２つの方法について検討した（図３）。前者の方法では、FNP **4** とそれぞれの scFv を混合し、超遠心分離を行って FNP 沈殿画分を回収し、S1TSCFR3-1 を固定化した scFv-FNP **4a** と S1TA3 を固定化した scFv-FNP **4b** を得た。一方、後者の方法では、FNP に **5** または **6** を用い、塩化ニッケルで処理後、それぞれの scFv を作用させ、S1TSCFR3-1 を固定化した scFv-FNP **5a**、**6a** と S1TA3 を固定化した scFv-FNP **5b**、**6b** を得た。scFv の固定化は、SDS-PAGE、ウェスタンブロッティングを行って、沈殿画分に scFv 由来のバンドが見られることで確認した（図４）。

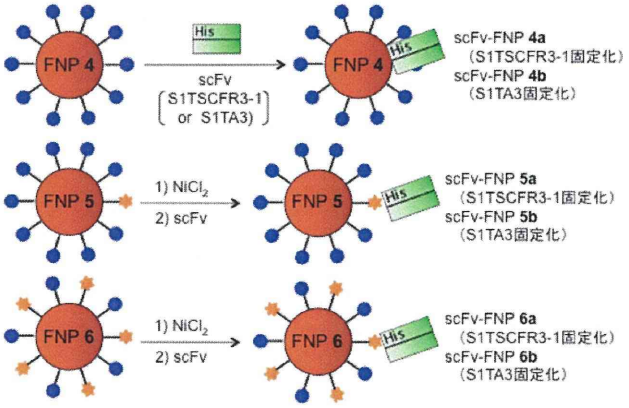

図３． scFv-FNP の調製

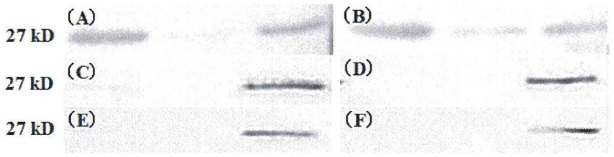

図４．調製した scFv-FNP のウェスタンブロッティング．(A) scFv-FNP **4a**、(B) scFv-FNP **4b**、(C) scFv-FNP **5a**、(D) scFv-FNP **5b**、(E) scFv-FNP **6a**、(F) scFv-FNP **6b**．左から上清画分１、上清画分２、沈殿画分．

1-4 scFv-FNP の細胞結合性解析

ATL 細胞への scFv-FNP の結合性はフローサイトメトリー（FACS）と共焦点顕微鏡による細胞の蛍光観察により解析した（図５）。細胞は ATL 細胞株である S1T 細胞および非 ATL 細胞株である CEM 細胞を使用した。遠心分離で細胞画分を回収し懸濁した後、scFv-FNP を加えて 4 ℃ で 30 分インキュベートした。遠心分離後、PBS で洗浄して FACS または蛍光観察により解析を行った。

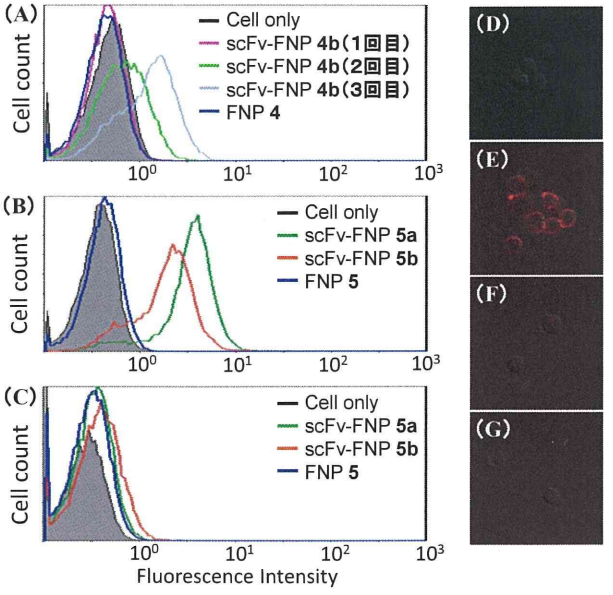

図５． FACS 解析．(A) S1T 細胞に対する scFv-FNP **4b** の結合性、(B) S1T 細胞に対する scFv-FNP **5a**、**5b** の結合性、(C) CEM 細胞に対する scFv-FNP **5a**、**5b** の結合性．共焦点顕微鏡解析による細胞観察（オーバーレイ画像）．Ex. 488 nm ; (D) S1T 細胞、(E) scFv-FNP **5b** を加えた S1T 細胞、(F) CEM 細胞、(G) scFv-FNP **5b** を加えた CEM 細胞．

C　研究結果および、
D　考察

半導体金属に直接 His タグの親和性で固定化した scFv-FNP **4b** の S1T 細胞に対する結合性は、細胞への結合性において再現性が得られなかった（図５A）。scFv-FNP **4a** も同様であった。この結果から、FNP 上の scFv は非特異的に脱着していると考えられる。一方、NTA、ニッケル、His タグを介して固定化した scFv-FNP **5a**、**5b** では、FACS 解析と共焦点顕微鏡による蛍光観察により、再現性よく、S1T 細胞に結合性を示し（図５B、E）、非 ATL 細胞である CEM 細胞には結合性を示さなかった（図５C、G）。このことから、NTA-ニッケル-His タグ結合を介することによって、強固に FNP 上に固定化されており、また、scFv の方向性も規定されて抗原を認識できていると考えられる。

E　結論

本研究で調製した NTA-ニッケル-His タグ結合を介した scFv-FNP は ATL 細胞に特異的に結合した。今後、低毒性の金属で構成されたナノ粒子を用いた scFv-FNP の開発と、これらを用いた ATL 診断薬・プローブとして応用を図る。

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H　知的財産権の出願・登録状況

特許出願

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特許取得

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厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

幹細胞生物学での技術開発と解析

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《研究要旨》
がん幹細胞を標的にした遺伝子治療の実現化には、日本人由来のがん幹細胞株を用いてその効果を最終確認する必要がある。しかし、安定したがん幹細胞株の樹立はいまだに困難であり、現在までに脳腫瘍患者組織から樹立され安定に維持されているものは少ない。このような困難を克服するために、腫瘍細胞のモデルにもなり得るヒト iPS を多くの日本人から樹立することを目指し現在までに約３００人分の親知らず由来の歯髄細胞を培養し、凍結保存してきた。この細胞を用いて１０以上の日本人細胞から iPS 細胞を安定して樹立することに成功した。また、iPS 細胞の樹立効率の個人差を基に、新規リプログラミング因子を発見した。

A.研究目的

小賊らにより開発が進められているがん幹細胞を標的にした遺伝子治療の実現化には、日本人由来のがん幹細胞株を用いてその効果を最終確認する必要がある。我々はこれまでの本事業の成果として日本人の悪性脳腫瘍組織から自己複製能を有し、二次腫瘍を形成する元となるがん腫幹細胞を単離した。しかし、安定したがん幹細胞株の樹立は困難であり、現在までに 40 以上の脳腫瘍患者組織から３株のみが樹立後も安定に維持されているにすぎない。このような困難を克服するために、腫瘍細胞のモデルにもなり得るヒト iPS を多くの日本人から樹立することを目指した。様々な遺伝的背景を持つ日本人由来 iPS からがん幹細胞を誘導することで、治療に最適なモデル系が提供できる。

