytes, therefore leading to induced release of mediators from the enterocytes via a p38-signaling pathway. It will be of interest to discern the molecular and cellular details of these events in the gut and to test whether tg-HD5 mice exhibit a similar downregulation of cryptdin gene expression.

Future perspectives

Much of Paneth cell biology and the role or responses of Paneth cells and their AMPs to immunopathologies remain open questions. For instance, the stem cells that continuously renew the small intestinal epithelia have not been identified or isolated. Therefore, the mechanisms that regulate transcription and signaling in regeneration and differentiation of individual cell lineages of the small intestine, including Paneth cells, remain obscure. The relationship between several diseases and AMPs has been reported. Among them, the β-defensin dysfunction in patients with cystic fibrosis and the protective role of a cathelicidin (LL37) against skin infections have been described [39–42]. In the intestine, associations exist between Paneth cell α-defensins and necrotizing enterocolitis in immature babies, but causal relations have not been established [43]. In Crohn's disease, mutations in the NOD2 gene (involved in cytoplasmic recognition of bacterial antigens) correlates with susceptibility to the disease [44,45]. Paneth cells express NOD2 in the small intestinal crypts of both healthy volunteers and patients with Crohn's disease [23]. Because NOD2 is not a membrane-bound molecule but a cytoplasmic molecule, it might not be a direct target for bacterial recognition. To clarify the signal transduction molecules that are associated with bacterial recognition in Paneth cells, intact Paneth cells are now tested at the single cell level. On the basis of current information regarding molecules found in

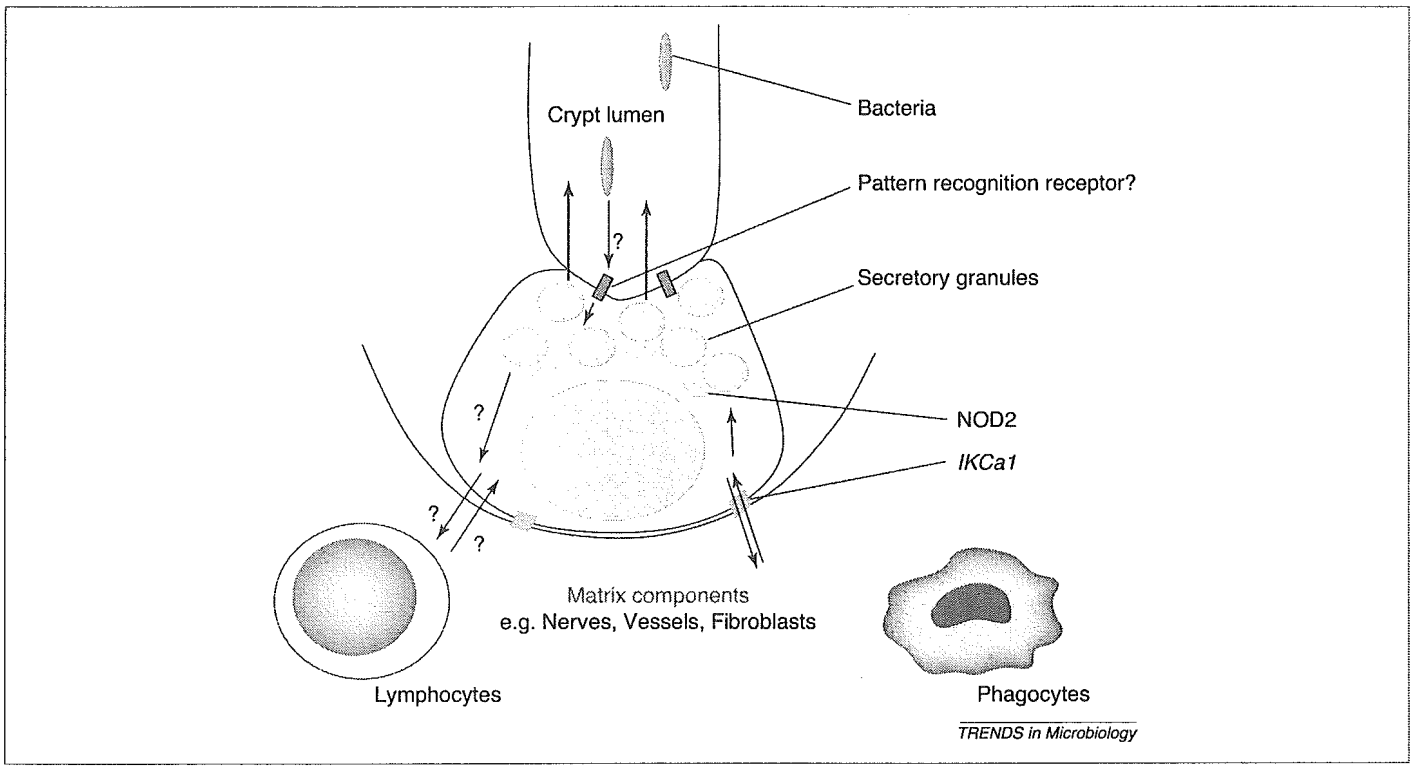


Figure 2. Possible biological and immunological responses via Paneth cells. Paneth cells reside in the bottom of small intestinal crypts and might regulate certain biological and immunological small intestinal responses. Secretory granules contain many bioactive factors. Crosstalks between microbes and Paneth cells, and between Paneth cells and matrix components, including cells in adaptive immunity and mesenchymal cells, might take place in the crypt microenvironment.

