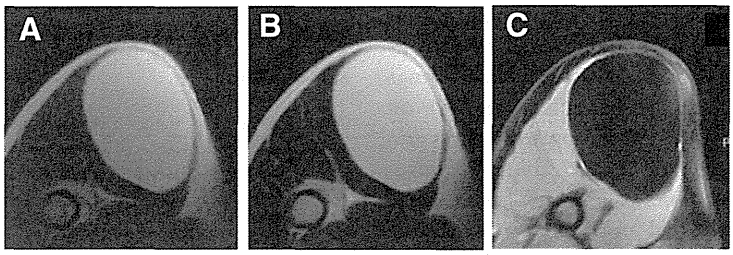

Figure 4 A 34-year-old male patient with a lipoma measuring 12 cm in diameter. Axial T1- and T2- weighted MRIs showed a homogenously high intramuscular mass **(A, B)**. T1-weighted fat saturation gadolinium-enhanced MRI showed no enhancement of the tumor **(C)**. This case obtained 2 points using the ALT score.

in outpatient clinics. It is well-known that adipocytic tumors, regardless of their benign or malignant status, could be large in size without inducing any symptoms. In this study, 31 out of 48 patients with lipomas had tumors larger than 5 cm. Patients with tumors less than 5 cm in size and superficially located may undergo resectional biopsy for diagnosis; however, diagnostic imaging studies are usually performed preoperatively. If computed tomography or MRI examinations reveal a soft tumor with lipomatous content in the majority of the tumor volume, lipoma or WDL/ALT is suspected rather than a high-grade soft tissue sarcoma (Figures 4 and 5). Even though the tumor may be large in size, asymptomatic lipomas do not necessarily require surgical resection. On the other hand, treatment of WDL/ALT is still controversial because of its very low malignancy potential [1,2,5,6]. Because WDL/ALTs have no potential for metastasis unless they undergo dedifferentiation, some pathologists suggest that the term "atypical lipomatous tumor" is more appropriate to use rather than "liposarcoma" [1]. The rate of dedifferentiation of WDL/ALT was previously reported to be 1–4% [6-8]. However, dedifferentiated liposarcoma (DDL) shows much more malignant potential than conventional WDL/ALT with a 5-year survival rate of 60–70% [9,10]. Okada et al. reviewed 18 cases of primary (*de novo*) DDL in the extremities and reported that the duration of the symptoms was an average of 38 months, and 9 patients showed rapid growth of long-standing tumors [10]. This result suggests that if preoperative diagnosis of WDL/ALT is easily made, surgeons could recommend resection of the tumor before it dedifferentiates.

Previously, other researchers reported on the significance of septal structures in WDL/ALT [4,11]. Gaskin et al. tried to differentiate WDL/ALTs from lipomas based upon the viewpoint that simple lipomas may contain thin, discrete septa, whereas WDL/ALTs usually contain thick or nodular septa or enhancement [12]. MRI analysis of 126 fatty masses by musculoskeletal radiologists reached the correct diagnosis in all 6 WDL/ALT cases (sensitivity, 100%); however, 10 of the suspected ALT tumor cases turned out to be variants of benign lipomas, such as chondroid lipoma, osteolipoma, or angiolipoma. The differential diagnosis of lipomatous tumors largely depended on the decisions made by the musculoskeletal radiologists. It would be useful for non-oncologist orthopedic surgeons if simplified diagnostic criteria were available. Therefore, we have created a scoring system to discriminate between lipoma and ALT by the combination of 4 values (Table 2). The score can be measured if enhanced MRI is performed (Figures 4 and 5). Based on this score, diagnosis of ALT is possible with 100% sensitivity and 77% specificity. This result is superior to MRI findings of intratumoral septa alone as a diagnostic finding, which showed 91.7% sensitivity and 74.2% specificity. Although the prevalence of hibernoma

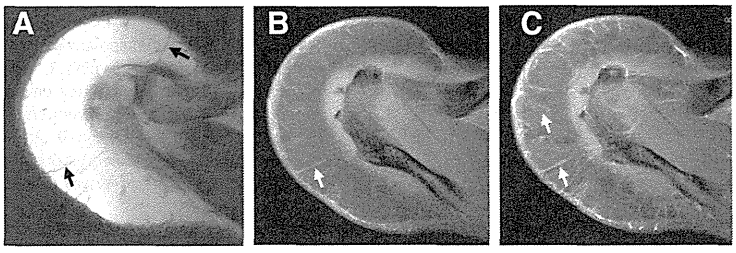

Figure 5 A 40-year-old male patient with ALT measuring 15 cm in diameter. Axial T1-weighted image **(A)** and T1- weighted fat saturation image **(B)** clearly demonstrated thick septa (arrows). T1-weighted fat saturation after gadolinium administration demonstrated enhancement of these septa **(C)**. This case obtained 5 points using the ALT score.