B.研究方法

岐阜大学医学部では、2005 年より、若年者を中心に親知らずの歯髄幹細胞(DPSC)を収集している。2013年12月までに、約３００人分の細胞を培養し、凍結保存した。その中で、iPS細胞誘導に関する同意が得られた２００人について、山中因子を発現するレトロウイルスベクターあるいは細胞のゲノムに組み込まれにくいプラスミドベクターを用いてiPS細胞を誘導した（現在１５人分程度）。その中には、日本人人口の約２０％以上に移植可能であると予測されている主要HLAローカスホモ細胞が３株含まれている。さらに、これまでのiPS細胞の誘導実験のデータを調べ、歯が完成する20歳前後を境に、iPS細胞誘導効率に差があることが確認され、若年齢層（CCおよびRFステージ）と成人層（RCステージ）の親知らずから得られた複数の細胞ラインを用いて遺伝子発現比較をおこない、若年齢層で高発現している遺伝子をunpaired t-testによって、統計学的に抽出した。これらの遺伝子はiPS細胞の誘導に関わる新規遺伝子である可能性があり、既知のリプログラミング因子とともに歯髄細胞に導入して確認した。

C.研究結果

歯髄細胞に導入したところ、そのうちのひとつDLX4をOCT3/4, SOX2とともに歯髄細胞に導入したところ（OSD）、c-MYC、KLF4無しでもiPS細胞が誘導できた。また、KLF4を加えることによって、さらなる誘導効率の上昇が観察された。また、遺伝子以外に、リプログラミング処理中の酸素濃度がiPS細胞の誘導効率に大きく影響することをDPSCを用いて明らかにし、国際誌に発表した。

市販の無血清培地をスクリーニングして、LONZA者のMSCGM-CDが、歯髄細胞培養に利用できることを明らかにした。そこで、無血清で樹立した歯髄細胞から、ゲノムへの組み込みとそれに伴う発がんリスクの少ないセンダイウイルスベクターを利用したCytoTune-iPS(DNAVEC)を用いて、iPS細胞を誘導し

たところ、充分な数のiPS細胞クローンを得られ、さらにこれらのiPS細胞は従来の方法と遜色ない分化能力を示した。

D.考察

抜歯された親知らずは、医療機関において医療廃棄物として処分されている。岐阜大学において収集されたヒトDPSC は特に再生治療に有用なHLAハプロタイプホモDPSCを含めて遺伝子改変を伴わない誘導法によってiPS 細胞に誘導可能であることが確認され、iPS細胞を用いた再生医療の実用化に不可欠なiPS細胞バンクの構築をおこなう際の有用なリソースとなることが示された。

iPS細胞は生体に移植するとテラトーマ（異形腫）を形成するという意味ではがん細胞であり、事実iPS細胞に由来するクローン動物には様々な頻度で悪性腫瘍が出現する。今後、再生医療にも治療可能な日本人由来のiPS細胞を、HLA等を指標に個人別に分類し、将来確立される可能性が高いiPS細胞からの確実な腫瘍幹細胞誘導法を利用して、腫瘍幹細胞の治療を初めとした様々な治療モデルを確立することが可能であろう。

さらに、m-CRAベクターの有効性や安全性を患者個人の様々な細胞を用いて検討する際の細胞のソースとして患者と同様の遺伝的バックグラウンドを持つiPS細胞から分化誘導した細胞を用いることで，m-CRAベクターを用いたガンの遺伝子治療に直接貢献することが可能であろう。

E.結論

再生治療に有用なHLAハプロタイプホモDPSCを含めて、DPSCがゲノム遺伝子への組み込みを伴わない誘導法によって従来の方法で誘導されたものと同等のiPS 細胞に誘導可能であることが確認され、iPS細胞を用いた再生医療の実用化に不可欠なiPS細胞バンクの構築をおこなう際の有用なリソースとなることが示された。

iPS 細胞の誘導効率の個体差を担う因子の発見は iPS 細胞の効率的な樹立に役立つとともに、再生能力の個体差を説明し、克服する有力な手段に発展する可能性がある。

m-CRA ベクターの有効性や安全性を患者個人の様々な細胞を用いて検討する際の細胞のソースとして、患者自信の歯髄細胞から誘導した iPS 細胞とともに、我々の細胞コレクションから選別した患者と同様の遺伝的背景を持つ他人の iPS 細胞から分化誘導した細胞を用いることも、m-CRA ベクターを用いたガンの遺伝子治療に貢献すると考えられる。

F．健康危険情報

G．研究発表

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H．知的財産権の出願・登録状況（予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

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分担研究報告書

人工グリオーマ幹細胞を用いた癌幹細胞制御機構の解明

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《研究要旨》
自己複製能、腫瘍形成能、抗がん剤・放射線療法耐性能を有するがん幹細胞は、がん治療の重要な標的であり、その性状解析を通した治療標的の同定は新規がん根治療法の創出に繋がると期待されている。しかし、がん幹細胞の精製は未だ難しく、私たちは試験管内でグリオブラストーマ（神経膠芽腫、GBM）形成能力を有する人工がん幹細胞（mGIC）を作製し、その性状解析から治療標的の同定を試みている。本年度は、mGIC と昨年度までに樹立したヒト GBM 浮遊細胞塊（hGIC）を用いて、新規 GIC 特異的マイクロ RNA（miRNA）群の同定とそれらの機能解析を行った。

A.研究目的

腫瘍に存在するがん幹細胞は、幹細胞能力・腫瘍形成能・治療抵抗性を有する。このためがん幹細胞を性状解析し治療標的を同定することは、新規がん根治療法の創出に繋がる。私たちは試験管内でがん幹細胞の作製を試み、神経幹細胞（NSC）とオリゴデンドロサイト前駆細胞を起源とするグリオブラストーマ幹細胞（mGIC）の作製に成功した。この mGIC は、１０個をヌードマウス脳内に移植することによりヒト GBM と同じ病理所見を示す悪性脳腫瘍を形成する。（Hide et al, Cancer Res. 2009; Nishide et al, PLoS One 2009; Hide et al, Stem Cells 2011)。

本研究では、新規 GIC マーカー・治療標的の同定を目的として、GIC 腫瘍形成能を関与する新規 miRNA 群の同定を試みた。

B.研究方法

DNA マイクロアレイを用いて、mGIC、hGIC および正常 NSC で発現している miRNA の網羅的な解析を行い、GIC で発現が増減する miRNA 群を同定した。抽出した候補 miRNA について、その強制発現による GIC への影響（細胞増殖・細胞死・運動能・腫瘍形成能）を検討した。

本研究は、北海道大学遺伝子組換え実験、動物実験、ヒト試料を用いた研究に関する倫理審査会の承認を得て遂行した。

C.研究結果

２種類のmGIC、３種類のhGIC、２種類のヒトGBM細胞株、ヒトとマウスNSCに発現しているmiRNAの網羅的な解析を行い、候補miRNA群を同定した。GBMにおける機能が報告されていないGIC-miRNA1について検討を進めた結果、それが腫瘍抑制因子として機能することを発見した。更に、GIC-miRNA1の標的因子が細胞外マトリックスのリモデリング制御因子であることも明らかにした。これらの結果から、GIC-miRNAの強制発現やその誘導が新規GBM治療法となることを示した。

D.考察

本研究ではGIC-miRNA1を含めた複数の抗腫瘍効果が期待できるmiRNAを同定しており、今後m-CRAに組み込むことにより新規抗癌作用を有する世界初の組替えアデノウイルスの創出が可能と考えられる。また、本研究で抽出したmiRNA群が他腫瘍にも有効であるかどうかの検討は非常に重要である。