Paneth cells as well as hypothetical molecules, Figure 2 describes the possibilities for crosstalk between microbes and Paneth cells, and between Paneth cells and matrix components, including cells involved in the adaptive immune response and mesenchymal cells. Residing in the bottom of crypts, Paneth cells might regulate certain biological and immunological small intestinal responses. Alternatively, these cells might arise in greater numbers during inflammatory episodes to provide higher local levels of α-defensins in response to the possibility of increased infectious threat.

The use of antibiotics saves a lot of human lives from many serious infections. Even so, there are disadvantages associated with such usage, including the increase in the number of multiple antibiotic-resistant microbes, the high rate of opportunistic infections that are appearing in hospitals and also cost-benefit issues. Some advantages for the use of AMPs can be highlighted by mechanisms that do not result in microbial resistance compared with the use of antibiotics, and also the relative safety of their use because they are host-endogenous peptides. In the USA, efforts have already commenced to investigate the potential use of AMPs in the treatment of infectious diseases or complications caused by pathogens that are resistant to conventional antibiotics.

Concluding remarks

Innate immunity plays a crucial role in intestinal host defense. The epithelial innate host defense is not a passive nonspecific process, but a potent well-regulated system. The molecular basis of signaling molecules in innate enteric immunity and possible pathological contributions of Paneth cells and their AMPs to human diseases has attracted increasing attention. The time might be opportune for testing cohorts of patients for evidence of genomic or proteomic polymorphisms that could alter the synthesis, function or delivery of innate immune mediators by this unique epithelial cell.

Acknowledgements

We thank A.J. Ouellette (University of California, Irvine, College of Medicine) for useful discussions. This paper is supported partially by Grant-in-Aid for Scientific Research on Priority Areas (C) from The Ministry of Education, Culture, Sports, Science (TA*) and Technology in Japan, and Industrial Technology Research Grant Program in #02A02013a from New Energy and Industrial Technology Development Organization of Japan (TA*).

References

1 Steiner, H. *et al.* (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 292, 246–268
2 Broekaert, W.F. *et al.* (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. *Plant Physiol.* 108, 1353–1358
3 Metz-Boutigue, M.H. *et al.* (2003) Innate immunity: involvement of new neuropeptides. *Trends Microbiol.* 11, 585–592
4 Cole, A.M. *et al.* (1997) Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. *J. Biol. Chem.* 272, 12008–12013
5 Zasloff, M. (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci. U. S. A.* 84, 5449–5453
6 Selsted, M.E. *et al.* (1985) Primary structure of three human neutrophil defensins. *J. Clin. Invest.* 76, 1436–1439
7 Ganz, T. and Lehrer, R.I. (1998) Antimicrobial peptides of vertebrates. *Curr. Opin. Immunol.* 10, 41–44
8 Schonwetter, B.S. *et al.* (1995) Epithelial antibiotics induced at sites of inflammation. *Science* 267, 1645–1648

