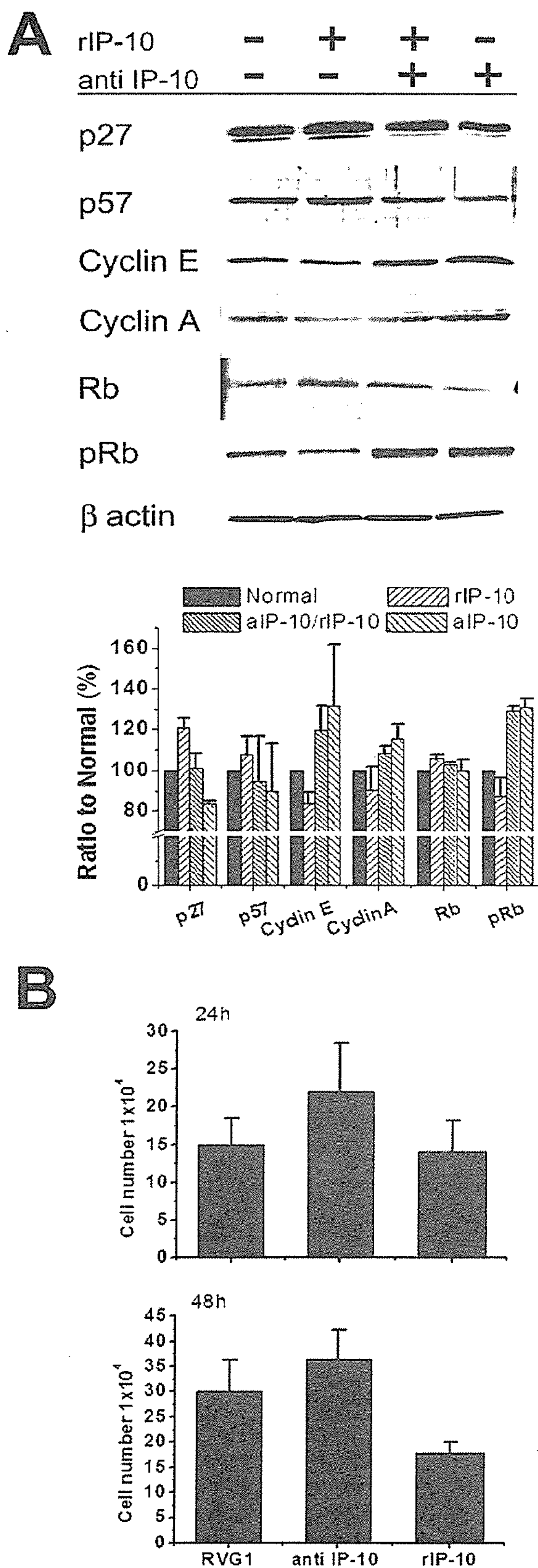

Figure 6. The effect of the treatments of rIP-10 and anti–IP-10 mAb on the expression of the cell-cycle regulatory proteins and on the proliferation. (A) Heightened expression of $p27^{Kip1}$ and lowered expression of cyclins E and A were detected in the nonpermissive conditioned cultured podocytes treated with rIP-10 for 24 h (second lane from the left). Anti–IP-10 mAb pretreatment inhibited this effect of rIP-10 (third lane). Anti–IP-10 mAb treatment without subsequent rIP-10 incubation lowered the $p27^{Kip1}$ level while clearly heightening the protein level of cyclin E, cyclin A, and retinoblastoma protein (pRb; fourth lane). No specific changes in the expression of $p57^{Kip2}$ and Rb were detected as a result of the intervention of IP-10. The ratios of their densitometric signals to that of the internal control (β-actin) were analyzed. The data are shown as ratios (%) relative to the normal group and are expressed as mean ± SD of three independent experiments. (B) Proliferation assay was carried out with permissive conditioned cultured podocyte. The treatment with anti–IP-10 for 24 and 48h enhanced the proliferation of the cells, whereas the treatment of rIP-10 for 48 h reduced the number of the cells.

expression is limited to the S/G2-M phase of the endothelial cell. They also reported that PF4, which shares the binding for CXCR3 with IP-10, upregulates the p21 level. However, no reports providing direct evidence that IP-10 is associated with the expression of cell-cycle regulatory proteins have been published. Our study is the first report to demonstrate that IP-10 plays a role in the regulation of the cell-cycle balance. More important, we have demonstrated here that anti–IP-10 antibody injection enhances the expression of cyclin A in the podocytes of proteinuric rats as well as normal rats. On the basis of these findings, we think that anti–IP-10 mAb treatment exacerbates podocyte injuries by disturbing the cell-cycle balance. We propose IP-10 as a possible therapeutic target candidate not only in podocyte injury but also in several diseases in which the negative regulation of the cell-cycle balance is broken down, although additional studies with other kinds of cell lines and tissues are needed to confirm this.

Finally, the question of whether IP-10 functions in the podocyte by binding its receptor, CXCR3, should be discussed. We have demonstrated here that the expression of CXCR3 increases in the injured podocyte in parallel with that of IP-10 (Figure 3), which suggests that IP-10 functions in the podocyte through CXCR3. It is reported that chemokines other than IP-10, which share the binding to CXCR3, can inhibit the proliferation of human microvascular endothelial cells as well and that the effect is inhibited by anti-CXCR3 antibody (36). Recently, Lasagni *et al.* (37) reported that CXCR3 has an alternative splicing variant (CXCR3-B) and that CXCR3-B mediates the inhibitory activity of IP-10 on the growth of human endothelial cells. Further characterization of CXCR3 may allow the development of new effective therapeutic strategies for podocyte injuries and other diseases that are caused by altered cell-cycle balance.

In conclusion, our study has demonstrated for the first time that IP-10 plays a pivotal role in maintaining the SD function by regulating the cell-cycle balance of the podocyte. IP-10/CXCR3 could be an attractive therapeutic target for nephrotic syndrome and a variety of diseases in which the negative cell-cycle balance has been disturbed.

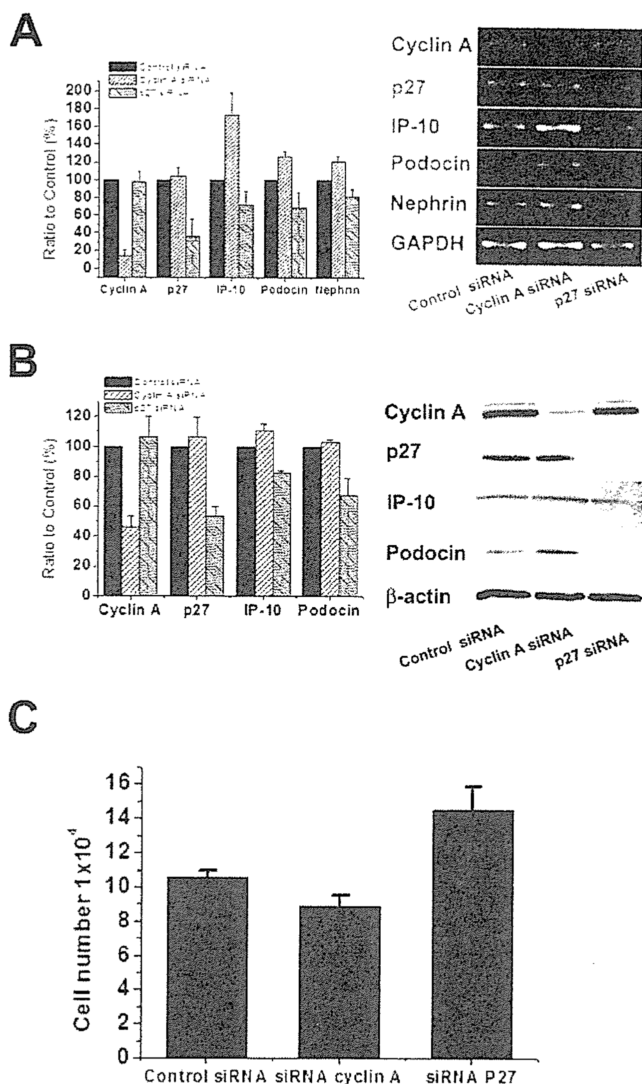

Figure 7. The effect of the treatment of small interfering RNA (siRNA) of cyclin A and $p27^{Kip}$ on the expression of IP-10 and SD components and on the proliferation. siRNA of cyclin A and $p27^{Kip1}$ clearly lowered the expression of each target molecule in both mRNA and protein levels in nonpermissive conditioned podocyte. siRNA treatment of cyclin A heightened the expression of IP-10 and SD components in both mRNA (A) and protein (B) levels, but the siRNA $p27^{Kip1}$ lowered the expression of these molecules. The treatment with siRNA for cyclin A for 48 h inhibited the proliferation of permissive conditioned podocyte, whereas the treatment with siRNA of $p27^{Kip1}$ for 48 h enhanced it (C).

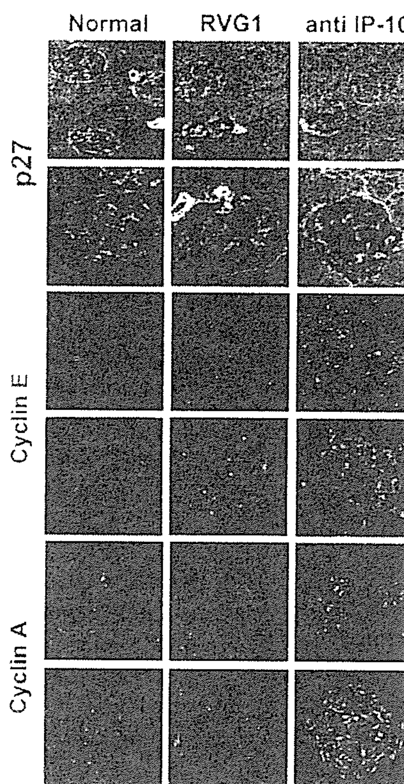

Figure 8. The effect of anti–IP-10 mAb treatment on the IF staining of $p27^{Kip1}$ and cyclins E and A. The expression of $p27^{Kip1}$ decreased in rats that received daily injections of anti–IP-10 mAb for 5 d, whereas the expression of cyclins E and A in the glomerular podocyte clearly heightened in rats treated with anti–IP-10 mAb. No altered expression of them was observed in rats treated with control IgG1 (RVG1). Magnification, ×200 in upper lane; ×400 in lower lane.