E.結論

人工 GIC とヒト GBM サンプルを用いて、GBM に有効な GIC-miRNA を同定し、その分子メカニズムを明らかにした。

F．健康危険情報

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1. 特許出願

グリオーマ形成阻害作用を有するmicroRNA　出願番号2013-238279

別紙４

研究成果の刊行に関する一覧表

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雑誌

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Intramuscular injection of adenoviral hepatocyte growth factor at a distal site ameliorates dextran sodium sulfate-induced colitis in mice

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Abstract. Inflammatory bowel disease (IBD) severely affects the quality of life of patients. At present, there is no clinical solution for this condition; therefore, there is a need for innovative therapies for IBD. Hepatocyte growth factor (HGF) exerts various biological activities in various organs. However, a clinically applicable and effective HGF-based therapy for IBD has yet to be developed. In this study, we examined the therapeutic effect of injecting an adenoviral vector encoding the human HGF gene (Ad.HGF) into the hindlimbs of mice with dextran sodium sulfate (DSS)-induced colitis. Plasma levels of circulating human HGF (hHGF) were measured in injected mice. The results showed that weight loss and colon shortening were significantly lower in Ad.HGF-infected mice as compared to control (Ad.LacZ-infected) colitic mice. Additionally, inflammation and crypt scores were significantly reduced in the entire length of the colon, particularly in the distal section. This therapeutic effect was associated with increased cell proliferation and an antiapoptotic effect, as well as a reduction in the number of $CD4^+$ cells and a decreased CD4/CD8 ratio. The levels of inflammatory, as well as Th1 and Th2 cytokines were higher in Ad.HGF-infected mice as compared to the control colitic mice. Thus, systemically circulating hHGF protein, produced by an adenovirally transduced hHGF gene introduced at distal sites in the limbs, significantly ameliorated DSS-induced colitis by promoting cell proliferation (i.e., regeneration), preventing apoptosis, and immunomodulation. Owing to its clinical feasibility and potent therapeutic effects, this method may be developed into a clinical therapy for treating IBD.

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Key words: hepatocyte growth factor, inflammatory bowel disease, gene therapy, adenoviral vector, antiapoptosis, immunomodulation, regeneration, cytokine, clinical application

Introduction

The breakdown of normal mucosal immunity causes the development of inflammatory bowel disease (IBD), which can be classified as Crohn's disease (CD) and ulcerative colitis (UC) (1). IBD is a chronically relapsing and remitting condition of unknown origin that exhibits various features of immunological inflammation and affects at least 1 in 1,000 people in western countries. IBD is characterized by inflammation in the intestine, and is associated with diarrhea, occult blood, abdominal pain, weight loss, anemia and leukocytosis. IBD primarily affects young adults, and the disease initially manifests in childhood in 15-25% of cases. Therefore, IBD patients often develop severe symptoms that decrease their quality of life (2). Consequently, there is a need for innovative therapies for IBD.

Current treatments for IBD focus on suppressing inflammation or modulating the immune response using corticosteroids, mercaptopurines, 5-ASA, or monoclonal antibodies against inflammatory cytokines, e.g., the anti-tumor necrosis factor (TNF)-α antibody infliximab (3). However, despite the wide variety of pharmacologic options for patients with IBD, consistent cures and prolonged remissions have yet to be achieved.

Hepatocyte growth factor (HGF) was originally identified (4-7) and cloned (8,9) as a potent mitogen for hepatocytes, but has since been established as a multifunctional cytokine

that exhibits mitogenic, motogenic, morphologic, angiogenic, antiapoptotic and organotrophic effects in a variety of tissues (10). HGF is upregulated in inflamed colonic mucosal tissue in patients with CD or UC (11-13), and plasma HGF levels are elevated in animal models of acute colitis (14). *In vitro*, HGF modulates intestinal epithelial cell proliferation and migration (15), thereby enhancing epithelial cell restitution, which is the initial step of gastrointestinal wound healing. In addition, administration of recombinant human HGF (hHGF) protein reduces the severity of colitis and accelerates colonic mucosal repair in models of TNBS-induced and DSS-induced colitis (16-19), as well as in HLA-B27 transgenic rats with colitis (20). Mukoyama *et al* (21) showed that the intrarectal administration of an adenoviral (Ad) vector carrying the HGF gene prevented TNBS-induced colitis. Additionally, Hanawa *et al* (22) demonstrated the attenuation of mouse DSS colitis by naked gene transfer of rat HGF into the liver, and Kanbe *et al* (23) reported the amelioration of mucosal damage in DSS colitis by the intrarectal administration of the naked HGF gene. In their study, Kanayama *et al* (24) demonstrated the promotion of colonic epithelial regeneration by HGF gene transfer through electroporation. Findings by those authors suggest that HGF is potentially an important new treatment modality for promoting the repair of intestinal mucosa in patients with IBD.

In the majority of previous studies, HGF was provided in the form of recombinant hHGF protein. However, due to the rapid clearance of the HGF protein, large doses and frequent administration of recombinant hHGF were required. Naked gene transfer is a simple and easy method, but the efficiency of gene transduction is extremely low, possibly leading to insufficient clinical effectiveness in human patients. By contrast, the intrarectal administration of an Ad carrying the HGF gene is considered to be extremely stressful for patients. Therefore, in this study we injected an Ad carrying the hHGF gene in single rounds of injections into both hindlimbs of mice 1 day after administration of DSS. We then investigated the therapeutic effects and mechanisms of systemically circulating HGF protein, produced by a gene introduced into the limbs, in the DSS-induced acute colitis model.

Materials and methods

Recombinant Ad. The Ad expressing hHGF under the transcriptional control of the cytomegalovirus immediate-early enhancer and a modified chicken β-actin promoter (Ad.HGF) was generated as described previously (25). The Ad.HGF and the control Ad expressing the LacZ gene (Ad.LacZ) were amplified in HEK-293 cells, purified twice on CsCl gradients, and desalted as described previously (26-29).

Animal studies. Six- to 7-week-old female BALB/c mice weighing 17-20 g (Japan SLC, Inc., Hamamatsu, Japan) were housed in cages in a temperature-controlled environment under a 12-h light-dark cycle with free access to food and water. The animal studies were performed in accordance with the National Institutes of Health guidelines, as specified by the Animal Care Facility at Gifu University School of Medicine.

To induce dextran sodium sulfate (DSS) colitis, the mice were provided with distilled drinking water containing 5% (w/v) DSS (MW, 36,000-50,000; ICN Biomedicals Inc., Aurora, OH, USA) for 7 days. Subsequently, colitis was maintained by feeding the mice 1% DSS (30-32) in the drinking water.

One day after the administration of DSS, Ad.HGF was injected into both hindlimbs of each mouse for a total dose of $1x10^{11}$ particles/mouse (i.e., $5x10^{10}$ particles each into the left and right thigh muscles) (n=8). Ad.LacZ was injected in a similar manner into control mice (n=8). These groups were followed until day 15 (i.e., 8 days after the end of the 7-day period of 5% DSS administration). To evaluate the severity of colitis, body weight was examined on a daily basis. On day 15, all the mice were sacrificed by inhaled anesthetics, and colon samples were collected for examination. In other experiments, on day 5 of 5% DSS administration, 5-bromo-2'-deoxyuridine (BrdU, 100 mg/kg) was administered intraperitoneally to mice (n=8) infected with Ad.HGF or Ad.LacZ, and the animals were sacrificed by inhaled anesthetics 2 h later. These samples were used for analyses of HGF signal transduction, cell proliferation, apoptosis, cytokines and lymphocyte surface markers. The concentration of exogenous hHGF in serum was analyzed using the same dose (i.e., $1x10^{11}$ particles/mouse) of Ad.LacZ or Ad.HGF in intact mice (n=16).