is very low, its MRI findings are similar to those of WDL/ALT. Vassos recently reported that hibernomas show spotty areas of contrast enhancement as well as prominent fibrovascular septa on MRI [13]. Hibernomas exhibit very high standard uptake values (SUVs) on [18F]fluorodeoxyglucose (FDG)-based positron emission tomography (PET) because they contain abundant mitochondria and are highly metabolically active [14]. One of our cases of hibernoma showed an SUV >40, suggesting that PET might be useful to distinguish hibernomas from WDL/ALTs (manuscript in preparation).

Treatment for WDL/ALT is still controversial because the recurrence rate after surgical resection of WDL/ALT is variable, ranging from 0–69% [15-17]. The recurrence of ALT in our series was seen in only 1 case (8.3%), similar to the findings in the report by Sommerville et al. showing an 8% local recurrence rate after marginal resection of 61 cases of ALT [6]. We agree with Sommerville et al. and Kubo et al. in the idea of "conservative" surgery for ALT to preserve the major vessels or nerves [18]. However, for recurrent ALT cases, we recommend as wide of a resection as possible because tumor margins are not usually clear and there is an increased chance of dedifferentiation. An increased number of intratumoral angiogenic vessels was revealed to be a significant factor that differentiates ALTs from lipomas in this study. Because angiolipomas are characterized by rich vasculature in mature adipose tissue, vascularity alone is not useful for the differentiation of ALTs from lipomas. As Folkmann's group proposed, angiogenesis could be a switch that turns on the malignant phenotype in adipocytic tumors [19]. In our study, the highest number of intratumoral vessels (21.4 vessels per field) was observed in the case of ALT recurrence, which eventually dedifferentiated. Contrast enhancement MRI definitely reflects the vascular supply in the tumor and also supports the theory.

Conclusion

Our ALT score (0–6 points) can be used to differentiate ALTs from lipomas based on MRI. If the score is equal to or higher than 3, we recommend marginal resection of the tumor to confirm the pathological diagnosis. Cut-off value should be validated by the future study because of the number of the case is not large in this study. Once the diagnosis of ALT is established, careful follow-up is recommended, especially for cases with increased vascularity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SN, MY, HSa and HSh participated in the diagnosis and surgical treatment of the patient. TS and YI helped draft and finalize the manuscript. SK performed the pathological examination and proof reading of the manuscript. All authors read and approved the manuscript.

Acknowledgements

We thank Shinichi Kitajima and Akihide Tanimoto for performing pathological analysis. This work was supported by JSPS KAKENHI Grant Number 24592237.

Author details

[1]Department of Orthopaedic Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima-city, Kagoshima 890-8520, Japan. [2]The Near-Future Locomotor Organ Medicine Creation Course (Kusunoki Kai), Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan. [3]Department of Medical Joint Materials, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan.

Received: 2 May 2013 Accepted: 2 February 2015
Published online: 22 February 2015

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

KENTARO YUGE[1,2], TOMOYUKI TAKAHASHI[1,3], NGIN CIN KHAI[1,3,4], KAZUKO GOTO[1], TAKAKO FUJIWARA[6], HISAYOSHI FUJIWARA[1,5,7] and KEN-ICHIRO KOSAI[1,3,4]

[1]Department of Gene Therapy and Regenerative Medicine, Graduate School of Medicine, Gifu University, Gifu 502-1194; [2]Division of Respirology, Neurology and Rheumatology, Department of Medicine, Kurume University School of Medicine, [3]Division of Gene Therapy and Regenerative Medicine, Cognitive and Molecular Research Institute of Brain Diseases, Kurume University, Kurume 830-0011; [4]Department of Gene Therapy and Regenerative Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544; [5]Department of Cardiology, Graduate School of Medicine, Gifu University, Gifu 502-1194; [6]Department of Food Science, Kyoto Women's University, Kyoto 605-8501; [7]Department of Cardiology, Hyogo Prefectural Amagasaki Hospital, Amagasaki, Hyogo 660-0828, Japan

Received November 4, 2013; Accepted February 19, 2014

DOI: 10.3892/ijmm.2014.1686

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.