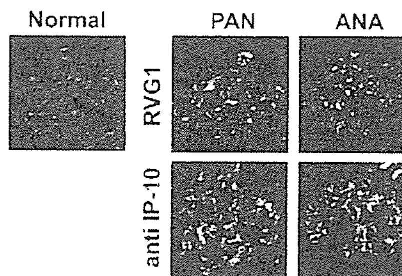

Figure 9. The effect of anti–IP-10 mAb treatment on the IF staining of cyclin A in PAN and ANA nephropathy. Heightened expression of cyclin A was observed on day 9 and day 5 after the induction of PAN and ANA nephropathy, respectively, when massive proteinuria was observed (irrelevant IgG1, RVG1-treated group). Daily injection with anti–IP-10 mAb enhanced the increase of cyclin A expression of both PAN and ANA nephropathy. The left top figure (normal) indicates the finding of normal rat. Magnification, ×400.

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RESEARCH ARTICLE

Attenuation of mouse acute colitis by naked hepatocyte growth factor gene transfer into the liver

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Abstract

Background Hepatocyte growth factor (HGF) has multiple biological effects on a wide variety of cells. It modulates intestinal epithelial proliferation and migration, and critically regulates intestinal wound healing.

Aims To investigate the therapeutic effect of HGF gene transfer, we introduced the HGF gene into the liver of mice with acute colitis.

Methods The rat HGF expression plasmid vector, pCAGGS-HGF, was injected via the tail vein into C57BL/6 mice, followed by dosing with dextran sulfate sodium in distilled water. Firstly, the HGF gene was injected once on day 0. Secondly, the HGF gene was injected on day 0 and again on day 2.

Results Injection of the HGF gene ameliorated colitis with inhibition of both loss of body weight and shortening of colon length. It protected the colon from epithelial erosions and cellular infiltration. Expression of mRNAs for IFN-γ, IL18, and TNF-α was reduced in the colon. In contrast, expression of mRNA for IL-10 was increased. The numbers of BrdU-positive intestinal epithelial cells were increased, and the numbers of TUNEL-positive apoptotic cells were decreased. Furthermore, a second injection prolonged the elevation of serum HGF levels, and ameliorated the symptoms better than a single injection. The empty pCAGGS plasmid did not ameliorate acute colitis.

Conclusions HGF gene transfer attenuated acute colitis by facilitating intestinal wound repair as well as inhibiting inflammation, suggesting a new strategy for treatment of IBD. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords HGF; c-Met; inflammatory bowel disease; DSS colitis; naked gene transfer

Introduction

Inflammatory bowel disease, which comprises ulcerative colitis and Crohn's disease, is characterized by chronically relapsing inflammation of the bowel of unknown origin [1]. Therapy for inflammatory bowel disease has been aimed predominantly at the regulation of the inflammatory cells and their production of various inflammatory mediators, such as arachidonic acid metabolites, chemokines, and pro-inflammatory cytokines [2,3]. Conventional therapy for inflammatory bowel disease involves 5-aminosalicylates, corticosteroids, and immunosuppressive drugs such as azathioprine. Tumor necrosis factor (TNF)-α and interleukin (IL)-6 have been investigated as target molecules for the development of newer therapeutic approaches for inflammatory bowel disease [4]. Anti-TNF-α antibodies have been established

for the treatment of Crohn's disease [5], and anti-IL-6 receptor antibodies have been tried clinically with great success [6]. These agents are beneficial and established as a standard therapy for inflammatory bowel disease; however, they have limited response rate and serious side effects. Therefore, newer therapeutic approaches are required.

Epidermal growth factor (EGF) is a potent mitogenic peptide produced by the salivary and duodenal Brunner's glands and stimulates several components of the healing response [7,8]. In a recent report, EGF enema has been shown to be an effective treatment for active left-sided ulcerative colitis [9]. Thus, newer therapeutic approaches for inflammatory bowel disease should be aimed at regeneration and repair of the wounded intestinal epithelial cells of the bowel as well as inhibition of inflammation.

Hepatocyte growth factor (HGF) is a pleiotropic factor initially identified as a growth factor for hepatocytes [10–12]. It has mitogenic, motogenic, and morphogenic functions in various types of cells, including gastrointestinal epithelial cells [13–15], through its high-affinity receptor tyrosine kinase, Met, that is encoded by the c-*met* proto-oncogene [16]. HGF activator, HGF activator inhibitor type-1, and HGF-associated molecules involved in the activation of HGF in injured tissues are associated with colonic mucosal repair [17]. HGF expression has been reported to be up-regulated in the inflamed colonic mucosal tissue in patients with ulcerative colitis [18], and plasma HGF levels are increased in animal models of acute colitis [19]. Recently, the administration of recombinant human HGF has been shown to facilitate colonic mucosal repair in rats with dextran sulfate sodium (DSS)-induced colitis [20], and in HLA-B27 transgenic rats [21].

Liver is an important target organ for gene transfer because of its high capacity for synthesizing serum proteins and its involvement in numerous genetic and acquired diseases [22]. Among the various gene delivery systems available, naked DNA-mediated gene transfer is the simplest, and techniques for introducing DNA into hepatocytes have been the most intensely studied methods for generating therapeutic amounts of gene product [23]. High levels of foreign gene expression can be achieved in mouse hepatocytes by rapid tail vein injection of a large volume of naked DNA solution, the 'hydrodynamics-based procedure' [24]. In this study, we investigated the therapeutic effect of rat HGF gene transfer into the liver by the hydrodynamics-based method in a murine model of colitis.

Materials and methods

Animals and induction of colitis

Female C57BL/6 (B6) mice (7–8 weeks old) were purchased from Charles River Japan (Atsugi, Kanagawa, Japan) and maintained in the Animal Center of Niigata University School of Medicine under specific pathogen-free conditions. Colitis was induced in the mice by the administration of 3–5% DSS (molecular weight 36 000–50 000; Wako, Osaka, Japan) in distilled water *ad libitum* for 7 days. Three hours before the administration of DSS at day 0 and/or day 2, we injected HGF or control vector into the mice as described below. Body weight, stool consistency (scores: 0, normal stools; 1, soft stools; 2, liquid stools), hemoccult positivity and the presence of gross blood (scores: 0, negative fecal occult blood; 1, positive fecal occult blood; 2, visible rectal bleeding) were assessed daily. The disease activity index (DAI) was determined as a combination of the above parameters according to the scoring criteria as we described previously [25]. All animal experiments were performed according to the 'Guide for Animal Experiments' of Niigata University School of Medicine.

Plasmid DNA injection techniques

Plasmid pCAGGS-rat-HGF was constructed by inserting the rat HGF cDNA into a unique EcoRI site in the pCAGGS expression vector, which has the CAG (cytomegalovirus immediate-early enhancer/chicken β-actin hybrid) promoter, and grown in *Escherichia coli* DH5α (Toyobo, Osaka, Japan). The plasmid was prepared using a Qiagen Endofree plasmid Giga kit (Qiagen GmbH, Hilden, Germany), as described previously. The empty pCAGGS plasmid was used as a control. The plasmid DNA was diluted in 2 ml (approximately 1/10 of the body weight) of Ringer's solution (Ohtsuka, Tokushima, Japan) at room temperature. At day 0 and/or day 2, we anesthetized the mice with diethyl ether, and injected 10 μg of either pCAGGS-HGF or pCAGGS plasmid into the tail vein through a 27-gauge needle with a <3 s injection time.

Measurement of HGF in plasma

To determine the concentration of HGF at various time points after injection, we used an enzyme-linked immunosorbent assay (ELISA) kit for rat HGF (Institute of Immunology, Tokyo, Japan) including an antibody that cross reacts with mouse HGF. Values are expressed in ng/ml.

Evaluation of histology

The degree of colonic injury was assessed by colon length and histological score. The entire colons (10 mice per group) were sampled and their lengths were recorded immediately. The entire colon was fixed in 4% formalin, embedded in paraffin, and transverse sections were stained with hematoxylin and eosin. According to the preliminary histological interpretation, we analyzed the distal colon tissue section located approximately 10 mm from the anal verge to calculate the number of infiltrating

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cells in the lamina propria of the colon. The crypt length of the colon of each mouse was also calculated as a mean value of five different crypts.

Quantitative reverse-transcription polymerase chain reaction (RT-PCR) to detect cytokine mRNAs

Total RNA was extracted from colon specimens with Trizol (Gibco BRL) according to the standard protocol then reverse-transcribed. Thereafter, cDNA was amplified using the ABI 7700 sequence-detector system (Applied Biosystems, Foster City, CA, USA) with a set of primers and probes corresponding to IFN-γ, TNF-α, IL-1β, IL-12, IL-18, IL-4, IL-10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described previously.

X-gal and immunohistochemical staining

pCAGGS-lacZ expresses *E. coli* β-galactosidase. The lung, heart, liver, spleen, and kidneys were harvested for X-gal staining 1 day after the injection of 10 µg of pCAGGS-lacZ, embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan), and then frozen in a mixture of dry ice and acetone. Serial sections (5-µm thick) were cut with a cryostat and placed on glass slides coated with 3-aminopropyltriethoxysilane. Then the sections were fixed in 1.5% glutaraldehyde at room temperature for 10 min, washed three times in cold phosphate-buffered saline (PBS) (5 min/wash), and incubated in an X-gal staining solution containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 0.5% Nonidet P-40 in PBS, pH 7.4, at 37 °C for 3 h, followed by counterstaining with nuclear fast red.

Immunohistochemical staining

All mice were injected with BrdU (Sigma) (500 µg/100 µl in PBS) 1 h before killing. To detect replicating cells, tissue sections were reacted with the antibody and reagents using a BrdU staining kit (ZYMED, South San Francisco, CA, USA) according to the manufacturer's instructions. Crypts that had five or more BrdU-labeled nuclei were defined as surviving crypts. The numbers of BrdU-labeled nuclei of colonic epithelial cells for each group were compared.

Terminal deoxynucleotide transferase labeling

Apoptotic cells were identified using an *in situ* apoptosis detection kit (Takara Biomedicals, Japan) according to the manufacturer's instructions. In brief, acetone-fixed 5-mm fresh-frozen colon sections were permeabilized on ice and incubated with the terminal deoxynucleotide transferase mixture for 1 h at 37 °C. FITC-labeled dNTP were treated with anti-FITC HRP for 30 min, visualized with DAB, and counterstained with hematoxylin.