Enzyme-linked immunosorbent assay. The plasma concentration of hHGF following adenoviral intramuscular gene transduction (IMGT) was measured in mice at each time point (n=4) using the Quantikine human HGF Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA). TNF-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ, IL-2, IL-4 and IL-5 levels in the colons of colitic mice were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Inc., Camarillo, CA, USA) according to the manufacturer's instructions.

Immunoprecipitation and c-Met receptor phosphorylation assay. The phosphorylation and activation of the c-Met receptor in colon tissues were detected by immunoprecipitation, as described previously (33,34). In brief, 1 g of colon tissue was homogenized in 4 ml of lysis buffer [1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 10% glycerol, 1 mM vanadate, and 1 mM phenylmethylsulfonyl fluoride] with a protease-inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan). Following centrifugation, the supernatant was incubated with 0.5 μg/ml anti-mouse c-Met antibody (sc-162; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 4 h, and then sequentially incubated with 5 μl of protein G-Sepharose beads for 3 h. After washing, proteins bound to the beads were dissolved in sample buffer and subjected to SDS-PAGE. Phosphorylated c-Met was immunoblotted using the anti-phosphotyrosine antibody PY20 (Transduction Laboratories, Lexington, KY, USA).

Histopathological analysis. After each mouse was sacrificed, the intestine was dissected from the anus to the cecum and rinsed with physiological saline. The colon length was measured, and the colon sample was divided into three sections (cecum, proximal colon and distal colon), with the cecum being separated first, and then the remaining part of the colon being divided into two equal segments (proximal colon and distal colon). The cecum, proximal colon and distal colon were opened longitudinally, and the proximal and distal colon

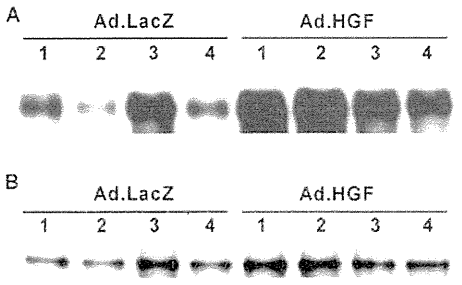

Figure 1. Tyrosine phosphorylation of c-Met in the colon epithelium. Colonic mucosal tissue of dextran sodium sulfate (DSS)-treated mice injected with Ad.LacZ (n=4) or Ad.HGF (n=4) was solubilized in lysis buffer. Lysates were immunoprecipitated with anti-c-Met antibody and blotted with (A) anti-phosphotyrosine antibody or (B) anti-c-Met antibody. Each lane represents the colonic tissue lysate of individual animals. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) led to the strong stimulation of c-Met phosphorylation in colonic mucosal tissue.

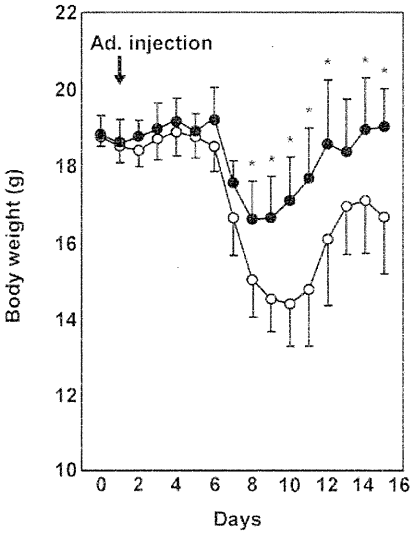

Figure 2. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) ameliorated weight loss. Mice were given distilled drinking water containing 5% dextran sodium sulfate (DSS) for 7 days and 1% DSS for 8 days, *ad libitum*. One day after DSS administration, Ad.HGF (closed circles; n=8) was injected into both hindlimb muscles of 8 mice. As a control, Ad.LacZ (open circles; n=8) was injected into both hindlimb muscles of another group of 8 mice. Ad.HGF injection significantly prevented weight loss in colitic mice. *P<0.05.

were equally divided longitudinally and transversely. Thus, the cecum was divided into two sections, and the proximal and distal colon were divided into four sections. The colon tissues were fixed in 10% formalin and embedded in paraffin, and 4-μm sections were cut and stained with hematoxylin and eosin (H&E) to determine the inflammation and crypt scores (35). Briefly, the sections were graded on a scale of 0-3 to indicate the severity of inflammation: 0, none; 1, mucosa; 2, mucosa and submucosa; and 3, transverse, and on a scale of 0-4 to indicate the severity of crypt damage: 0, none; 1, basal 1/3 damage; 2, basal 2/3 damage; 3: loss of the entire crypt with the surface epithelium remaining intact; and 4, loss of the entire crypt and surface epithelium. The changes were also scored with regard to the extent of tissue involvement, measured as a percentage: i) 1-25%, ii) 26-50%, iii) 51-75%, and iv) 76-100%. Each section was then separately scored for each feature by taking the product of the severity score and the score for the extent of tissue involvement. Thus, the inflammation score ranged from 0 to 12, and the crypt score ranged from 0 to 16. Apoptotic cells were detected using a light microscope (Olympus, Tokyo, Japan) and the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate biotin nick end-labeling (TUNEL) assay (ApopTag kit; Intergen Co., Purchase, NY, USA), as described previously (25,33,36). To detect proliferating cells, BrdU incorporation was measured using a staining kit (Zymed Laboratories, Inc., South San Francisco, CA, USA) according to the manufacturer's instructions.

Endothelial cells, $CD4^+$ T lymphocytes, and $CD8^+$ T lymphocytes were detected *in situ* using an anti-vWF antibody (Dako Cytomation Co., Ltd., Kyoto, Japan), anti-CD4 antibody and anti-CD8 antibody (both from Zymed Laboratories, Inc.), respectively, as described previously (25,36).

Statistical analysis. Values provided are the means ± SEM values. The significance of differences was evaluated using the Student's t-test.

Results

Intramuscular injection of Ad.HGF produces circulating plasma hHGF, leading to c-Met activation in the colonic mucosa. DSS-induced colitis was induced in 6- to 7-week-old female BALB/c mice. One day after DSS administration, Ad.HGF was administered in a single procedure involving injections into both hindlimbs (total dose, $1x10^{11}$ particles/mouse; as mentioned in Materials and methods). In the hHGF-overexpressing mice, the plasma levels of hHGF were 1,140±101, 634±341 and 33.9±15.8 pg/ml at 2, 4 and 6 days after injection, respectively. No hHGF was detected in the Ad.LacZ-treated mice at any time point, demonstrating that this method accurately detected only hHGF protein expressed from the hHGF transgene, without a cross-reaction resulting in detection of the endogenous mouse HGF protein. These results indicate that hHGF expression was effectively induced by the intramuscular injection of Ad.HGF, leading to the presence of hHGF in the plasma of the mice.

The biological effects of HGF are mediated by its receptor c-Met, which is capable of activating multiple intracellular transducers and signaling pathways. Therefore, we examined c-Met tyrosine phosphorylation in the colonic mucosal epithelium by western blotting (Fig. 1). Phosphorylated c-Met was detected at low or moderate levels in the injured colonic mucosa of mice treated with Ad.LacZ, presumably as a result of a DSS-induced increase in endogenous HGF in response to colonic mucosal injury (14). By contrast, the injured colonic mucosa of mice treated with Ad.HGF exhibited high levels of c-Met tyrosine phosphorylation.