9 Ouellette, A.J. *et al.* (1994) Mouse Paneth cell defensins: primary structures and antibacterial activities of numerous cryptdin isoforms. *Infect. Immun.* 62, 5040–5047
10 Jones, D.E. and Bevins, C.L. (1992) Paneth cells of the human small intestine express an antimicrobial peptide gene. *J. Biol. Chem.* 267, 23216–23225
11 Hooper, L.V. *et al.* (2003) Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nat. Immunol.* 4, 269–273
12 Medzhitov, R. *et al.* (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388, 394–397
13 Akira, S. *et al.* (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2, 675–680
14 Takeda, K. *et al.* (2003) Toll-like receptors. *Annu. Rev. Immunol.* 21, 335–376
15 Sadlack, B. *et al.* (1993) Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* 75, 253–261
16 Watanabe, M. *et al.* (1995) Interleukin-7 is produced by human intestinal cells and regulates the proliferation of intestinal mucosal lymphocytes. *J. Clin. Invest.* 95, 2945–2953
17 Kanai, T. *et al.* (2001) Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterology* 121, 875–888
18 Paneth, J. (1888) Ueber die secernierenden Zellen des Duenndarmepithels. *Archiv. Mikr. Anat.* 31, 113–191
19 Ouellette, A.J. (1997) Paneth cells and innate immunity in the crypt microenvironment. *Gastroenterology* 113, 1779–1784
20 Porter, E.M. *et al.* (1997) Broad-spectrum antimicrobial activity of human intestinal defensin 5. *Infect. Immun.* 65, 2396–2401
21 Stamp, G.W. *et al.* (1992) Lysozyme gene expression in inflammatory bowel disease. *Gastroenterology* 103, 532–538
22 Keshav, S. *et al.* (1990) Tumor necrosis factor mRNA localized to Paneth cells of normal murine intestinal epithelium by *in situ* hybridization. *J. Exp. Med.* 171, 327–332
23 Morita, Y. *et al.* (2001) Identification of xanthine dehydrogenase/xanthine oxidase as a rat Paneth cell zinc-binding protein. *Biochim. Biophys. Acta* 1540, 43–49
24 Porter, E.M. *et al.* (2002) The multifaceted Paneth cell. *Cell. Mol. Life Sci.* 59, 156–170
25 Lala, S. *et al.* (2003) Crohn's disease and the NOD2 gene: a role for paneth cells. *Gastroenterology* 125, 47–57
26 Ouellette, A.J. and Bevins, C.L. (2001) Paneth cell defensins and innate immunity of the small bowel. *Inflamm. Bowel Dis.* 7, 43–50
27 Satoh, Y. *et al.* (1986) Quantitative light microscopic observations on Paneth cells of germ-free and ex-germ-free Wistar rats. *Digestion* 34, 115–121
28 Satoh, Y. and Vollrath, L. (1986) Quantitative electron microscopic observations on Paneth cells of germfree and ex-germfree Wistar rats. *Anat. Embryol.* 173, 317–322
29 Satoh, Y. *et al.* (1995) Carbamylcholine- and catecholamine-induced intracellular calcium dynamics of epithelial cells in mouse ileal crypts. *Gastroenterology* 108, 1345–1356
30 Garabedian, E.M. *et al.* (1997) Examining the role of Paneth cells in the small intestine by lineage ablation in transgenic mice. *J. Biol. Chem.* 272, 23729–23740
31 Wilson, C.L. *et al.* (1999) Regulation of intestinal α-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 286, 113–117
32 Ayabe, T. *et al.* (2002) Activation of Paneth cell α-defensins in mouse small intestine. *J. Biol. Chem.* 277, 5219–5228
33 Ghosh, D. *et al.* (2002) Paneth cell trypsin is the processing enzyme for human defensin-5. *Nat. Immunol.* 3, 583–590
34 Cunliffe, R.N. *et al.* (2001) Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disese. *Gut* 48, 176–185
35 Ayabe, T. *et al.* (2000) Secretion of microbicidal -defensins by intestinal Paneth cells in response to bacteria. *Nat. Immunol.* 1, 113–118
36 Ayabe, T. *et al.* (2002) Modulation of mouse Paneth cell α-defensin secretion by mIKCa1, a Ca^{2+}-activated, intermediate conductance Potassium channel. *J. Biol. Chem.* 277, 3793–3800
37 Salzman, N.H. *et al.* (2003) Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* 422, 522–526
38 Salzman, N.H. *et al.* (2003) Enteric *Salmonella* infection inhibits Paneth cell antimicrobial peptide expression. *Infect. Immun.* 71, 1109–1115
39 Smith, J.J. *et al.* (1996) Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* 85, 229–236
40 Goldman, M.J. *et al.* (1997) Human β-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* 88, 553–560
41 Nizet, V. *et al.* (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414, 454–457
42 Ong, P.Y. *et al.* (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* 347, 1151–1160
43 Salzman, N.H. *et al.* (1998) Enteric defensin expression in necrotizing enterocolitis. *Pediatr. Res.* 44, 20–26
44 Hugot, J.P. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 31, 599–603
45 Ogura, Y. *et al.* (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 31, 603–606
46 Kisich, K.O. (2001) Antimycobacterial agent based on mRNA encoding human β-Defensin 2 enables primary macrophages to restrict growth of Mycobacterium tuberculosis. *Infect. Immun.* 69, 2692–2699
47 Bals, R. (1999) Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect. Immun.* 67, 6084–6089
48 Bechinger, B. *et al.* (1993) Structure and orientation of the antibiotic peptide magainin in membranes by solid-state nuclear magnetic resonance spectroscopy. *Protein Sci.* 2, 2077–2084
49 Maloy, W.L. and Kari, U.P. (1995) Structure-activity studies on magainins and other host defense peptides. *Biopolymers* 37, 105–122
50 Matsuzaki, K. (1999) Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochim. Biophys. Acta* 1462, 1–10
51 Ouellette, A.J. and Selsted, M.E. (1996) Paneth cell defensins: endogenous peptide components of intestinal host defense. *FASEB J.* 10, 1280–1289
52 Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415, 389–395
53 Selsted, M.E. *et al.* (1992) Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. *J. Biol. Chem.* 267, 4292–4295
54 Tang, Y.Q. *et al.* (1999) A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* 286, 498–502

nature medicine

Induction of interleukin-8 preserves the angiogenic response in HIF-1α–deficient colon cancer cells