Monoclonal antibodies

The following monoclonal antibodies were used for immunofluorescence and flow cytometric analyses: anti-CD4 (clone GK1.5, IgG2b), anti-CD8 (clone 53-6.7, IgG2a), anti-B220 (clone RA3-6B2, IgG2a), anti-Mac-1 (clone M-70.15, IgG2b), anti-mouse INF-γ (clone XMG1.2), and anti-mouse IL-10 (clone JES5-16E3).

For immunostaining of HGF and c-Met, a goat polyclonal IgG antibody raised against a peptide mapping at the carboxyl terminus of HGFα (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or an affinity purified rabbit polyclonal antibody raised against a peptide mapping at the carboxyl terminus of c-Met p140 of mouse origin (SP260; Santa Cruz Biotechnology) was used.

IF staining procedure

Frozen sections of the colon were prepared in a cryostat and stained with several fluorescent dye-conjugated anti-mouse antibodies as described above. The sections were observed by fluorescence microscopy.

Ex vivo colonic tissue culture

Ex vivo colon tissue culture was done by the established method with modification described as below [26]. The entire colon was taken from B6 mice with DSS colitis on days 2 and 5, or from those without colitis. The left side of the colon was cut into small pieces, each of which weighed 15 mg. Each colon sample was washed three times using RPMI 1640 culture medium containing 10% FBS (fetal bovine serum), penicillin G (100 IU/ml), and streptomycin (10 ng/ml), and placed in a center well of an organ culture dish (Falcon; Becton Dickinson Labware, NJ, USA). The center well of the dish was filled with 1 ml of the same medium, and incubated for 2 h and 24 h at 37 °C under 95% air and 5% CO_2. To evaluate the direct effect of HGF on *ex vivo* colon tissue, each sample was treated with recombinant human HGF (IBL, Gunma, Japan) at a concentration of 10 ng/ml at the beginning of tissue culture. The total RNA was extracted from each colon tissue and analyzed as described above for determining the mRNA expression levels for IFN-γ, IL-10, mouse HGF, and c-Met.

Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical analyses were performed using the unpaired

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Student's t test or the nonparametric Mann-Whitney test. Differences were considered significant at $p < 0.05$.

Results

Localization of pCAGGS-lacZ gene expression and serum levels of rat HGF after pCAGGS-HGF injection

To clarify the site of transgene expression, we delivered 10 µg of pCAGGS-lacZ or pCAGGS into the tail vein of normal mice. LacZ gene expression was assessed in various organs including the liver, heart, lungs, kidney, spleen, and colon. X-gal stained only in the liver of the pCAGGS-lacZ-injected mice (Figure 1A), but not in the liver of the pCAGGS-injected mice (data not shown). The stained cells were predominantly hepatocytes, which were identifiable by their polygonal shape and round nuclei, visible at a higher magnification. We did not find convincing examples of X-gal-stained cells in the heart, lungs, kidney, spleen, or colon of the pCAGGS-lacZ-injected mice (data not shown).

We also confirmed the expression of HGF in hepatocytes in the liver of mice 2 days after injection of 10 µg pCAGGS-HGF by immunofluorescence using anti-HGF antibody (Figure 1C). Expression of HGF was not detected in the liver of mice injected with pCAGGS (Figure 1D). In the liver of mice injected with pCAGGS-HGF, the expression levels of rat HGF mRNA were significantly increased, but these were not increased in mice injected with control pCAGGS (data not shown).

We next evaluated the time course of rat HGF expression after the injection of 10 µg of DNA, using a less than 3-s injection time and a volume of 2 ml. Mice were injected with either 10 µg of pCAGGS-HGF (n = 5) or 10 µg of pCAGGS (n = 5). After injection of pCAGGS-HGF, the peak plasma HGF level was 15 ± 5 ng/ml at 12 h; then it decreased gradually, and became undetectable at 60 h (Figure 1B). The plasma rat HGF levels in the control mice were not significantly increased (Figure 1B).

HGF and c-Met expression after pCAGGS-HGF injection in the colons of mice with DSS colitis

Administration of DSS did not change the time course of the plasma rat HGF level after either pCAGGS or pCAGGS-HGF injection. In other words, after injection of pCAGGS-HGF into mice with DSS colitis, the peak plasma HGF levels were at 15 ng/ml at 12 h, and then decreased gradually and became undetectable at 72 h. The plasma rat HGF levels were not significantly increased in the DSS colitis mice injected with the empty pCAGGS.

Next we evaluated the expression of HGF and c-Met in colon tissues of mice with DSS colitis by immunofluorescence at day 2 after gene transfer. HGF expression was not detected in the colons of mice injected with either pCAGGS or pCAGGS-HGF. In contrast, c-Met expression was detected in epithelial cells of the crypts of the colons of both groups (Figures 1E and 1F). The number of c-Met-positive epithelial cells was greater in pCAGGS-gene transferred mice, and stronger expression was detected in the epithelial cells of the apical side of the crypt.

HGF gene transfer protected mice from DSS colitis

To determine the effect of HGF gene transfer on colon injury, we injected 10 µg of pCAGGS-HGF or pCAGGS into the tail veins of mice before DSS administration. Mice injected with control vector pCAGGS showed 10–20% weight loss, together with diarrhea, and gross bleeding; however, pCAGGS-HGF injection ameliorated clinical disease severity from day 1 to day 4 (Figure 2A). The decrease in colon length reflects the extent of colon damage in the model, and it was minimal in mice with HGF gene transfer at days 2, 5, and 6 compared with the mice injected with control vector (Figure 2B).

In mice injected with pCAGGS, broad mucosal ulceration and degeneration, and inflammatory cellular infiltration were observed, and these lesions progressed with time (Figures 2C–2I). Inflammatory cells were infiltrated to the colon progressively with time in mice with DSS colitis which were injected with pCAGGS; however, pCAGGS-HGF injection significantly reduced the number of inflammatory cells in the colon of mice with DSS colitis (Figure 2J).

The crypt length was decreased in colitis mice injected with pCAGGS. In contrast, HGF gene transfer prevented the shortening of crypt in DSS colitis (Figure 2K).

The effect of HGF gene transfer on immune cell trafficking into the colon of DSS colitis

An immunofluorescence study revealed that DSS-induced mucosal inflammation was accompanied by a significant infiltration of Mac-1+ macrophages, CD4+ T cells, CD8+ T cells, and B220+ cells in the lamina propria of mice injected with the empty pCAGGS (Figures 3A–3D, 3I).The numbers of these immune cells were significantly reduced in mice after HGF gene therapy (Figures 3E–H, 3I).

The effect of HGF gene transfer on cytokine mRNA expression in the colons of mice with DSS colitis

To reveal the immune response in the colon of mice with DSS colitis, we analyzed the expression of mRNA of several cytokines. At day 2 after induction of colitis, the levels of expression of the mRNAs for proinflammatory

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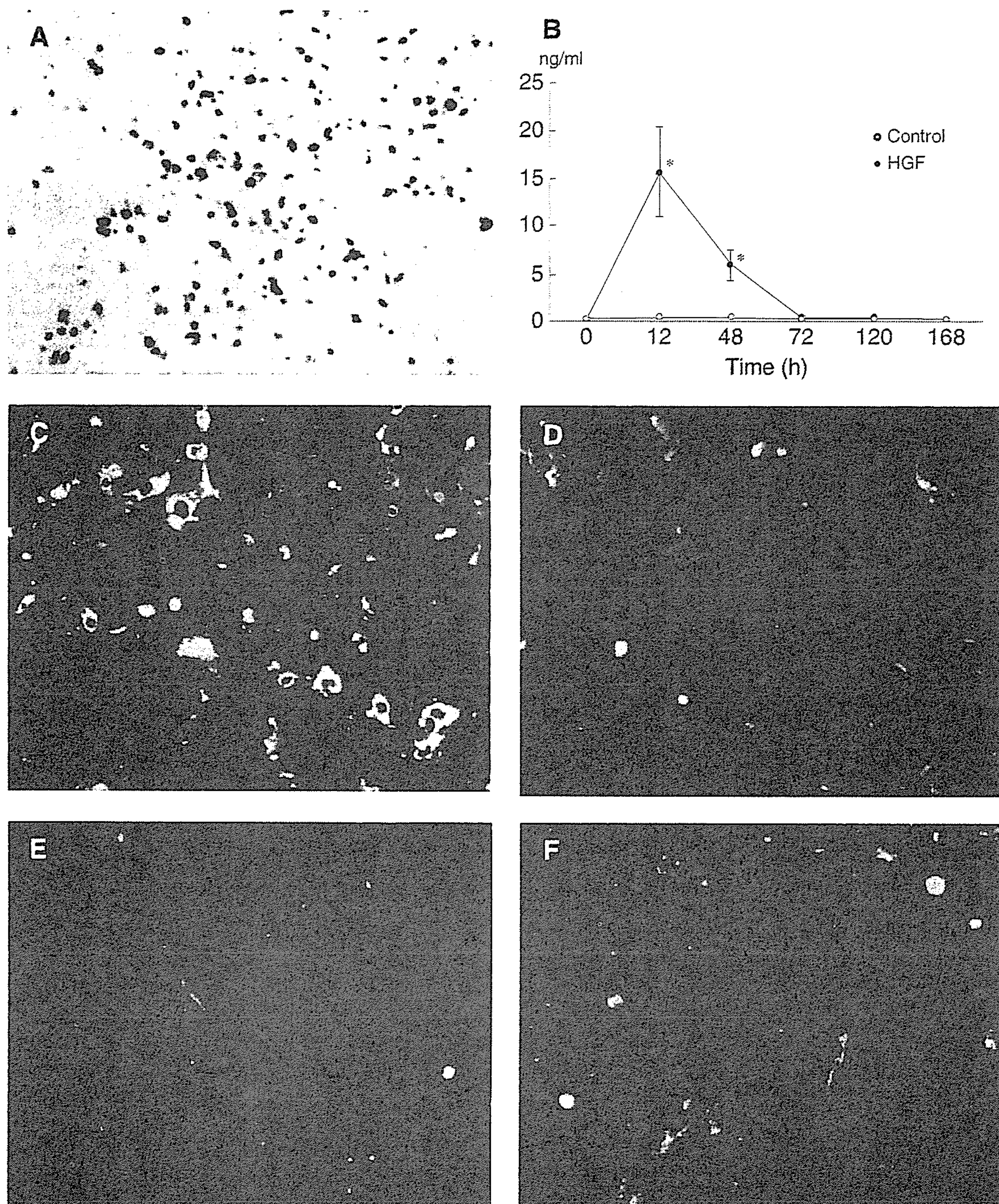