Adenoviral hHGF IMGT prevents weight loss in DSS-induced colitis mice. DSS-induced colitis is characterized by bloody stools and severe weight loss (30). In mice treated with Ad.LacZ, we observed persistent liquid stool and waste with subsequent severe weight loss. By contrast, colitic mice that received a single round of injections of Ad.HGF exhibited

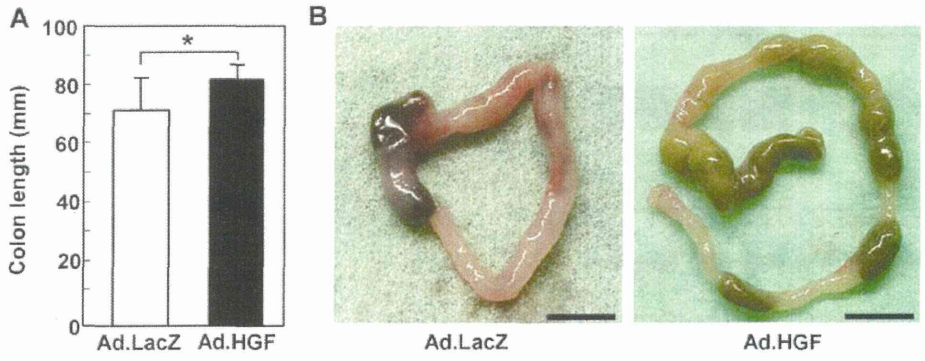

Figure 3. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) reduced inflammation in the colon and prevented colon shortening in dextran sodium sulfate (DSS)-induced colitis. Colon lengths were measured from the colocecal junction to the anal verge on day 15 (Ad.LacZ, n=8; Ad.HGF, n=8). (A) Ad.HGF treatment prevented shortening of the colon in mice with DSS-induced colitis. *P<0.05. Representative colon pictures from the Ad.LacZ- and Ad.HGF-injected groups are shown in (B). The scale bar indicates 1 cm.

significant reductions in liquid stool and gross bleeding from the rectum (data not shown). Fig. 2 shows the mean weight change, and that the body weights of Ad.HGF-treated mice were significantly higher than those of the Ad.LacZ-treated mice. In the Ad.LacZ-treated control mice, weight loss occurred 6-7 days after the initiation of DSS administration. Ad.HGF treatment significantly prevented this weight loss.

Adenoviral hHGF IMGT reduces colitis-induced intestinal shortening and pathological scores. Shortening of the colon correlates well with histologic changes, and colon length is therefore frequently used as a morphologic parameter to indicate the degree of inflammation (35). The colon lengths of mice treated with Ad.LacZ and Ad.HGF were 72.0±10.6 and 82.0±4.7 mm, respectively (Fig. 3A). In contrast to the colons in the Ad.HGF-treated group, the colons in the Ad.LacZ-treated group were short and severely inflamed, with evident hemorrhages (Fig. 3B).

To validate this finding, we evaluated the effect of Ad.HGF on DSS-induced colonic mucosal injury in mice by histological analysis at day 15. In the cecum and proximal part of the colon (i.e., towards the end of the cecum), the inflammation and crypt scores appeared to be decreased by Ad.HGF administration although this difference was not statistically significant (Figs. 4A and B, 5A and B). By contrast, treatment with Ad.HGF significantly decreased the inflammation and crypt scores in the distal part (i.e., towards the anus) and in the colon overall (Figs. 4C and D, 5C and D).

Kinetics of inflammation in colitic mice. To elucidate the mechanism underlying the therapeutic effect of hHGF, we studied the expression of TNF-α and IL-1β in the colon and evaluated the inflammation and crypt scores at days 4, 7, 10 and 14 of the experimental colitis model (Fig. 6). The expression of TNF-α and IL-1β peaked as early as day 4 (Fig. 6A and B). The inflammation and crypt scores peaked as early as day 7 (Fig. 6C and D). Given that the plasma concentration of hHGF protein peaked on day 2 and decreased thereafter, colon tissue were sampled and hHGF functions were analyzed on day 5.

Adenoviral hHGF IMGT suppresses apoptosis and enhances regeneration of the colonic epithelium. In DSS-induced colitis, loss of colonic mucosal epithelial cells is closely associated with apoptosis (37,38). To evaluate the role of Ad.HGF in preventing apoptosis in colonic epithelial cells, we performed the TUNEL assay to detect apoptotic cells (Fig. 7A). Ad.HGF-treated colitic mice had significantly (2.1-fold) fewer TUNEL-positive cells per high-power field (HPF) than Ad.LacZ-treated colitic mice.

To determine whether Ad.HGF-injection stimulated the proliferation of colonic epithelial cells, we measured the DNA labeling index in the colonic mucosal epithelium. As shown in Fig. 7B, the average number of BrdU-positive cells in the colonic mucosal epithelium was significantly (1.8-fold) higher in Ad.HGF-treated as compared to Ad.LacZ-treated mice, suggesting that hHGF stimulates proliferation in the colonic epithelial cells of colitic mice. These results suggested that adenoviral hHGF IMGT promoted survival and regeneration of the colonic mucosal epithelium in mice with DSS-induced colitis. HGF is known to promote angiogenesis (10). Therefore, we hypothesized that the angiogenic effect of HGF may contribute to the repair of the damaged colonic epithelium. However, when we analyzed angiogenesis in the distal part of the colon by anti-vWF immunohistochemistry, the number of blood vessels in the colon did not differ significantly between Ad.HGF-treated mice and controls, although a few more vessels appeared to be present in Ad.HGF-treated animals (Fig. 7C).

Effects of adenoviral hHGF IMGT on immunoreactive cells and inflammatory cytokines in DSS-induced colitis. To determine whether IMGT of hHGF affected the immune system of DSS-treated mice, we directly detected immune cells in the colon. Adenoviral hHGF IMGT decreased the number of $CD4^+$ T cells and the CD4/CD8 ratio, but not the number of $CD8^+$ T cells (Fig. 8).

The inflammatory cytokine cascade plays an important role in the pathogenesis of DSS-induced colitis. Therefore, we analyzed the cytokine profile of the entire colon by ELISA. In general, we observed upregulation of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in the colitic mice (39,40). The expression levels of TNF-α, IL-1β and IL-6 were further increased by hHGF IMGT (Fig. 9).