Yusuke Mizukami[1], Won-Seok Jo[1], Eva-Maria Duerr[1], Manish Gala[1], Jingnan Li[1], Xiaobo Zhang[1], Michael A Zimmer[2], Othon Iliopoulos[2], Lawrence R Zukerberg[3], Yutaka Kohgo[4], Maureen P Lynch[5], Bo R Rueda[5] & Daniel C Chung[1]

Hypoxia inducible factor-1 (HIF-1) is considered a crucial mediator of the cellular response to hypoxia through its regulation of genes that control angiogenesis[1–4]. It represents an attractive therapeutic target[5,6] in colon cancer, one of the few tumor types that shows a clinical response to antiangiogenic therapy[7]. But it is unclear whether inhibition of HIF-1 alone is sufficient to block tumor angiogenesis[8,9]. In HIF-1α knockdown DLD-1 colon cancer cells (DLD-1$^{HIF\text{-}kd}$), the hypoxic induction of vascular endothelial growth factor (VEGF) was only partially blocked. Xenografts remained highly vascularized with microvessel densities identical to DLD-1 tumors that had wild-type HIF-1α (DLD-1$^{HIF\text{-}wt}$). In addition to the preserved expression of VEGF, the proangiogenic cytokine interleukin (IL)-8 was induced by hypoxia in DLD-1$^{HIF\text{-}kd}$ but not DLD-1$^{HIF\text{-}wt}$ cells. This induction was mediated by the production of hydrogen peroxide and subsequent activation of NF-κB. Furthermore, the *KRAS* oncogene, which is commonly mutated in colon cancer, enhanced the hypoxic induction of IL-8. A neutralizing antibody to IL-8 substantially inhibited angiogenesis and tumor growth in DLD-1$^{HIF\text{-}kd}$ but not DLD-1$^{HIF\text{-}wt}$ xenografts, verifying the functional significance of this IL-8 response. Thus, compensatory pathways can be activated to preserve the tumor angiogenic response, and strategies that inhibit HIF-1α may be most effective when IL-8 is simultaneously targeted.

We subcutaneously injected DLD-1 cells, which contained either wild-type HIF-1α or HIF-1α stably knocked down by siRNA[10] (DLD-1$^{HIF\text{-}wt}$ or DLD-1$^{HIF\text{-}kd}$, respectively), into CD1 nude mice. Four weeks after inoculation, tumor volumes and weights were significantly lower in DLD-1$^{HIF\text{-}kd}$ tumors (**Fig. 1a,b**), indicating an important role for HIF-1 in tumor growth *in vivo*. We confirmed this finding in an independent colon cancer cell line, Caco2 (**Supplementary Fig. 1** online). Large necrotic areas were much more prevalent in DLD-1$^{HIF\text{-}wt}$ xenografts (**Fig. 1c**). Furthermore, a prominent inflammatory infiltrate composed predominantly of neutrophils was observed only in DLD-1$^{HIF\text{-}kd}$ xenografts (**Fig. 1c**). Although there were larger areas of necrosis in DLD-1$^{HIF\text{-}wt}$ xenografts, the cross-sectional surface area of non-necrotic viable tumor was still significantly greater when compared to DLD-1$^{HIF\text{-}kd}$ xenografts (0.33 cm^2 versus 0.16 cm^2, respectively, $P = 0.025$). Thus, the difference in size of the tumors cannot be entirely attributed to the larger area of necrosis in the DLD-1$^{HIF\text{-}wt}$ tumors. A persistent silencing effect of the HIF-1α siRNA construct was confirmed *in vivo* (**Fig. 1d**).

There was a significant decrease in the Ki-67 labeling index in DLD-1$^{HIF\text{-}kd}$ xenografts (41.3 ± 3.2% in DLD-1$^{HIF\text{-}wt}$ tumors versus 27.4 ± 2.6% in DLD-1$^{HIF\text{-}kd}$ tumors; $P < 0.01$), suggesting that HIF-1α regulates cellular proliferation *in vivo*. We calculated the apoptotic index by counting TUNEL-positive cells in non-necrotic areas. We observed a small but statistically significant difference in the apoptotic index between the two groups (3.2 ± 0.53% in DLD-1$^{HIF\text{-}wt}$ tumors versus 1.9 ± 0.42% in DLD-1$^{HIF\text{-}kd}$ tumors; $P < 0.05$), but this difference is unlikely to counterbalance the considerable difference in proliferation rates.