Correspondence to: Dr Ken-Ichiro Kosai, Department of Gene Therapy and Regenerative Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
E-mail: kosai@m2.kufm.kagoshima-u.ac.jp

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

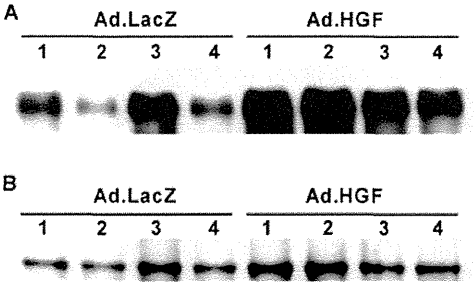

Figure 1. Tyrosine phosphorylation of c-Met in the colon epithelium. Colonic mucosal tissue of dextran sodium sulfate (DSS)-treated mice injected with Ad.LacZ (n=4) or Ad.HGF (n=4) was solubilized in lysis buffer. Lysates were immunoprecipitated with anti-c-Met antibody and blotted with (A) anti-phosphotyrosine 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.

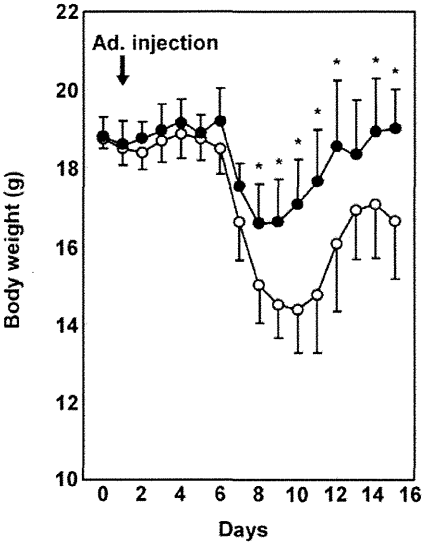

Figure 2. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) ameliorated weight loss. Mice were given distilled drinking water containing 5% dextran sodium sulfate (DSS) for 7 days and 1% DSS for 8 days, *ad libitum*. One day after DSS administration, Ad.HGF (closed 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

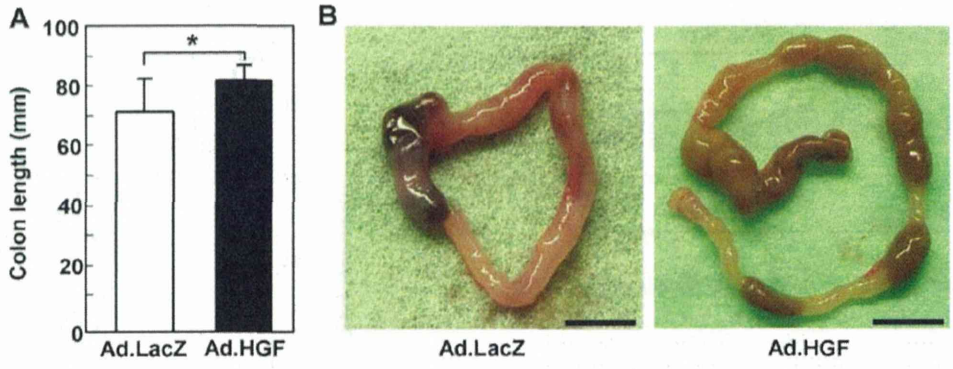

Figure 3. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) reduced inflammation in the colon and prevented colon shortening in dextran sodium sulfate (DSS)-induced colitis. Colon lengths were measured from the colocecal junction to the anal verge on day 15 (Ad.LacZ, 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-