Figure 1. HGF gene transfection into the liver elevated serum HGF in mice. (A) X-gal-stained mouse liver section 1 day after injection with pCAGGS-lacZ. Original magnification: ×30. (B) Plasma levels of HGF at various time points. Blood samples were collected at 12, 48, 72, 120, and 168 h after pCAGGS-HGF injection following administration of DSS, and were detected by polyclonal HGF ELISA. Data are expressed as mean (SD), n = 10. $^*p < 0.01$ versus control. (C) HGF expression was detected by immunofluorescence in the livers of mice with DSS colitis, which were injected with pCAGGS-HGF. (D) HGF was not detected by immunofluorescence in control, pCAGGS-injected mice. (E, F) c-Met, the receptor for HGF, was detected on colonic epithelial cells of mice with DSS colitis by immunofluorescence, and its expression was elevated slightly in mice injected with pCAGGS-HGF (E) compared with pCAGGS-injected mice (F)

cytokines such as IL1-β, IL18, and IFN-γ were increased, and those of TNF-α were increased at day 6 (Figure 3J). HGF gene transfer clearly reduced the expression of mRNAs for these cytokines (Figure 3K). Expression of IL-4 mRNA was increased slightly, but there was no statistical difference between mice injected with pCAGGS and those with pCAGGS-HGF (Figure 3K). Interestingly, expression of mRNA of IL-10, which is known to be an anti-inflammatory cytokine, was increased markedly at day 6 in the colons of both groups of mice; however,

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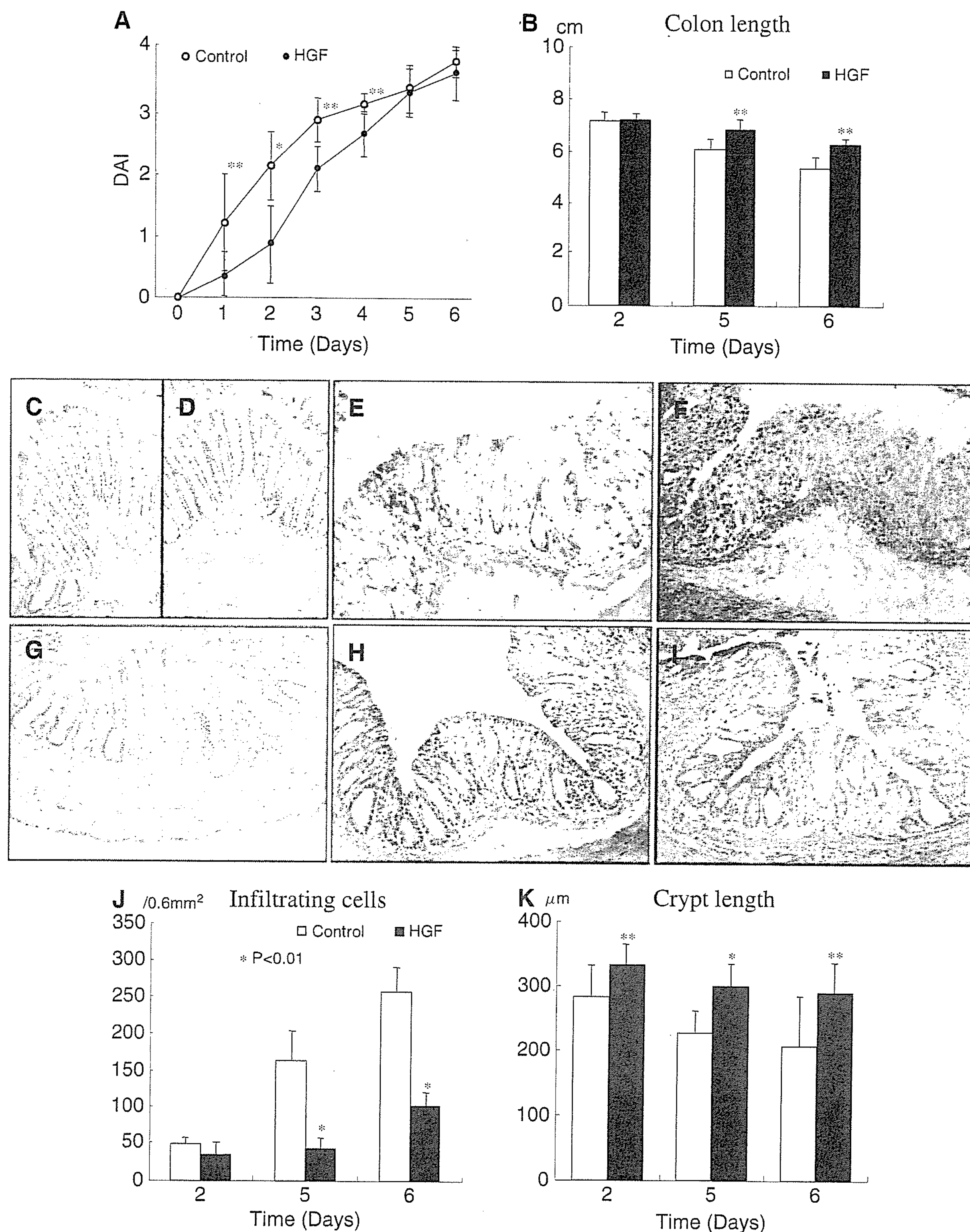

Figure 2. HGF gene transfer protected mice from acute colitis induced by DSS. (A) Disease activity index (DAI). Open circles, control pCAGGS-injected mice; closed circles, pCAGGS-HGF-injected mice. (B) Colon length, at days 2, 5, and 6. Open bars, control-pCAGGS; closed bars, pCAGGS-HGF. (C–I) Distal colon tissues: (C) from a normal mouse; (D) from pCAGGS-injected mice on day 2, (E) day 5, (F) and day 6 after DSS; and from pCAGGS-HGF-injected mice on day 2 (G), day 5 (H), and (I) day 6 after DSS. (J–K) Histological scores of colitic lesions. (J) The number of infiltrating cells in the lamina propria of the colon, at days 2, 5, and 6 after DSS. Open bars, from pCAGGS-injected mice; closed bars, pCAGGS-HGF-injected mice. (K) The crypt length of colon of mice, at days 2, 5, and 6 after DSS. Open bars, from pCAGGS-injected mice; closed bars, pCAGGS-HGF-injected mice. $^{*}p < 0.01$; $^{**}p < 0.05$

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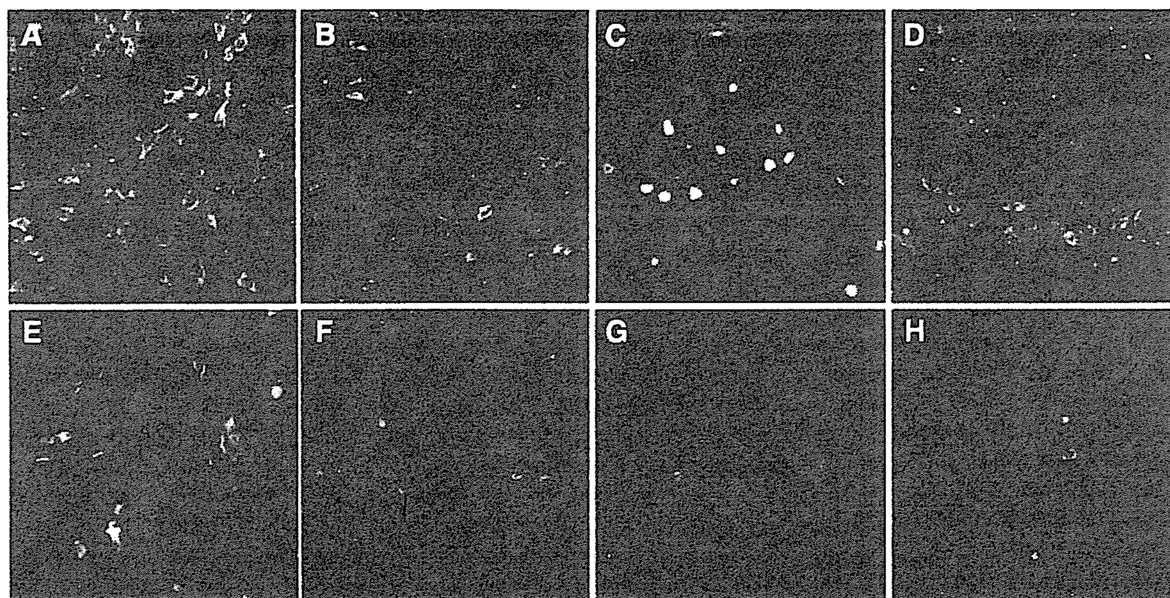