When we incubated DLD-1$^{HIF\text{-}kd}$ cells under hypoxic conditions (1% O_2) *in vitro*, we observed only a 25% reduction ($P = 0.11$) in the induced levels of *VEGF* mRNA and protein (**Fig. 1e**). In the DLD-1$^{HIF\text{-}kd}$ xenografts, *VEGF* mRNA and protein levels were also induced (**Fig. 1f**), though not to the same extent observed *in vitro*. Compared to the DLD-1$^{HIF\text{-}wt}$ xenografts, *VEGF* mRNA levels were 51% lower ($P = 0.028$) and protein levels were 52% lower ($P = 0.0024$) in DLD-1$^{HIF\text{-}kd}$ xenografts. This persistent expression of VEGF was not mediated by HIF-2α, as mRNA encoding HIF-2α and HIF-2α protein levels were barely detectable in normoxic conditions and the gene was not induced by hypoxia (**Supplementary Fig. 2** online).

To specifically address whether hypoxia regulates VEGF in the absence of HIF-1 *in vivo*, we identified hypoxic areas within the tumor mass using Hypoxyprobe-1 (pimonidazole hydroxychloride). There were large hypoxic regions surrounding the necrotic areas in the center of the DLD-1$^{HIF\text{-}wt}$ tumors (**Fig. 1g**). In contrast, DLD-1$^{HIF\text{-}kd}$ tumors showed only restricted regions of intratumoral hypoxia. Double immunofluorescence showed that VEGF was preferentially

[1]Gastrointestinal Unit, [2]Oncology Unit, Department of Medicine and [3]Department of Pathology, Massachusetts General Hospital and Harvard Medical School, 50 Blossom Street, Boston, Massachusetts 02114, USA. [4]Third Department of Internal Medicine, Asahikawa Medical College, 2-1 Midorigaoka-Higashi, Asahikawa, 078-8510 Japan. [5]Vincent Center for Reproductive Biology, Department of Obstetrics and Gynecology, Massachusetts General Hospital and Harvard Medical School, 50 Blossom Street Boston, Massachusetts 02114, USA. Correspondence should be addressed to D.C.C. (chung.daniel@mgh.harvard.edu).