We also examined the effect of hHGF IMGT on Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-5) cytokine expres-

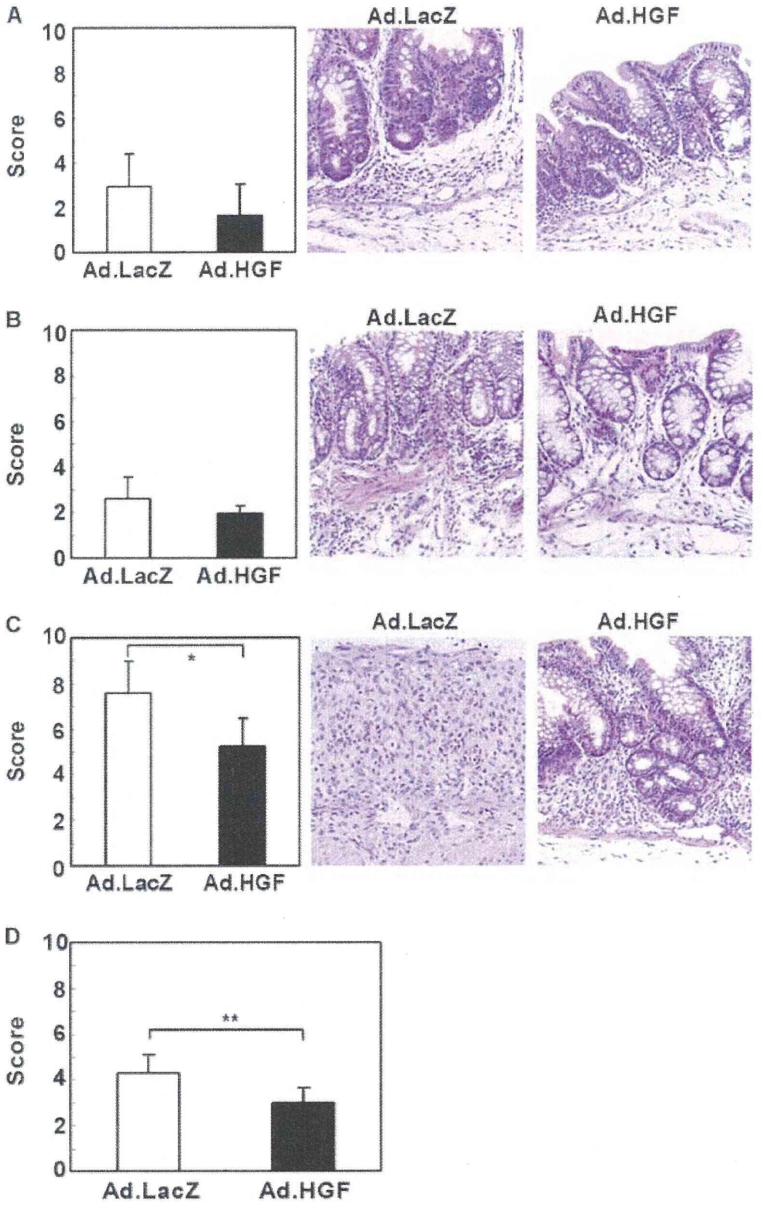

Figure 4. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) decreased colon inflammation in dextran sodium sulfate (DSS)-induced colitis. (A) Cecum, (B) proximal, (C) distal, and (D) total colon samples from the anal ring were used for histological evaluation. Colonic tissues taken on day 15 were stained with hematoxylin and eosin (representative histopathological images are shown on the right) (original magnification, x100). Histological scoring of the severity of inflammation was performed in a blind manner (graph on the left). Infiltration of inflammatory cells was significantly reduced in the adenoviral HGF treatment group. $^{*}P<0.05$ and $^{**}P<0.01$.

sion in the colons of colitic mice. IFN-γ, IL-2 and IL-4 were upregulated by hHGF treatment (Fig. 10).

Discussion

This study evaluated the therapeutic potential of the intramuscular injection of HGF-expressing Ad for treating IBD, using a mouse model of DSS-induced colitis. The therapeutic strategy of adenoviral HGF IMGT, in which hHGF protein was produced at distal sites (hindlimbs) and systemically delivered to the target organ (the injured colon epithelium), functioned well. Epithelial cell injury in DSS-induced colitis was potently prevented by this method, which is clinically feasible, less invasive, and does not suffer from the drawbacks associated with the direct treatment of colitic tissues. Although previous studies (16-18) have shown that HGF exerts protective effects in bowel disease, the regimens tested involved high levels of recombinant HGF protein (>100 μg/kg) and repeated injections.

Recent advances in molecular techniques have provided several strategies for *in vivo* gene delivery, including naked plasmid DNA, liposomes encapsulating DNA, and viral vectors (41,42). For instance, Hanawa *et al* (22) reported that administration of the naked HGF gene into the liver attenuated acute colitis in mice, and Kanbe *et al* (23) showed that intrarectal administration of a plasmid carrying the HGF gene

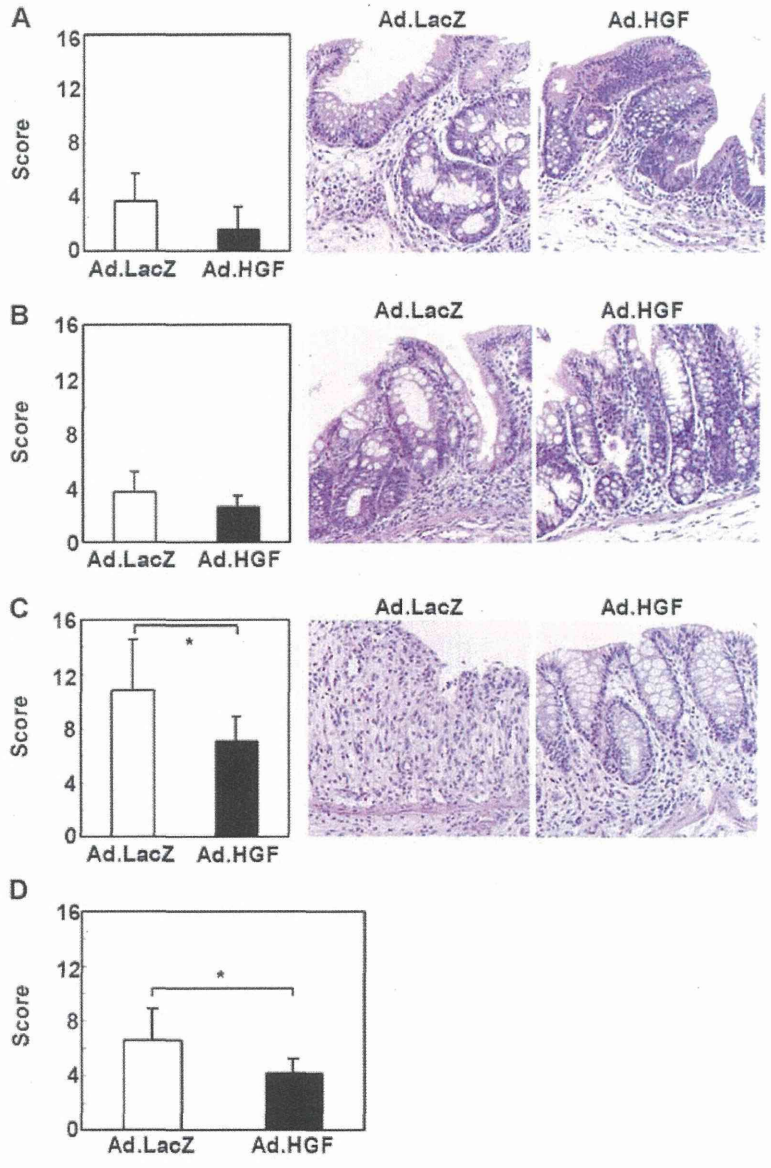

Figure 5. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) prevented crypt destruction in dextran sodium sulfate (DSS)-induced colitis. (A) Cecum, (B) proximal, (C) distal, and (D) total colon samples from the anal ring were used for histological evaluation. Colonic tissues taken on day 15 were stained with hematoxylin and eosin (representative histopathological images are shown on the right; original magnification, x100). Histological scoring of the severity of crypt damage was performed in a blind manner (graph on the left). Crypt damage was significantly reduced in the adenoviral hHGF treatment group. *P<0.05.

ameliorated DSS-induced colitis in mice. Kanayama *et al* (24) found that colonic epithelial regeneration is promoted by HGF gene transfer via electroporation. Oh *et al* (43) reported that HVJ liposomes encapsulating the hHGF gene ameliorated TNBS-induced colitis in mice, and that intrarectal administration of an Ad carrying the HGF gene improved colonic damage in TNBS-induced colitis (21). However, each type of gene therapy system used thus far has some associated limitations and concerns, particularly from the viewpoints of clinical applicability, feasibility and safety (41,42).