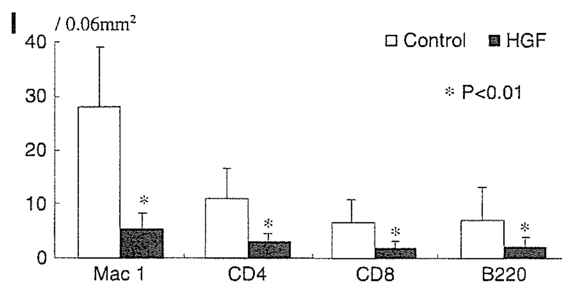

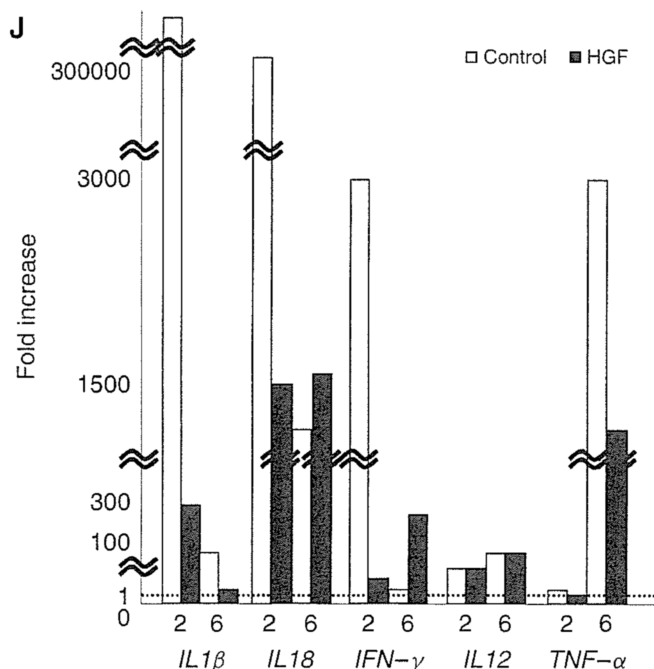

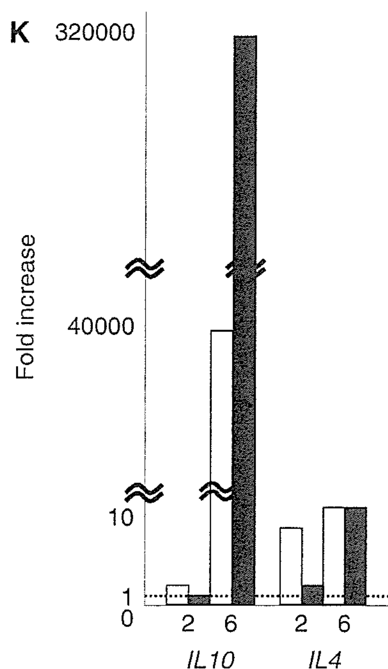

Figure 3. HGF transfection into the liver blocked immune cell traffic into the colon. (A–H) Immunostaining for Mac-1 (A, E), CD4 (B, F), CD8 (C, G), and B220 (D, H) of the distal colon tissues from pCAGGS-injected mice (A–D), and pCAGGS-HGF-injected mice (E–H). (I) Quantitative analysis of immune cells that infiltrated the colon. $^*p < 0.01$. (J, K) Real-time quantitative PCR of pro-inflammatory and anti-inflammatory cytokines mRNA expression in the colon tissue. Open bars, from pCAGGS-injected mice; closed bars, pCAGGS-HGF-injected mice. Each amount was normalized to the level of GAPDH and the final relative values were expressed relative to the calibrators on day 0

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HGF gene therapy significantly increased the expression level of IL-10 mRNA compared with the control vector (Figure 3K).

HGF gene transfer enhanced the proliferation of crypt epithelial cells in DSS colitis

We assessed the BrdU incorporation to evaluate the mitogenic effect of HGF on colonic epithelial cells in the amelioration of DSS colitis. BrdU+ cells were detected at the basal side of a crypt in the colon of mice with DSS colitis (Figures 4A and 4B); however, their numbers were much greater in mice that received HGF gene therapy than that of control vector (Figure 4C).

HGF gene transfer protected colonic mucosa from apoptosis in DSS colitis

As we have reported previously, apoptosis was observed in the colonic epithelial cells of mice with DSS colitis. HGF has an anti-apoptotic activity on several cells, and therefore we assessed the apoptotic epithelial cells in the colons of mice with DSS colitis using the terminal transferase uridyl nick endlabeling (TUNEL) method to detect DNA fragmentation *in situ*. TUNEL-positive colonic epithelial cells were observed along the crypts (Figures 4D and 4E), but their numbers were significantly reduced in mice that were transferred the HGF gene in comparison with that in control vector transferred mice (Figures 4D–4F).

Repeated injection of pCAGGS-HGF maintained peak levels of serum HGF longer and enabled a better cure of DSS colitis by facilitating regeneration and inhibiting apoptosis of crypt epithelial cells

Although HGF gene therapy could ameliorate acute colitis as described above, the benefit of the therapy is limited. The level of serum HGF is elevated within 3 days of a single injection of pCAGGS-HGF, and we speculated that maintenance of the elevated HGF level could induce a better therapeutic effect on colitis. We next injected pCAGGS-HGF twice, and the serum HGF level reached a peak at 12 h after the first gene injection, and decreased slightly, but was maintained at a level of 8 ng/ml during day 2 and day 5 after a second administration, 48 h after the first injection (Figure 5).

Clinical scores such as the DAI and the colon length of mice with DSS colitis were significantly ameliorated with the repeated HGF gene transfer compared to a single time administration (Figures 6A and B). Histologically, repeated injection of the HGF gene ameliorated the colitic lesions better than a single injection (Figures 6C–6F).

The numbers of BrdU-positive epithelial cells in the crypts of the colons were greater in mice injected with HGF twice than that of single injection (Figures 7A–7C). The

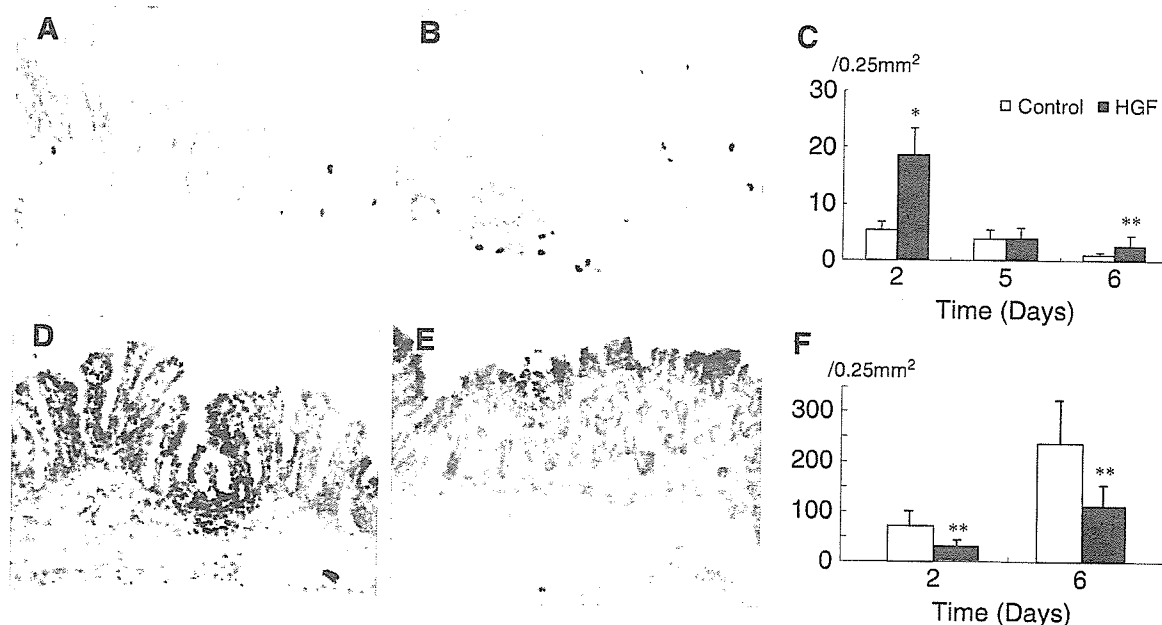

Figure 4. HGF gene transfection into the liver promoted proliferation and inhibited apoptosis of epithelial cells of the colon. (A–C) BrdU staining of distal-colon tissues from pCAGGS-injected mice (A), and pCAGGS-HGF-injected mice (B) at day 2 after DSS administration. The number of BrdU+ colonic epithelial cells (C). Open bars, from pCAGGS-injected mice; closed bars, pCAGGS-HGF-injected mice. (D–F) TUNEL staining of the distal colon tissues from pCAGGS-injected mice (D), and pCAGGS-HGF-injected mice (E) at day 2 after DSS administration. The number of TUNEL-positive apoptotic colonic epithelial cells (F). Open bars, from pCAGGS-injected mice; closed bars, pCAGGS-HGF-injected mice. *$p < 0.01$; **$p < 0.05$

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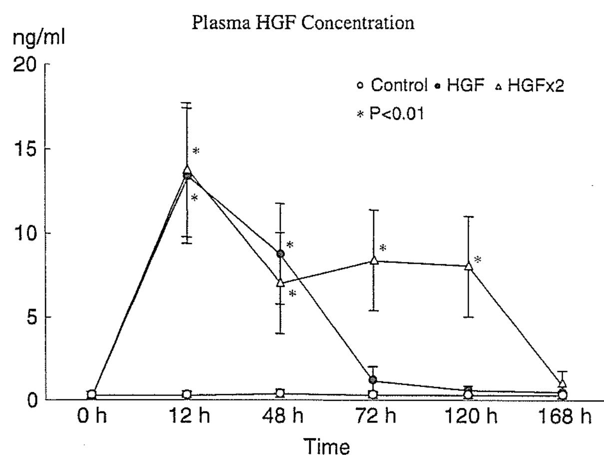

Figure 5. Repeated injection of pCAGGS-HGF kept the elevated level of plasma HGF. Plasma levels of HGF at various time points. Blood samples were collected at 12, 48, 72, 120, and 168 h after the first pCAGGS-HGF injection, and were detected by polyclonal HGF ELISA. pCAGGS-HGF was injected at 0 and 48 h after DSS administration. Open circles, pCAGGS-injected mice; closed circles, pCAGGS-HGF-injected once on day 0; open triangles, pCAGGS-HGF was injected on days 0 and 2 after DSS administration. Data are expressed as mean (SD), n = 10. $*p < 0.05$ versus control

number of TUNEL-positive apoptotic colonic epithelial cells was also reduced markedly in mice injected twice compared with mice with a single injection (Figures 7D–F).

The effect of HGF on cytokine mRNA expression in the colonic explants of mice with DSS colitis

To reveal the direct effect of HGF on immune response in the colon, we analyzed the expression of mRNA of IFN-γ and IL-10 in the colonic explants of mice with DSS colitis. In explants from mice with DSS colitis at days 2 and 5, the levels of expression of the mRNA of IFN-γ, a pro-inflammatory cytokine, was increased (Figure 8). The expression of mRNA of IFN-γ was clearly reduced by the treatment with HGF on explants (Figure 8). Expression of mRNA of IL-10, which is a known anti-inflammatory cytokine, was increased markedly at days 2 and 5 in the *ex vivo* colon tissue cultured with HGF (Figure 8). Interestingly, the expression of mRNAs for intrinsic mouse HGF and c-Met was clearly increased in explants cultured with HGF (Figure 8).