Published online 28 August 2005; doi:10.1038/nm1294

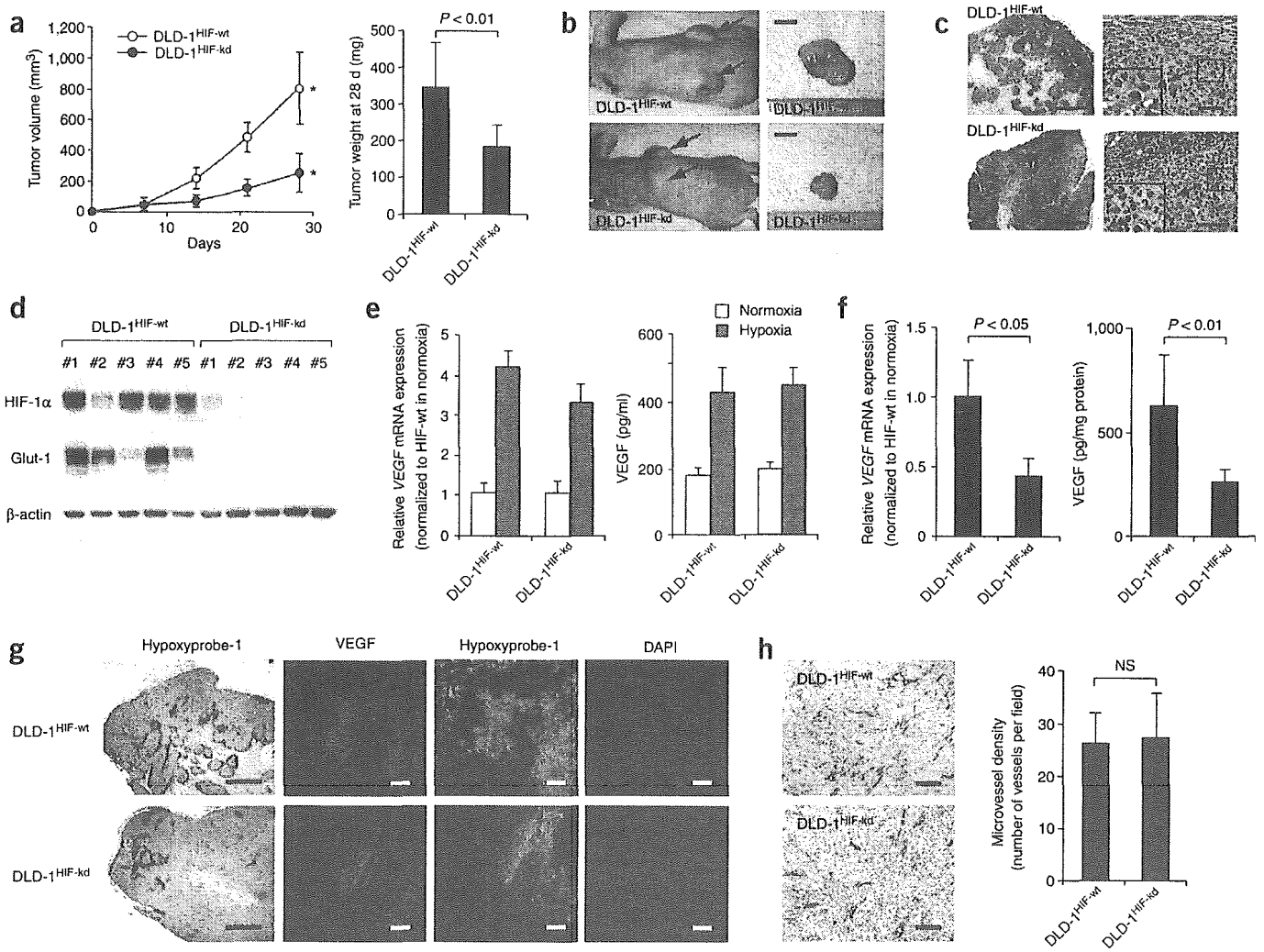


Figure 1 Growth of DLD-1$^{HIF\text{-}kd}$ cells *in vivo*. (**a**) Tumor volume and weight of DLD-1$^{HIF\text{-}wt}$ and DLD-1$^{HIF\text{-}kd}$ xenografts. *$P < 0.05$. (**b**) Gross appearance of xenografts and excised tumors at 4 weeks. Scale bar, 5 mm. (**c**) H&E staining of resected tumors. Left scale bars, 1 mm; right scale bars, 50 μm. (**d**) Immunoblotting for HIF-1α and Glut-1 in DLD-1$^{HIF\text{-}kd}$ xenografts. *VEGF* mRNA and protein levels in cultured DLD-1 cells (**e**) and in tumor xenografts (**f**) were measured. (**g**) Intratumoral 'hypoxia' was detected by immunohistochemistry for Hypoxyprobe-1. Scale bar, 1 mm. Immunofluorescent staining for VEGF (Texas red) and Hypoxyprobe-1 (FITC). Scale bar, 100 μm. (**h**) Immunohistochemistry for CD31 and quantification of microvessel density in DLD-1 xenografts. Scale bar, 100 μm.

expressed in the hypoxic areas of both DLD-1$^{HIF\text{-}kd}$ and DLD-1$^{HIF\text{-}wt}$ xenografts (**Fig. 1g**).

It is possible that the difference in growth between the xenografts resulted from impaired angiogenesis, potentially attributable to lower levels of VEGF in DLD-1$^{HIF\text{-}kd}$ tumors. But immunostaining for the endothelial cell marker CD31 showed abundant microvascular networks in all tumors (**Fig. 1h**). We observed no quantitative difference in microvessel density (26.1 ± 6.3/field in DLD-1$^{HIF\text{-}wt}$ and 28.7 ± 8.6/field in DLD-1$^{HIF\text{-}kd}$ xenografts), suggesting that high levels of HIF-1 may not be required to stimulate angiogenesis or maintain vessel integrity in DLD-1 tumors.

Although upregulation of VEGF was preserved in DLD-1$^{HIF\text{-}kd}$ xenografts, the absolute levels of VEGF were reduced. We therefore determined whether other angiogenic factors may be induced in a compensatory manner to maintain tumor vascularity in the absence of HIF-1. cDNA microarray analysis identified genes that were upregulated at least twofold by hypoxia but whose expression was attenuated less than 30% when HIF-1 was silenced. *VEGF* was upregulated fourfold in DLD-1$^{HIF\text{-}wt}$ cells by hypoxia, and this induction was decreased only 10.6% by HIF-1 silencing (**Supplementary Table 1** online). In addition, expression of the proangiogenic cytokine *IL-8* was increased twofold in DLD-1$^{HIF\text{-}kd}$ cells cultured in hypoxic conditions compared to DLD-1$^{HIF\text{-}wt}$ cells.