In this study, we assessed for the first time the therapeutic potential of a unique method of adenoviral hHGF IMGT for treating IBDs. In accordance with the results obtained in our previous studies of a mouse model of myocardial infarction (25,36), we successfully detected circulating hHGF in the plasma of colitic mice after adenoviral hHGF IMGT. In the colons of colitic mice that received adenoviral hHGF IMGT, the c-Met/HGF receptor was highly phosphorylated on tyrosine, demonstrating the functional efficacy of the adenoviral hHGF IMGT system. Furthermore, hHGF IMGT stimulated proliferation and inhibited apoptosis in the disrupted intestinal epithelial barrier. These results indicate that our hHGF IMGT system induces protection and regeneration in the colon, suggesting that it would be useful in clinical treatments for bowel diseases.

The effects of HGF on carcinogenesis remain unclear. Some studies suggest that HGF may promote the growth and metastasis of some cancer types, probably via the stimulation

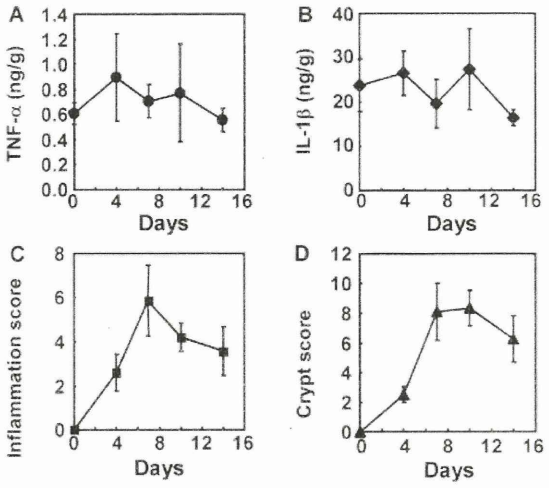

Figure 6. Expression of tumor necrosis factor (TNF)-α and interleukin (IL)-1β, and inflammation and crypt scores, in dextran sodium sulfate (DSS)-induced colitis. Twenty mice were given distilled drinking water containing 5% DSS for 7 days and 1% DSS for 7 days, *ad libitum*. Five mice were sacrificed at days 4, 7, 10 and 14. Analyses were performed to determine (A) TNF-α and (B) IL-1β expression in the colon per gram of total colon tissue, (C) inflammation score, and (D) crypt score. TNF-α and IL-1β expression increased on days 4 and 10, the inflammation score peaked at day 7, and the crypt score peaked at days 7 and 10.

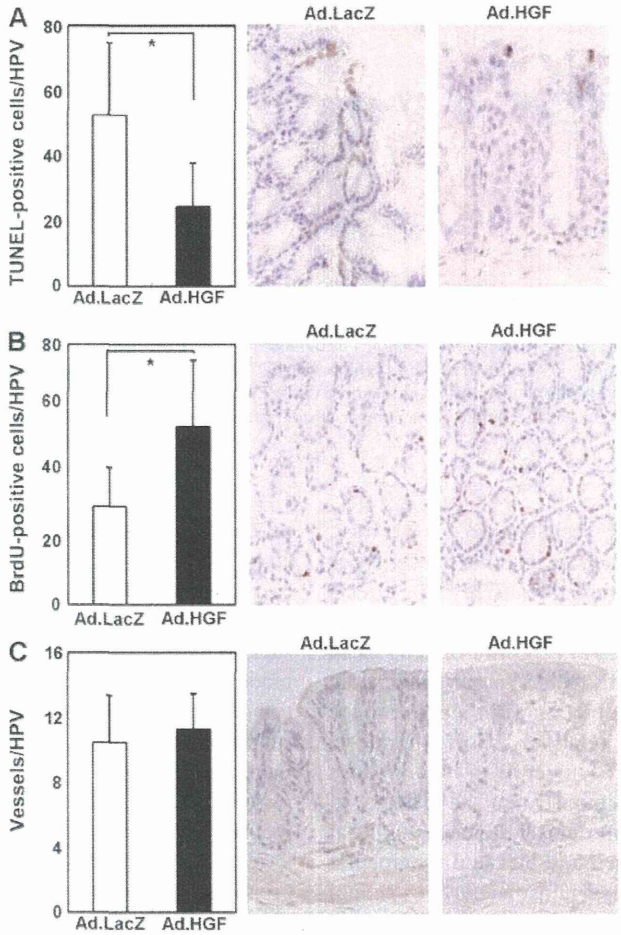

Figure 7. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) prevented apoptosis and stimulated intestinal epithelial regeneration in dextran sodium sulfate (DSS)-induced colitis. Colon tissues were stained by immunohistochemistry (representative histopathological images are shown on the right) (original magnification, x100). The graphs indicate the average number of positive cells or vessels per high-power field (left column). (A) TUNEL staining of the distal colon from Ad.LacZ-treated and Ad.HGF-treated mice. The graph indicates the number of apoptotic cells detected in the epithelial crypts. A single round of Ad.HGF injection into both hindlimbs almost completely prevented apoptosis in the colon epithelium. (B) 5-Bromo-2'-deoxyuridine (BrdU) staining of the distal colon from Ad.LacZ-treated and Ad.HGF-treated mice. In the Ad.HGF-treated mice, a significant increase in the amount of BrdU-incorporating cells was observed in the colon epithelium. (C) vWF staining of the distal colon from Ad.LacZ-treated and Ad.HGF-treated mice. No significant difference was observed in the number of vessels between the two groups. *P<0.05.

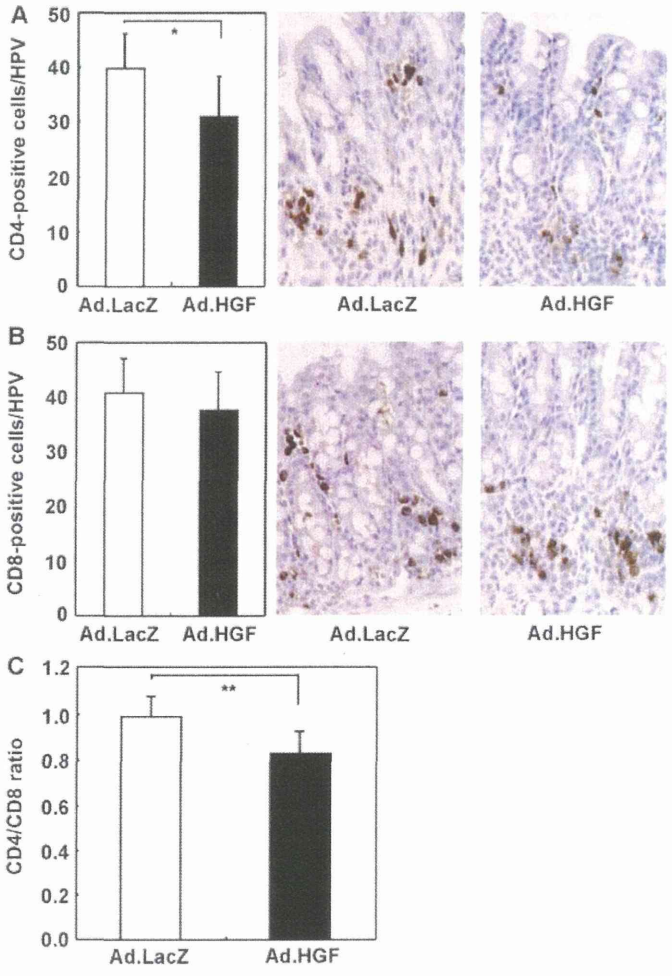

Figure 8. Effects of adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) on inflammatory cells in dextran sodium sulfate (DSS)-induced colitis. (A) CD4 immunostaining of the distal colon. (B) CD8 immunostaining of the distal colon. The two images on the right are representative of immunostaining of $CD4^+$ and $CD8^+$ (original magnification, x200), and the graphs on the left indicate the average number of positive cells per high-power field. (C) The graph indicates the CD4/CD8 ratio. The number of infiltrating $CD4^+$ T cells and the CD4/CD8 ratio were decreased by adenoviral HGF IMGT. $^{**}P<0.001$ and $^{*}P<0.05$.