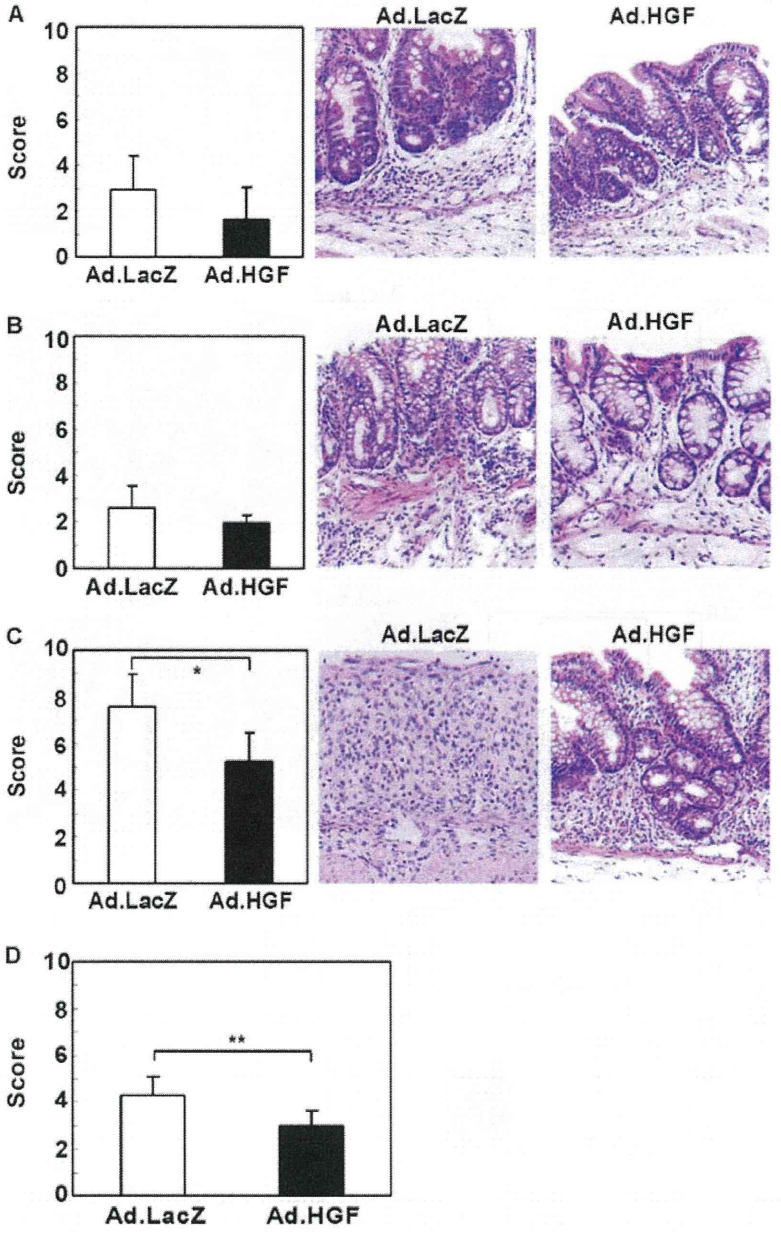

Figure 4. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) decreased colon inflammation in dextran sodium sulfate (DSS)-induced colitis. (A) Cecum, (B) proximal, (C) distal, and (D) total colon samples from the anal ring were used for histological evaluation. Colonic 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