Hypoxia upregulated *IL-8* mRNA >2.5-fold in DLD-1$^{HIF\text{-}kd}$ cells, but there was no induction in DLD-1$^{HIF\text{-}wt}$ cells (**Fig. 2a**). Consistent with this result, the level of IL-8 in the supernatant of DLD-1$^{HIF\text{-}kd}$ cells was increased almost threefold compared to DLD-1$^{HIF\text{-}wt}$ cells. We obtained similar results with previously established, independent DLD-1$^{HIF\text{-}kd}$ clones[10] (data not shown). Extracts from DLD-1$^{HIF\text{-}kd}$ xenografts also showed significantly higher *IL8* mRNA and protein levels when compared to DLD-1$^{HIF\text{-}wt}$ tumors (**Fig. 2b**). *IL-8* promoter reporter constructs showed higher basal activity in DLD-1$^{HIF\text{-}kd}$ cells (**Fig. 2c**), and there was further induction of promoter activity in hypoxia that was not observed in the DLD-1$^{HIF\text{-}wt}$ cells. There was also a 2.1-fold induction of the *IL-8* promoter when HIF-1α was transiently knocked down in parental DLD-1 cells, indicating this

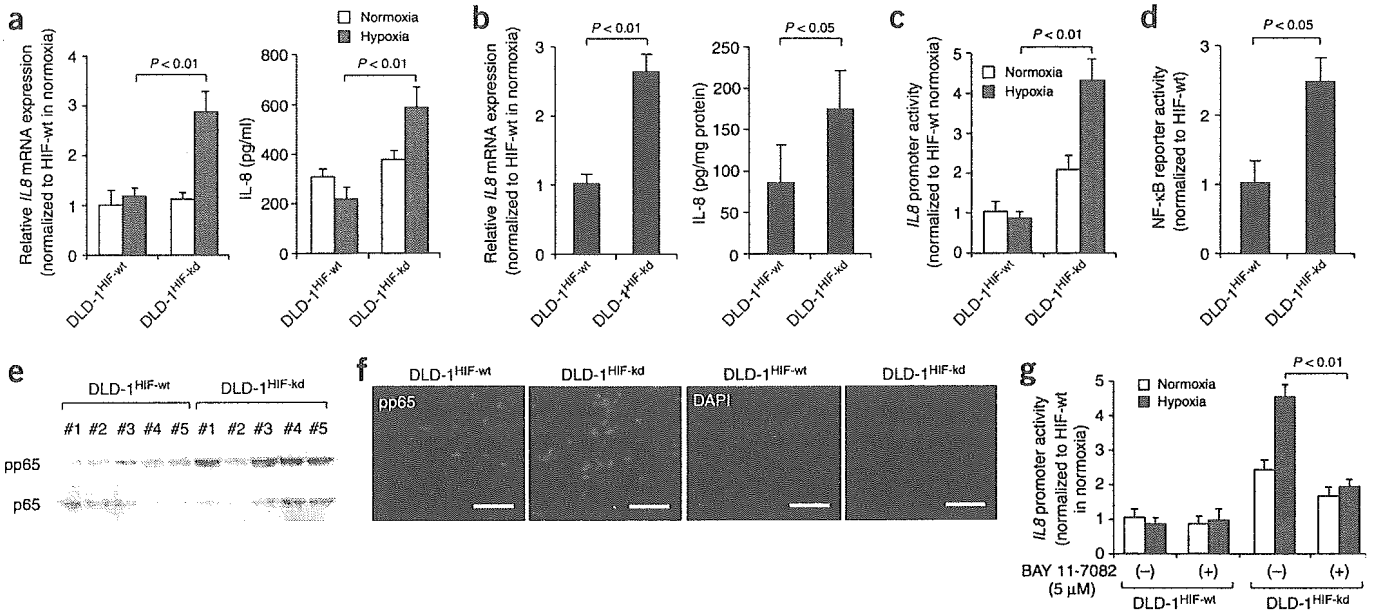


Figure 2 Knockdown of HIF-1 facilitates the induction of IL-8 by NF-κB during hypoxic conditions. *IL8* mRNA and protein levels in (**a**) cultured DLD-1 cells and (**b**) DLD-1 xenografts. (**c**) *IL8* promoter activity during hypoxia in DLD-1$^{HIF\text{-}kd}$ and DLD-1$^{HIF\text{-}wt}$ cells. (**d**) NF-κB reporter activity in hypoxic conditions in DLD-1$^{HIF\text{-}kd}$ cells. (**e**) Immunoblotting for NF-κB, p65 subunit and Ser536-phosphorylated p65 (p-p65), in DLD-1 tumor lysates. (**f**) Immunohistochemistry for phosphorylated p65 in DLD-1 xenografts (shown as Texas Red). Scale bar, 50 μm. (**g**) Effect of NF-κB inhibition on *IL8* promoter activity with BAY 11-7082.

phenomenon was not an artifact of the stable transfection process. In addition, expression of a constitutively active HIF-1α in which the proline at position 564 was changed to an alanine in DLD-1 cells did not induce the *IL-8* promoter (1.01 ± 0.14-fold increase), indicating that HIF-1 does not directly regulate *IL-8* gene expression. This hypoxic effect was not unique to DLD-1 cells. Knockdown of HIF-1α in additional colon cancer cells (ColoHSR, SW 480 and HCT116), pancreatic cancer cells (Panc-1, CAPAN-1), breast cancer cells (MDA-MB-453) and lung cancer cells (HOP-92) showed a similar induction of IL-8 in hypoxia (**Supplementary Fig. 3** online). Finally, we confirmed specificity of these siRNA constructs by observing expression of HIF-1α synonymous codon mutants (**Supplementary Fig. 4** online). The absence of HIF-1 can therefore stimulate IL-8 on a transcriptional level, and this is further enhanced in hypoxia.