of cancer cell growth and angiogenesis (44,45). By contrast, carcinogenesis or malignant phenotypes in other cancer types are potently inhibited by overexpressed HGF (33). The effects of HGF on IBDs are also unclear. In general, tumor development may be caused by long-term exposure of cells to an abnormally overexpressed growth factor. In our therapeutic system, the duration of hHGF secretion after single rounds of intramuscular injection was relatively short; therefore, we consider the risk of cancer occurrence to be very low. In addition, a previous study demonstrated the efficacy of repeated administration of Ad into muscles, suggesting that this approach may yield sustained and elevated therapeutic efficiency: neutralizing antibodies against adenovirus should hinder only Ad circulating in the bloodstream, but not Ad administered into the muscle (46). These findings are encouraging with regard to the potential safety and clinical applicability of this approach.

With regard to the therapeutic mechanism, previous studies have reported that administration of recombinant HGF protein (16) and vector encoding HGF gene (43) ameliorate TNBS-induced colitis and reduced inflammation, decreasing the levels of inflammatory cytokines such as TNF-α. In particular, Oh *et al* (43) showed that administration of a plasmid carrying the HGF gene reduced the invasion of $CD4^+$ cells and neutrophils and suppressed the expression of Th1 cytokines such as IL-12, IL-1β and IFN-γ in a TNBS-induced colitis model. Hanawa *et al* (22) showed that administration of an HGF gene-containing plasmid in the liver by intravenous injection suppressed the mRNA levels of IFN-γ, IL-18 and TNF-α, and increased the mRNA levels of anti-inflammatory cytokines such as IL-10. Jeschke *et al* (47) found that recombinant HGF reduced burn-related damage to the small intestine. The serum levels of TNF-α, IL-1β and IL-6 were higher in the HGF-treated group than in the control group. However, Jeschke *et al* (47) did not explain why the levels of these cytokines were increased by HGF. Our data indicate that the number of $CD4^+$ cells decreased, but the levels of TNF-α, IL-1β and IL-6, as well as those of Th1 and Th2 cytokines such as IL-2, IFN-γ and IL-4, were elevated in the Ad.HGF-treated group. We hypothesize that the reasons for the differences between our findings and those of previous studies may involve differences among mouse strains, our use of intramuscular gene administration

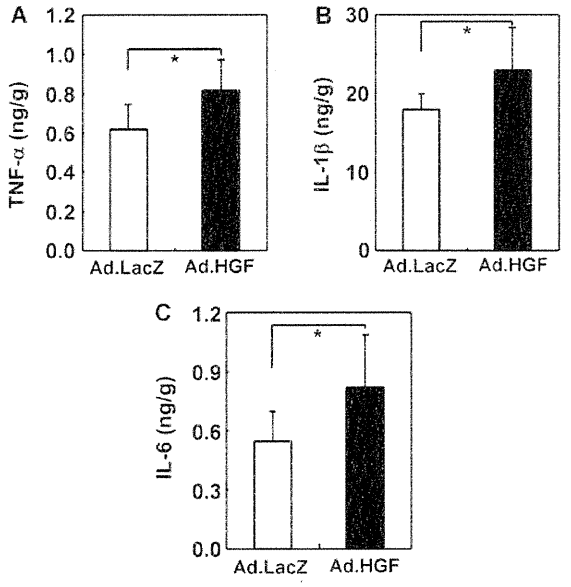

Figure 9. Effects of adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) on inflammatory cytokines in dextran sodium sulfate (DSS)-induced colitis. On day 5 of DSS administration, the expression of inflammatory cytokines was evaluated by enzyme-linked immunosorbent assay (ELISA). The graphs indicate the level of each cytokine per gram of total colon tissue. The expression of inflammatory cytokines, (A) tumor necrosis factor (TNF)-α, (B) interleukin (IL)-1β, and (C) IL-6 increased after administration of adenoviral HGF IMGT. *P<0.05.

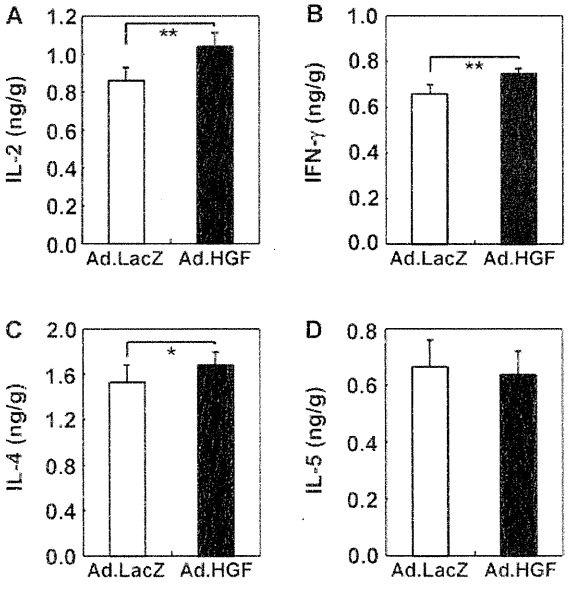

Figure 10. Effects of adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) on Th1 and Th2 cytokines in dextran sodium sulfate (DSS)-induced colitis. The expression of the Th1 [(A) interleukin (IL)-2 and (B) interferon (IFN)-γ] and Th2 [(C) IL-4 and (D) IL-5] cytokines was determined by enzyme-linked immunosorbent assay (ELISA). The graphs indicate the expression of each cytokine per gram of total colon tissue. The expression of IL-2, IFN-γ and IL-4 increased after the administration of adenoviral HGF IMGT. *P<0.05 and **P<0.001.

mediated by an Ad, and our selection of the early phase of DSS colitis for analysis of inflammation and cytokine expression.

Futamatsu *et al* (48) reported that HGF suppressed T-cell proliferation and IFN-γ production and increased IL-4 and IL-10 secretion from $CD4^+$ T cells *in vitro*, and also reduced the severity of experimental autoimmune myocarditis *in vivo* by inducing Th2 cytokines and suppressing apoptosis of cardiomyocytes. Kuroiwa *et al* (49) demonstrated that HGF gene delivery inhibited Th2 immune responses and ameliorated lupus nephritis, autoimmune sialadenitis, and cholangitis in chronic GVHD mice. Another study indicated that treatment with HGF potently suppressed dendritic cell functions such as antigen-presenting capacity, both *in vitro* and *in vivo*, thus downregulating antigen-induced Th1 and Th2 immune responses in a mouse model of allergic airway inflammation (50). HGF has been suggested to suppress