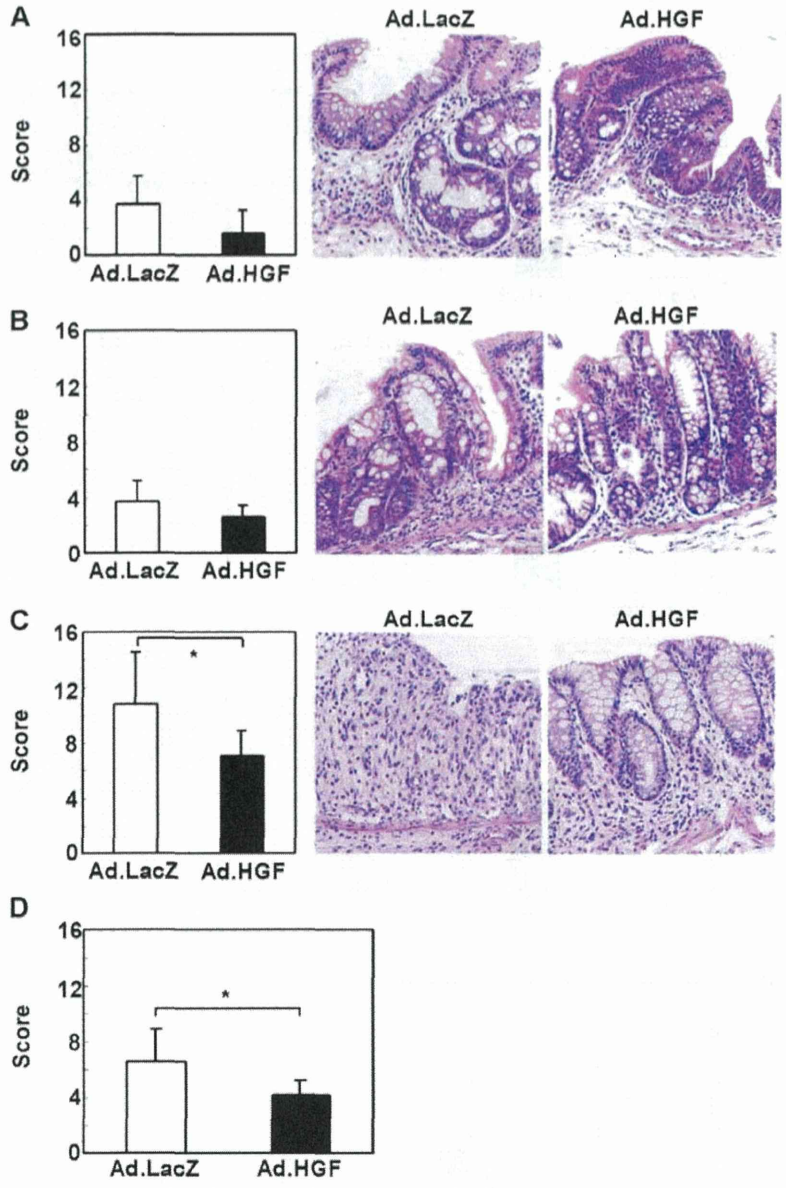

Figure 5. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) prevented crypt destruction in dextran sodium sulfate (DSS)-induced colitis. (A) Cecum, (B) proximal, (C) distal, and (D) total colon samples from the anal ring were used for histological evaluation. Colonic 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

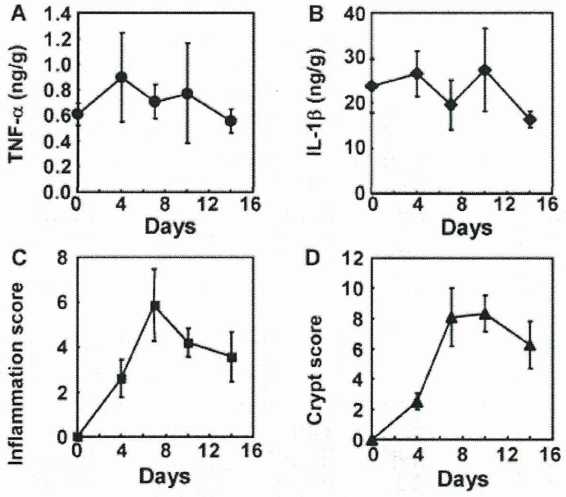

Figure 6. Expression of tumor necrosis factor (TNF)-α and interleukin (IL)-1β, and inflammation and crypt scores, in dextran sodium sulfate (DSS)-induced colitis. Twenty mice were given distilled drinking water containing 5% DSS for 7 days and 1% DSS for 7 days, *ad libitum*. Five mice were sacrificed 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.

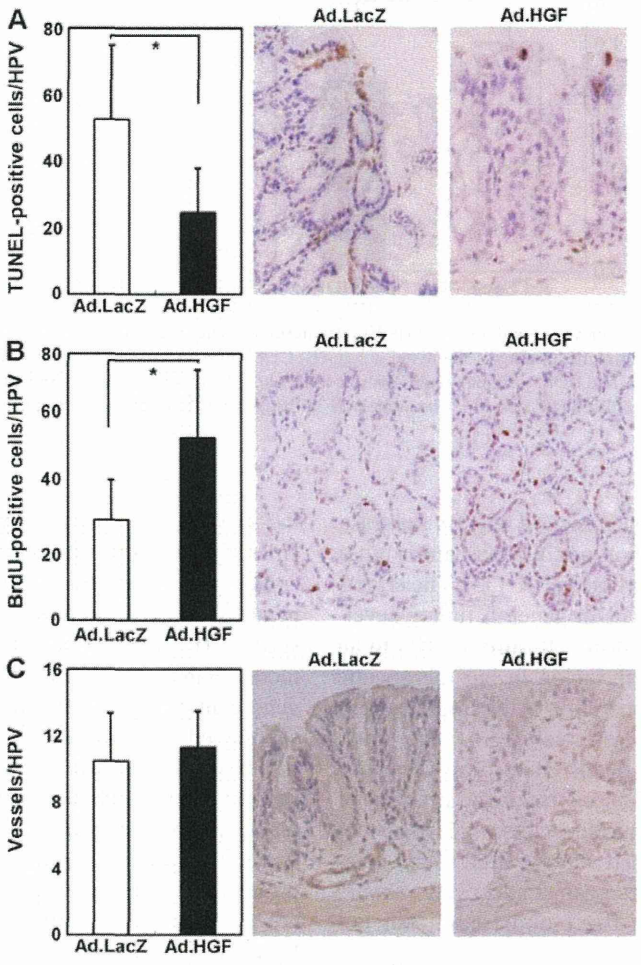

Figure 7. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) prevented apoptosis and stimulated intestinal epithelial regeneration in dextran sodium sulfate (DSS)-induced colitis. Colon tissues were stained by immunohistochemistry (representative histopathological 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. *$P<0.05$.