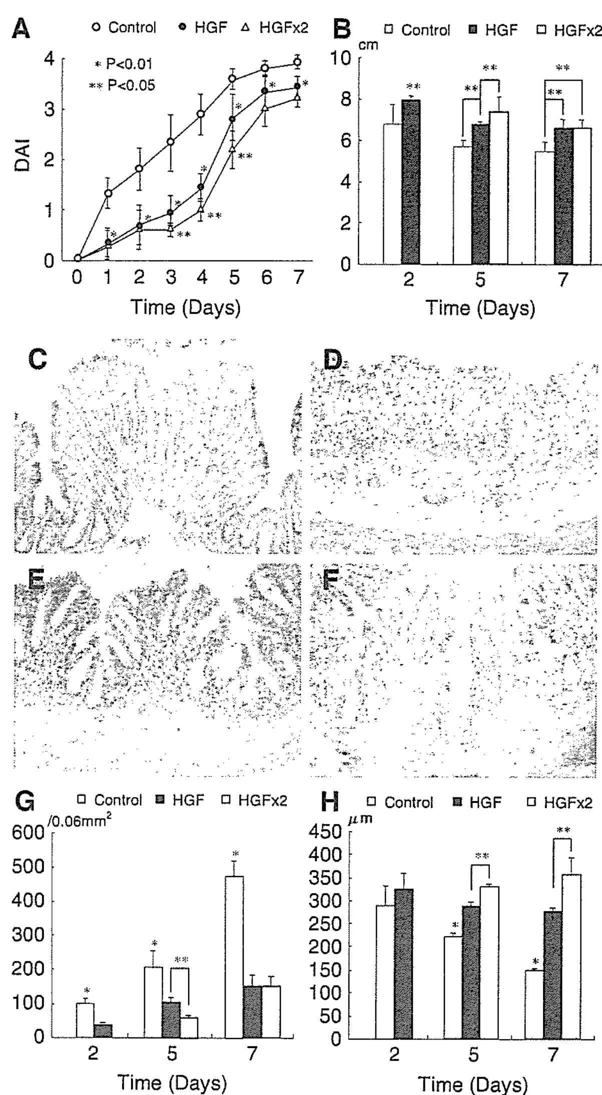

Figure 6. Repeated HGF gene transfer protected mice better from acute colitis induced by DSS. (A) Disease activity index (DAI). Open circles, control pCAGGS-injected mice; closed circles, pCAGGS-HGF-injected mice; open triangles, pCAGGS-HGF was injected on days 0 and 2 after DSS administration. (B) Colon length, at days 2, 5, and 7. Open bars, control pCAGGS; closed bars, pCAGGS-HGF; gray bars, pCAGGS-HGF was repeatedly injected on day 0 and day 2 after DSS administration. (C–F) Distal colon tissues: from a normal mouse (C); from pCAGGS-injected mice on day 2 after DSS (D); from mice with pCAGGS-HGF injection once on day 0 (E); and from mice with pCAGGS-HGF injections on days 0 and 2 (F). (G, H) Histological scores of colitic lesions. (G) The numbers of infiltrating cells in the lamina propria of the colon on day 6 after DSS. Open bars, control-pCAGGS; closed bars, pCAGGS-HGF; gray bars, pCAGGS-HGF was injected on day 0 and day 2 after DSS administration. (H) The crypt length of colons of mice on days 2, 5, and 6 after DSS. Open bars, control-pCAGGS; closed bars, pCAGGS-HGF; gray bars, pCAGGS-HGF was injected on days 0 and 2 after DSS administration. $*p < 0.01$; $**p < 0.05$

Discussion

There are various gene delivery systems, and the most intensely studied methods utilize viral vectors. There are concerns about the possibility of recombination with endogenous virus to produce a deleteriously infectious form. It has also been reported that T-cell leukemia developed nearly 3 years after gene therapy for SCID using a defective retroviral vector [27,28]. Thus, there are several hurdles to overcome to enable the clinical application of gene therapy with viral vectors.

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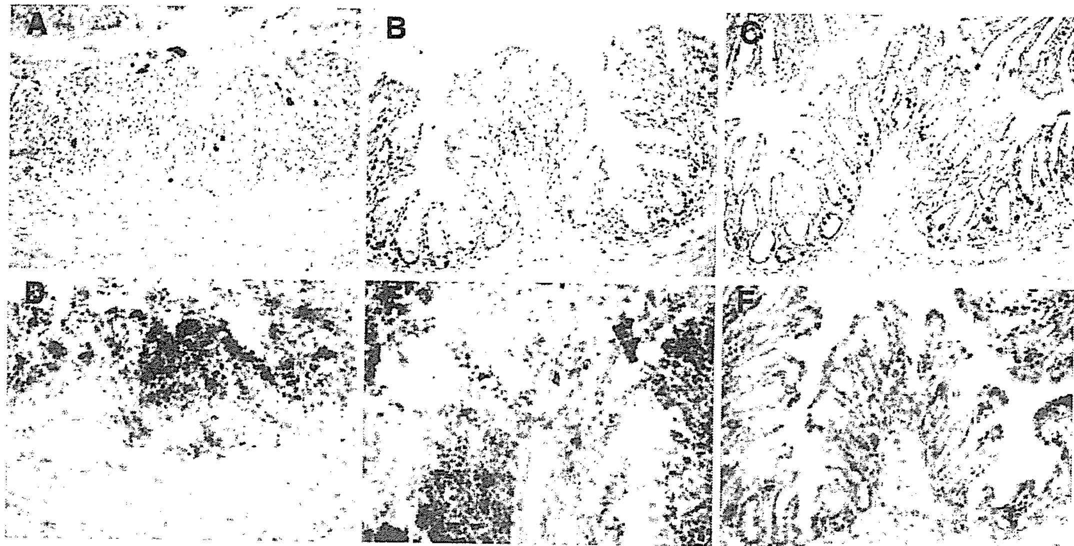

Figure 7. Repeated HGF gene transfection in the liver increasingly promoted proliferation and anti-apoptosis of epithelial cells of the colon. (A–C) BrdU staining of distal-colon tissues from a control pCAGGS-injected mouse (A), from a pCAGGS-HGF mouse injected once (B), and from a pCAGGS-HGF mouse injected twice (C). (D–F) TUNEL staining of the distal-colon tissues from a pCAGGS-injected mouse (D), from a pCAGGS-HGF mouse injected once (E), and from a pCAGGS-HGF mouse injected twice (F)

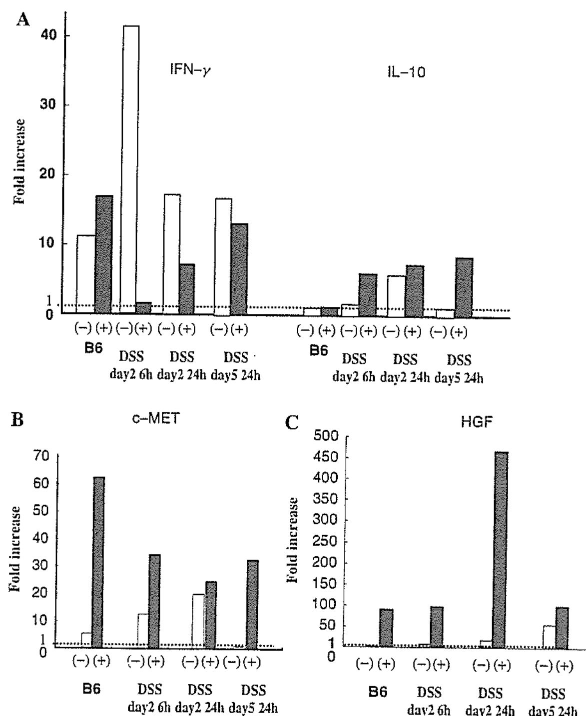

Figure 8. HGF directly regulated the expression of mRNA with decrease for IFN-γ and increase for IL-10 in *ex vivo* colonic tissue culture of mice with DSS colitis. Real-time quantitative PCR of mRNA expression for pro-inflammatory IFN-γ (A), anti-inflammatory IL-10 (A), c-Met (B), and mouse endogenous HGF (C) in the *ex vivo* colon tissue cultured with HGF. The colonic explant samples were taken from mice with DSS colitis on day 2 and day 5, or from those without DSS colitis. The explants were cultured in an organ culture dish for 6 h and 24 h. Open bars, from pCAGGS-injected mice; closed bars, pCAGGS-HGF-injected mice. Each amount was normalized to the level of GAPDH and the final relative values were expressed relative to the calibrators on day 0

Among the alternative methods, naked DNA-mediated gene transfer is the simplest, and techniques for introducing naked DNA into hepatocytes have been the most intensely studied methods for generating therapeutic amounts of gene product [23,24]. Liver-targeted gene transfer is an important tool for expanding the treatment options for diseases of several organs, as well as the liver, because the liver has a great capacity to synthesize serum proteins and is involved in numerous genetic and acquired diseases [29,30]. In this study, we used hydrodynamics-based gene transfer by tail vein injection to transfect the rat HGF gene into hepatocytes to over-express HGF in serum [31–33]. An important feature of the method is that it can achieve liver-targeted gene transfer with naked DNA that is driven by strong, non-tissue-specific, viral promoters, such as the CAG promoter [34].We could effectively transfect the HGF gene into the liver (Figure 1), and induce an ideal elevation of serum HGF levels using this method (Figures 1 and 5). Over-expression of HGF by gene transfer into the liver is an effective method for HGF production, reaching the target organ, the colon, and acting as a drug to ameliorate DSS colitis (Figure 2).

Liver-targeted gene transfer by tail vein injection has a high efficacy, but results in an accelerated decline in gene expression levels (Figure 1) compared with transfer into the muscles of mice by electroporation of the HGF gene *in vivo*. One of the mechanisms of this decline might be an immune response by the mice to the foreign protein, rat HGF. To solve this problem, we injected naked DNA twice, and succeeded in maintaining an elevated serum level of HGF (Figure 5). However, prolonged serum elevation of HGF might have unpredicted side effects on the body such as carcinogenesis [35]. Therefore, we should search for a local method of induction of naked DNA into the intestine, which could exert its therapeutic effects for the

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treatment of IBD only in the colon, without increasing the serum HGF level.

This study demonstrated that (1) pretreatment with HGF gene therapy ameliorated the clinical symptoms of DSS colitis; (2) over-expressed HGF in serum ameliorated DSS colitis lesions, associated with an increase of BrdU-positive crypt epithelial cells, and decrease of apoptotic epithelial cells in the colonic crypts of DSS mice; (3) elevated serum HGF inhibited immune cell trafficking into the colon, and suppressed the pro-inflammatory response of DSS colitis; (4) these therapeutic effects of HGF depended on the duration of elevated serum HGF levels; and (5) HGF directly regulated the expressions of mRNA of IL-10 and IFN-γ in colonic explants from mice with DSS colitis.