NF-κB is a major regulator of IL-8. NF-κB reporter activity was increased 151% ($P < 0.01$) in HIF-1α knockdown cells (**Fig. 2d**). Western blotting (**Fig. 2e**) and immunohistochemistry (**Fig. 2f**) of tissue xenografts showed that phosphorylation of the p65 subunit was greater in DLD-1$^{HIF\text{-}kd}$ xenografts, suggesting that HIF-1 inhibition does upregulate the NF-κB pathway *in vivo*. Densitometry of western blots quantified a 2.0 ± 0.4-fold increase in the ratio of phosphorylated p65 to unphosphorylated p65 ($P < 0.01$). The hypoxic induction of the *IL-8* promoter in DLD-1$^{HIF\text{-}kd}$ cells was significantly downregulated by BAY 11-7082, a specific inhibitor of NF-κB[11] (**Fig. 2g**). Thus, activation of the NF-κB pathway is important for the induction of IL-8 in the absence of HIF-1.

We then speculated that HIF-1 inhibition may enhance the production of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) that can activate NF-κB[12,13]. Hypoxic conditions can lead to the increased production of ROS[14,15], and scavenging of ROS is often achieved by increased production of pyruvate[16] that occurs when cells shift from oxidative to glycolytic metabolism. This shift depends upon HIF-1α[17]. DLD-1$^{HIF\text{-}kd}$ cells released more H_2O_2 *in vitro*, and hypoxia further enhanced its production (**Fig. 3a**). Four distinct chemical inhibitors of ROS production (*N*-acetyl-L-cysteine, pyrrolidinedithiocarbamate, rotenone and diphenylene iodonium) each strongly blocked the induction of NF-κB promoter activity by hypoxia in DLD-1$^{HIF\text{-}kd}$ cells (**Fig. 3b**). Finally, exogenous administration of the long-acting H_2O_2 analog, *t*-butyl hydroperoxide, stimulated the production of IL-8 in parental DLD-1 cells. This induction was inhibited by BAY 11-7082 (**Fig. 3c**), again showing that NF-κB mediates this effect of ROS.

In contrast to DLD-1$^{HIF\text{-}kd}$ cells, we did not observe hypoxic induction of *IL8* mRNA (**Fig. 3d**) and protein (data not shown) in Caco2$^{HIF\text{-}kd}$ colon cancer cells[10]. Given that DLD-1 cells harbor the Gly13Asp mutation in the *KRAS* oncogene (*KRAS*38g→a), whereas Caco2 cells are wild-type (*KRAS*$^{35g/35g}$), we speculated that oncogenic *KRAS* may have a role in the hypoxic induction of IL-8 (ref. 18). When we induced the expression of the Gly12Val *KRAS* mutation (KRAS35g→t) in Caco2$^{HIF\text{-}kd}$ cells , hypoxia upregulated *IL-8* mRNA 2.5-fold, whereas the effect was not observed in Caco2$^{HIF\text{-}wt}$ cells or in Caco2$^{HIF\text{-}kd}$ cells exposed to hypoxia only (**Fig. 3d**). *KRAS*^{35t} only modestly induced *IL-8* mRNA in Caco2$^{HIF\text{-}kd}$ cells in normoxic conditions. Expression of *KRAS*^{35t} in Caco2$^{HIF\text{-}wt}$ cells also upregulated the *IL-8* promoter, but this activation was more pronounced in Caco2$^{HIF\text{-}kd}$ cells under conditions of hypoxia (**Fig. 3e**). BAY 11-7082 blocked the induction of the *IL-8* promoter by hypoxia and *KRAS*^{35t} (**Fig. 3e**).

Exogenous expression of oncogenic *KRAS* may act supraphysiologically. Endogenous *KRAS*^{38a} in DLD-1 cells was therefore silenced by siRNA and this resulted in a 50% reduction of KRAS protein levels, consistent with a silencing effect of the one mutant allele[19]. Knockdown of *KRAS*^{38a} attenuated the hypoxic induction of an NF-κB reporter and *IL8* promoter activity (**Fig. 3f**) as well as *IL8* mRNA levels (**Fig. 3g**) in DLD-1$^{HIF\text{-}kd}$ but not in DLD-1$^{HIF\text{-}wt}$ cells. These observations were confirmed in the Panc-1 pancreatic and PC3 prostate cancer cell lines, indicating the broader importance of *KRAS* on this alternative regulation of IL-8 (**Supplementary Fig. 5**