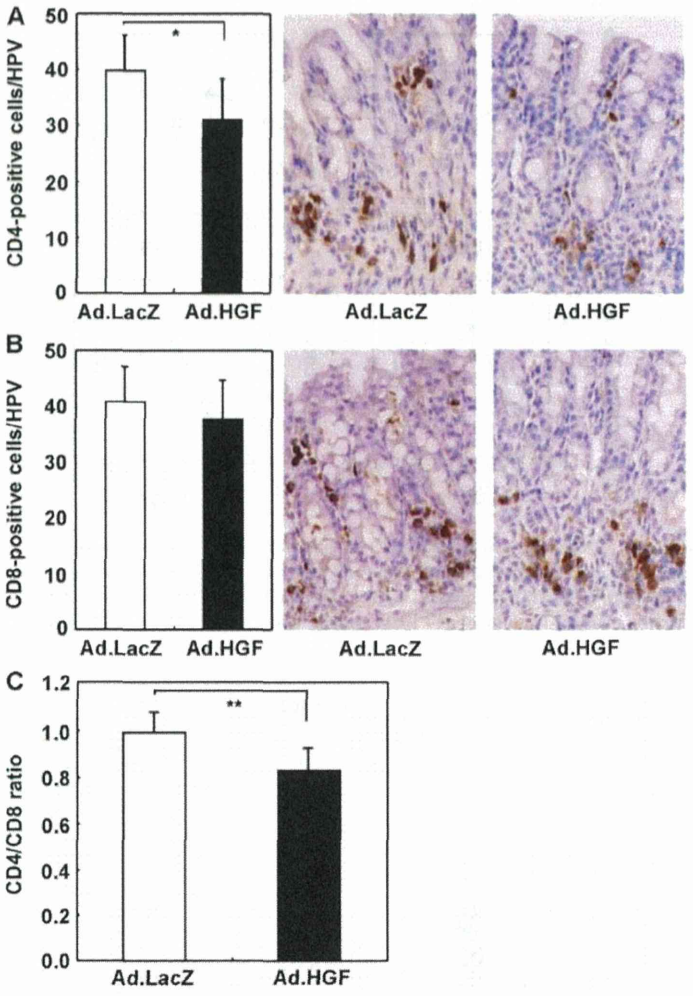

Figure 8. Effects of adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) on inflammatory cells in dextran sodium sulfate (DSS)-induced colitis. (A) CD4 immunostaining of the distal colon. (B) CD8 immunostaining of the distal colon. The two images on the right are representative 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

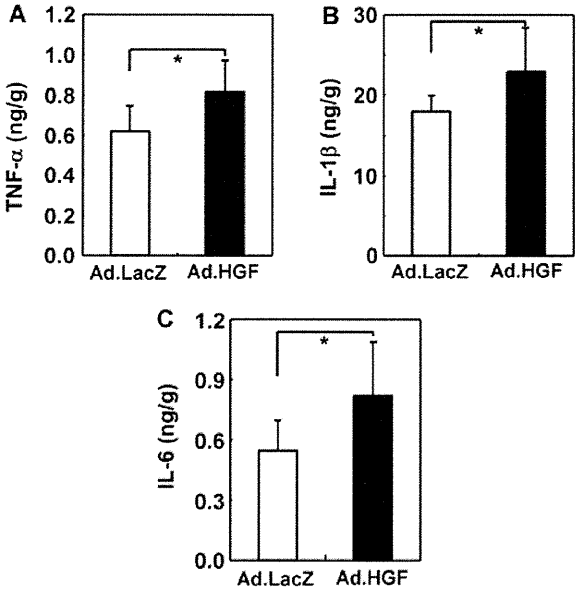

Figure 9. Effects of adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) on inflammatory cytokines in dextran sodium sulfate (DSS)-induced colitis. On day 5 of DSS administration, the expression of inflammatory cytokines was evaluated by enzyme-linked immunosorbent 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.