HGF modulates intestinal epithelial proliferation and migration, serving as a critical regulator of intestinal wound healing, and several studies have reported that this growth factor is useful for the treatment of inflammatory bowel disease. Tahara *et al.* reported that administration of recombinant human HGF released by intraperitoneally transplanted osmotic pumps ameliorated DSS colitis in the rat [20]. Arthur *et al.* also reported that HGF administration by the same method ameliorated diarrhea and colitis in HLA-B27 transgenic rats [21]. Our gene therapy with HGF ameliorated DSS colitis clinically and pathologically through elevation of serum HGF (Figure 2). This could be an alternative drug-delivery method for recombinant HGF treatment of inflammatory bowel disease.

Epithelial repair is immediately accompanied by an immediate inflammatory reaction, and epithelial stem cells are induced to enter DNA synthesis as a result of wounding. HGF has been found to stimulate epithelial proliferation in the lung, stomach, and liver as well as the intestine. We have shown here that HGF gene transfer increased the numbers of BrdU-positive epithelial cells following acute DSS-induced colon injury. This might have caused rapid epithelial regeneration, as demonstrated by histological analysis showing typical regenerative changes and by higher number of surviving crypts compared with untreated mice (Figures 2 and 4).

In addition to its mitogenic activity, HGF has anti-apoptotic properties, and several reports revealed that recombinant HGF therapy ameliorated experimental colitis partially by inhibiting apoptosis of colonic epithelial cells. In this study HGF gene therapy decreased TUNEL-positive colonic epithelial cells in DSS colitis (Figure 4).

In the majority of tissues, HGF is secreted from mesenchymal cells, such as fibroblasts, and its receptor, c-Met, is found typically on epithelial cells. In our study, weak c-Met expression was observed in the crypt epithelial cells in DSS colitis, in both the pCAGGS-injected and pCAGGS-HGF-injected groups (Figure 1). Expression of c-Met mRNA was higher in the DSS colitis group injected with pCAGGS and marginal in the DSS colitis group injected with pCAGGS-HGF (unpublished observation). In the murine model of TNBS colitis, HGF gene transfer into the muscle showed an increased c-Met expression in the colon of untreated mice than those of treated mice, but phosphorylated c-Met expression was stronger in the colon of mice after HGF gene therapy than in untreated mice [36]. We are now investigating the expression of c-Met and its phosphorylation in the colons of mice with HGF gene therapy.

This study demonstrated that HGF gene therapy reduced inflammatory cell trafficking into the colon and suppressed the expression of inflammatory cytokines (Figure 3). In DSS colitis, the first insult of colonic injury is epithelial damage by DSS, resulting in destruction of the gut barrier and influx of intestinal bacterial toxins, and then the inflammatory cascade follows. HGF exerts its mitogenic and anti-apoptotic activity primarily, and thereby restores the gut barrier, leading to a reduction in the infiltration of inflammatory cells and the production of inflammatory cytokines [37,38]. Thus, the anti-inflammatory effect of HGF on DSS colitis might be the secondary effect of HGF in amelioration of DSS colitis. Interestingly, this study showed that expression of the mRNA of the anti-inflammatory cytokine IL-10 is increased markedly in mice with DSS colitis and injected with pCAGGS-HGF (Figure 3). We also observed that the numbers of IL-10-positive cells were not changed in the colons of mice with DSS colitis after HGF gene therapy (unpublished observation). These results might suggest that HGF augments the anti-inflammatory response in the inflamed colon, not by secondary effect but by its own primary effect. To address the point, we performed *ex vivo* organ culture experiments, which revealed the direct effect of HGF on immune response in the colon. We analyzed the expression of mRNA of IFN-γ and IL-10 in the colonic explants of mice with DSS colitis. In the colonic explants of mice with DSS colitis, the levels of expression of the mRNA for the pro-inflammatory cytokine IFN-γ were increased (Figure 8). The expression of mRNA for IFN-γ was clearly reduced by the treatment with HGF on explants (Figure 8), whereas expression of mRNA of IL-10 was increased markedly in the *ex vivo* colon tissue cultured with HGF (Figure 8). Thus, it is likely that HGF directly regulates the transcription of pro-inflammatory and anti-inflammatory cytokines in the diseased colonic tissue. Interestingly, the expression of mRNAs for intrinsic mouse HGF and c-Met was clearly increased in explants cultured with HGF (Figure 8).

However, the present study has not yet revealed the target cells of the HGF in the colon tissue, especially in the context of the anti-inflammatory effect of HGF. In a future study, we should identify the target cells of HGF and the molecular mechanism of how such regulation of cytokine expression works through the stimulation of c-Met, the receptor for HGF, using methods such as reporter gene expression assay.

Finally, our study showed that these therapeutic effects of HGF described above depended on the duration of elevated serum HGF level (Figures 5–7). As mentioned above, repeated injection of the HGF gene solved the problem of a short duration of response following a single injection (Figures 1 and 5). In our previous report, we

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showed that repeated injection of the rat Epo gene lost its efficacy of expression of protein in the serum in rats, and the mechanism remains unclear [34]. In addition to the duration of elevated serum HGF level, Arthur *et al.* reported that there is a plateau above which a further increase in HGF levels provides no added benefit in HLA-B27 transgenic rats treated with recombinant HGF [21]. Recently, Mukoyama *et al.* reported the therapeutic effect of adenoviral-mediated HGF gene administration via the intrarectal route on TNBS-induced colitis [39]. The method could transfect the gene successfully into the epithelial cells of the colon without elevation of serum HGF levels. This could avoid the side effect of overly expressed serum HGF in serum on organs other than the intestine. On the contrary, the direct transfection of the gene into epithelial cells carries the risk of carcinogenesis of the colon [35], especially considering the chronic intestinal inflammatory status of patients with inflammatory bowel disease. We must be cautious when choosing the best method for HGF gene transfer, to enable HGF to work maximally in the target organ of the intestine.

In conclusion, this study demonstrated that hydrodynamics-based transfection of the rat HGF gene into the liver via tail vein injection ameliorated DSS colitis in mice, and its effect appears to depend on the duration of elevated serum HGF level. These results suggest that HGF gene therapy could be a new therapeutic approach to inflammatory bowel disease.

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REVIEW

Novel strategies for the treatment of inflammatory bowel disease: Selective inhibition of cytokines and adhesion molecules

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Abstract

The etiology of inflammatory bowel disease (IBD) has not yet been clarified and immunosuppressive agents which non-specifically reduce inflammation and immunity have been used in the conventional therapies for IBD. Evidence indicates that a dysregulation of mucosal immunity in the gut of IBD causes an overproduction of inflammatory cytokines and trafficking of effector leukocytes into the bowel, thus leading to an uncontrolled intestinal inflammation. Such recent advances in the understanding of the pathogenesis of IBD created a recent trend of novel biological therapies which specifically inhibit the molecules involved in the inflammatory cascade. Major targets for such treatment are inflammatory cytokines and their receptors, and adhesion molecules. A chimeric anti-TNF-α monoclonal antibody, infliximab, has become a standard therapy for CD and it is also likely to be beneficial for UC. Several anti-TNF reagents have been developed but most of them seem to not be as efficacious as infliximab. A humanized anti-TNF monoclonal antibody, adalimumab may be useful for the treatment of patients who lost responsiveness or developed intolerance to infliximab. Antibodies against IL-12 p40 and IL-6 receptor could be alternative new anti-cytokine therapies for IBD. Anti-interferon-γ and anti-CD25 therapies were developed, but the benefit of these agents has not yet been established. The selective blocking of migration of leukocytes into intestine seems to be a nice approach. Antibodies against α4 integrin and α4β7 integrin showed benefit for IBD. Antisense oligonucleotide of intercellular adhesion molecule 1 (ICAM-1) may be efficacious for IBD. Clinical trials of such compounds have been either recently reported or are currently underway. In this article, we review the efficacy and safety of such novel biological therapies for IBD.

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Key words: Inflammatory bowel disease; Cytokine; Adhesion molecule; Treatment

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INTRODUCTION

Although the etiology of inflammatory bowel disease (IBD), such as, Crohn's disease (CD) and ulcerative colitis (UC), has not yet been fully addressed, there has been remarkable progress in the understanding of this field in the past decade. In normal bowels, the immune reaction is sophisticatedly regulated while keeping a balance between the effectors and the regulators, and as a result, the homeostasis of the gut is maintained. A lot of evidences indicate that mucosal immunity is dysregulated in the bowel of IBD[1]. Two forms of IBD show distinct profiles of T cell mediated immunity. In the gut of CD, a strong Th1 reaction is induced, while the Th2 response is upregulated in the colon of UC[2]. Particularly in CD, it is evident that Th1 dominant immunity plays an important role in the pathogenesis. In UC, although the relevance of elevated Th2 cytokines to the colonic inflammation has not yet been clarified, an increased proinflammatory cytokine production is also observed which seems to be related to the inflammation. For the development of intestinal inflammation, leukocyte trafficking to the gut is an important step[3]. Adhesion molecules, such as integrins, mediate the selective binding between the leukocytes and the endothelial cells and thus the migration of leukocytes into the normal and inflamed intestine. The main classical medical treatments for IBD are steroids and immunosuppressive agents which non-specifically reduce immunity and inflammation. Recent advances in the understanding of the mechanism of bowel inflammation have led to a recent trend in the development of biological therapies which selectively inhibit the action of molecules essential to the inflammatory process. Major targets for such therapies are inflammatory cytokines and their receptors, and adhesion molecules. Re-

cently, numerous challenges have been performed to generate anti-cytokine and anti-integrin compounds to treat IBD. This article reviews the efficacy and safety of such novel biological therapies for IBD targeting cytokines, cytokine receptors and adhesion molecules.

INHIBITION OF TNF

TNF-α is a proinflammatory cytokine which is abundantly expressed in the gut of CD[1-6]. In animal models of experimental colitis, treatment with anti-TNF-α antibody has been shown to be effective in the suppression of intestinal inflammation[7,8]. As a result, this cytokine was considered to be an attractive target for the treatment of IBD and several anti-TNF reagents have thus been developed. These reagents include infliximab, CDP571, CDP870, etanercept, onercept and adalimumab. Infliximab is a chimeric IgG1 monoclonal antibody against TNF-α, which was created in late 1980s, and it has been demonstrated to be effective in reducing intestinal inflammation in CD as described below. Most of the other anti-TNF reagents are modified by a reduction of the mouse peptide sequence or are completely humanized in order to reduce the immunogenecity. Not all of the other anti-TNF reagents have been proven to be as effective in the treatment of CD as infliximab, and the efficacy of such reagents seems to be dependent, not only on the ability to neutralize soluble TNF-α, but also on the capacity to bind to the membrane-bound TNF-α on the cell surface, thereby mediating the apoptosis of the effector cells[9].

Infliximab

Infliximab was demonstrated to be effective in both the induction and maintenance therapy for refractory luminal and fistulizing CD. In a randomized double-blind placebo-controlled trial, 108 patients with moderate-to-severe CD which is resistant to conventional therapy, were treated with the single intravenous infusion of either placebo or infliximab at a dose of 5 mg/kg, 10 mg/kg or 20 mg/kg. The rates of the clinical response at 4 wk were 81% for infliximab 5 mg/kg, 50% for infliximab 10 mg/kg and 64% for infliximab 20 mg/kg, all of which were significantly higher than that for the placebo-treated group. The clinical remission rate at 4 wk was also significantly higher in the infliximab-treated group than that in the placebo-treated group (33% *vs* 4%)[10]. In a randomized, double blind, placebo-controlled trial for the treatment of fistulizing disease, 94 CD patients with draining abdominal and perianal fistulas refractory to conventional therapy were treated with three intravenous infusions at wk 0, 2 and 6 of either a placebo or infliximab at a dose of 5 mg/kg or 10 mg/kg. The response rates were significantly greater in the infliximab 5 mg/kg group (68%) and in the infliximab 10 mg/kg group (56%) than that in the placebo-treated group (26%). The rates of a complete closure of the fistulas were also significantly higher in the infliximab 5 mg/kg group (55%) and in the infliximab 10 mg/kg group (38%) than in the placebo-treated group (13%)[11]. The effectiveness of infliximab for the maintenance therapy for inflammatory CD was assessed in a large trial called ACCENT I. Three hundred and thirty-five responders to a single infusion of infliximab were subsequently treated with 5 mg/kg infliximab at wk 2 and 6, followed by infusions of either 5 mg/kg or 10 mg/kg infliximab once every 8 wk until wk 54, or they were treated with placebo at wk 2 and 6, and subsequently every 8 wk. The rates of clinical response and remission at wk 30 and 54 was significantly greater in both groups receiving 5 mg/kg and 10 mg/kg infliximab every 8 wk than those in the placebo-treated group[12]. In addition, an analysis comparing the scheduled and episodic treatment strategies of infliximab for CD was conducted based on the ACCENT I data. The efficacy of the scheduled therapy was better than episodic strategy in terms of CDAI score, clinical remission and response rates, improvement in IBDQ score, mucosal healing and CD-related hospitalization and surgery[13]. For an evaluation of the infliximab maintenance therapy for fistulizing CD, ACCENT II trial was conducted. One hundred and ninety-six CD patients with draining perianal and enterocutaneous fistulas who responded to the induction therapy with three infusions of 5 mg/kg infliximab at wk 0, 2 and 6 received either a placebo or 5 mg/kg infliximab every 8 wk. The median time to the loss of response, response rate and complete fistula closure rate at wk 54 in the infliximab maintenance group were significantly greater than those in the placebo group[14].

Regarding the safety of infliximab treatment, it is well tolerated in the majority of the patients. In randomized controlled clinical trials, the rates of adverse events occurring in infliximab-treated patients were comparable to those in placebo-treated patients[10-12,14]. Serious side effects, however, have been reported and attention must be paid to the possible occurrence of serious infections and autoimmune disorders, as well as the theoretical threat of cancer and lymphoma. In an analysis of 500 infliximab-treated patients in Mayo Clinic, serious adverse events were observed in 8.6%, of which 6% was considered to possibly be related to infliximab[15]. Such events included serious infections, severe infusion reactions, serum sickness-like reactions, drug-induced lupus, cancer, non-Hodgkin's lymphoma and demyelinating process. The infectious complications included fatal sepsis, pneumonia, viral gastroenteritis, abdominal abscesses requiring surgery and histoplasmosis. Five deaths (1%) were observed which were likely or possibly related to infliximab. The reactivation of latent tuberculosis has been reported elsewhere[16], as a result, it is recommended that all patients be screened for latent tuberculosis before the initiation of this treatment regimen.

Infliximab is a mouse/human chimeric monoclonal antibody of which 25% is mouse peptide sequence. The murine component is ascribed to its immunogenecity, such as infusion-related reactions and serum sickness-like diseases. In such immunological reactions, the formation of antibodies against infliximab, called human anti-chimeric antibodies (HACA) is of particular concern as the presence of HACA is associated with an increased frequency of infusion reactions and the reduction in efficacy[17]. Concomitant immunosuppressive therapy and premedication with 200 mg of hydrocortisone reduce the frequency of HACA formation[17,18]. The scheduled infusions in the maintenance therapy have been shown to be associated

with the reduction of the rate of HACA formation[13]. As a result, the regular infliximab-treatment every 8 wk is likely to be beneficial for CD patients, not only for the maintenance of the remission state but also for the avoidance of infusion reactions.

The efficacy of infliximab for the treatment of UC remains controversial as two randomized controlled trials for steroid-refractory UC resulted in opposite results. A study indicated the benefit of infliximab for UC since 50% (4 of 8 patients) of the infliximab-treated patients showed treatment success, while none of the 3 patients receiving a placebo showed response[19]. However, this study was terminated prematurely because of a slow enrollment. Another study failed to show any benefit of infliximab over placebo as there was no significant difference between patients who received infliximab and placebo in the remission rates and an improvement in the activity scores[20]. Recently, two large multicenter randomized trials, ACT1 and ACT2, have been performed. In both trials, 364 patients with active UC were randomized to receive placebo or infliximab in a dose of 5 mg/kg or 10 mg/kg at wk 0, 2, 6, 14 and 22 in ACT1, and at wk 0, 2 and 6 then every 8 wk through wk 46 in ACT2. In both trials, both 5 mg/kg and 10 mg/kg infliximab showed significantly greater percentages in both the induction and maintenance of clinical remission and response, and in mucosal healing than placebo at both wk 8 and 30[21,22]. In addition, it was also recently demonstrated that infliximab is effective as a rescue therapy to avoid a colectomy or death in severe to moderately severe UC refractory to conventional therapies[23]. As a result, infliximab thus appears to also be efficacious for the treatment of UC as well as for CD.

CDP571

CDP571 is a "humanized" IgG4 antibody against TNF-α, created by genetic engineering to replace the murine component other than the binding domain with parts of a human IgG4 molecule. The resulting molecule is a chimera of 95% human and 5% mouse residues. The first study of 31 patients with active CD demonstrated that CDP571 5 mg/kg resulted in a greater decrease in the mean CDAI score at wk 2 compared with placebo[24]. After a promising pilot trial, CDP571 was tested in a placebo-controlled dose-finding trial[25]. In this study, 169 patients were randomized to receive a single intravenous infusion of either CDP571 in a dose of 10 or 20 mg/kg, or placebo. At wk 2, the clinical response rate was significantly higher in the patients treated with CDP571 (45%) than in those received a placebo (27%). Re-treatment was performed either every 8 wk with a placebo or CDP571 10 mg/kg, or every 12 wk with a placebo or CDP571 10 mg/kg (4 groups). The clinical remission rates at wk 24 in CDP571-treated groups were not significantly different from those of the placebo-treated groups. In a subsequent, randomized, double-blind, placebo-controlled, multicenter study[26], the efficacy and tolerability of CDP571 in 396 patients with active CD was evaluated. Among the patients treated with CDP571 10 mg/kg every 8 wk, the percentage of patients achieving a clinical response was significantly higher than in those receiving a placebo at wk 2 and 4. However, at wk 28 the difference was not statistically significant. As a result, CDP571 therapy showed a short term benefit in induction therapy, but it is not sufficient to maintain a long term effect. In a post-hoc exploratory analysis of a subgroup of patients with elevated baseline CRP levels, there was a significant difference in the number of patients showing a clinical response at wk 2 (CDP571, 49.5%; placebo, 15.5%), and at all time points from wk 12 to wk 28, thus leaving the possibility that CDP571 is more efficacious in a selected group of patients[26]. CDP571 failed to show a steroid sparing effect in patients with steroid-dependent CD[27]. CDP571 was well tolerated even in patients with CD who developed either infusion reactions or delayed-type hypersensitivity reactions to infliximab[28]. From these results, CDP571 was considered to be safe but not as effective as infliximab for CD and further clinical development of this antibody for the treatment of CD has thus been discontinued.

CDP870

CDP870 is a pegylated Fab fragment of humanized anti-TNF monoclonal antibody. In a placebo-controlled dose-finding study[29], 292 patients were randomized to receive a subcutaneous dose of CDP870 (100, 200, or 400 mg) or placebo at wk 0, 4, and 8. The group that received CDP870 400 mg showed greater clinical response rates than other groups at all time points. The clinical response rates of the CDP870 400 mg group were significantly higher than those of the placebo treated group at wk 2, 4, 8 and 10. The difference, however, did not reach statistical significance at wk 12. A greater dose separation was evident in the analysis of a patient subgroup with elevated CRP levels. In an exploratory analysis[30] in 119 patients with increased CRP levels (≥ 10 mg/L), the differences in the clinical response between the 400 mg/dose (53.1%) and placebo (17.9%) were significant at 12 wk. These studies, therefore, indicated that CDP870 may be more effective in patients with elevated CRP levels. CDP870 seems to be safer and less immunogenic than infliximab. The efficacy, however, is likely to be lower than that of infliximab. The question arises whether physicians want to compromise on efficacy in the scope of better long-term safety. Further clinical trials are ongoing.

Etanercept

Etanercept is a genetically engineered fusion protein consisting of two recombinant human TNF p75 receptors linked to an Fc portion of human IgG1 fragment. The subcutaneous injection of etanercept at a dose of 25 mg twice weekly, which is an effective dose for rheumatoid arthritis, is a safe but ineffective dose for the treatment of patients with moderate to severe CD[31].

Onercept

Onercept, a recombinant, fully human, soluble p55 TNF receptor has showed efficacy to CD in an open-label pilot study (n = 12)[32]. A large, placebo-controlled, dose-finding study has been completed but the data have not yet been published. A press release by Serono (Geneve, Switzerland) revealed that the primary endpoint of this trial was not met.