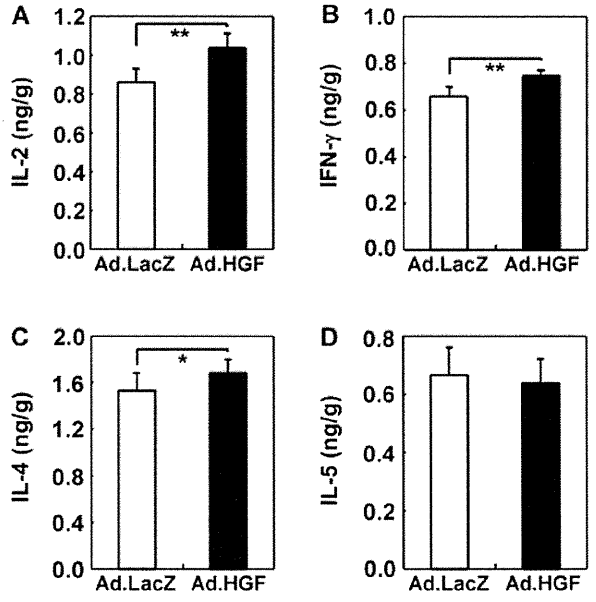

Figure 10. Effects of adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) on Th1 and Th2 cytokines in dextran sodium sulfate (DSS)-induced colitis. The expression of the Th1 [(A) interleukin (IL)-2 and (B) interferon (IFN)-γ] and Th2 [(C) IL-4 and (D) IL-5] cytokines 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

airway hyper-responsiveness, inflammation, remodeling, and eosinophil function in asthma (51). Okunishi *et al* (52) reported that HGF suppresses antigen-induced T-cell priming by regulating the functions of dendritic cells through IL-10 downregulation in the antigen-sensitization phase. By contrast, they found that repeated treatment with HGF induced Th2 immune responses with the upregulation of IL-10 by DCs in the chronic inflammation phase of a mouse model of collagen-induced arthritis. Thus, it is clear that HGF induces various immune responses in different disease models. However, further analysis is required to clarify the effects of HGF on the immune system.

In conclusion, we have shown that a single round of intramuscular injections of adenoviral hHGF is sufficient to inhibit apoptosis and reconstitute the epithelium in a mouse model of DSS-induced colitis. Based on these results, this approach shows promise for clinical application in IBD.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We would like to thank Hatsue Oshika for her technical assistance.

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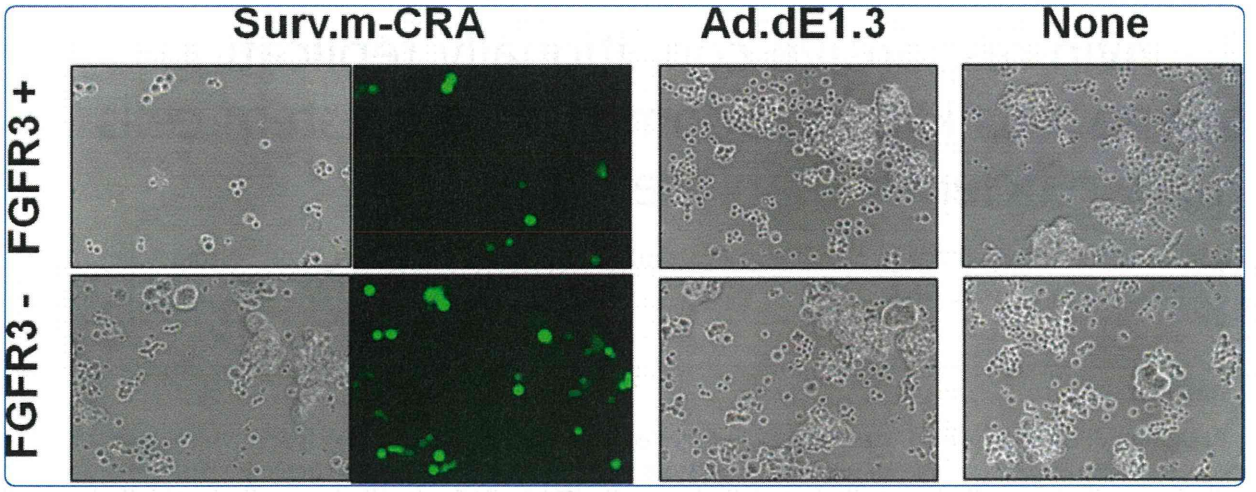

Survivin-responsive conditionally replicating adenovirus kills rhabdomyosarcoma stem cells more efficiently than their progeny

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Survivin-responsive conditionally replicating adenovirus kills rhabdomyosarcoma stem cells more efficiently than their progeny

Kiyonori Tanoue[1,2], Yuqing Wang[1], Minako Ikeda[1], Kaoru Mitsui[1], Rie Irie[1], Takao Setoguchi[3], Setsuro Komiya[4], Shoji Natsugoe[2] and Ken-ichiro Kosai[1*]

Abstract

Background: Effective methods for eradicating cancer stem cells (CSCs), which are highly tumorigenic and resistant to conventional therapies, are urgently needed. Our previous studies demonstrated that survivin-responsive conditionally replicating adenoviruses regulated with multiple factors (Surv.m-CRAs), which selectively replicate in and kill a broad range of cancer-cell types, are promising anticancer agents. Here we examined the therapeutic potentials of a Surv.m-CRA against rhabdomyosarcoma stem cells (RSCs), in order to assess its clinical effectiveness and usefulness.

Methods: Our previous study demonstrated that fibroblast growth factor receptor 3 (FGFR3) is a marker of RSCs. We examined survivin mRNA levels, survivin promoter activities, relative cytotoxicities of Surv.m-CRA in RSC-enriched (serum-minus) vs. RSC-exiguous (serum-plus) and FGFR3-positive vs. FGFR3-negative sorted rhabdomyosarcoma cells, and the *in vivo* therapeutic effects of Surv.m-CRAs on subcutaneous tumors in mice.

Results: Both survivin mRNA levels and survivin promoter activities were significantly elevated under RSC-enriched relative to RSC-exiguous culture conditions, and the elevation was more prominent in FGFR3-positive vs. FGFR3-negative sorted cells than in RSC-enriched vs. RSC-exiguous conditions. Although Surv.m-CRA efficiently replicated and potently induced cell death in all populations of rhabdomyosarcoma cells, the cytotoxic effects were more pronounced in RSC-enriched or RSC-purified cells than in RSC-exiguous or progeny-purified cells. Injections of Surv.m-CRAs into tumor nodules generated by transplanting RSC-enriched cells induced significant death of rhabdomyosarcoma cells and regression of tumor nodules.

Conclusions: The unique therapeutic features of Surv.m-CRA, *i.e.*, not only its therapeutic effectiveness against all cell populations but also its increased effectiveness against CSCs, suggest that Surv.m-CRA is promising anticancer agent.

Keywords: Cancer stem cells, Conditionally replicating adenovirus, Fibroblast growth factor receptor 3, Gene therapy, Oncolytic adenovirus, Promoter, Rhabdomyosarcoma, Survivin, Tumor-initiating cell, Virotherapy

Background

Accumulating data have suggested that cancer stem cells (CSCs), also called tumor-initiating cells, are a small but specialized population of tumor cells that possess high capacity for tumor initiation, invasion, and metastasis, as well as for self-renewal [1]. After most cells in the tumor are killed by conventional chemotherapy or radiotherapy, residual CSCs are believed to give rise to the bulk populations of tumor-cell progeny and recapitulate the original tumor nodule [2]. From the standpoints of clinical oncology and therapeutics, the most critical feature of CSCs is that they are highly resistant to conventional chemoradiotherapies [3,4], because they are predominantly in a dormant or slow-growing phase of the cell cycle [3] and they express high levels of multiple drug-resistance transporters [4]. Because the poor prognosis of patients with malignant tumors is caused, at least in part, by CSCs, the development of effective therapies against CSCs is urgently needed.

Rhabdomyosarcoma is the most common soft-tissue malignancy in children and adolescents [5]. Metastatic rhabdomyosarcoma is often incurable, and is associated with poor prognosis; approximately 20% of rhabdomyosarcoma

* Correspondence: kosai@m2.kufm.kagoshima-u.ac.jp
[1]Department of Gene Therapy and Regenerative Medicine, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan
Full list of author information is available at the end of the article

patients have disseminated disease at the time of diagnosis [6]. Whereas current treatment for rhabdomyosarcoma relies on chemotherapy, the cytotoxic actions of chemotherapeutic agents are not only ineffective but also non–tumor-specific in treatment of advanced and metastatic tumors. Therefore, these agents can impair normal development and cause secondary cancers in some growing children [5]. To develop a novel and innovative therapy against malignant rhabdomyosarcoma, we previously identified rhabdomyosarcoma stem cells (RSCs) and showed that fibroblast growth factor receptor 3 (FGFR3) is a marker of RSCs [7]. For instance, implantation of a single FGFR3-positive KYM-1 rhabdomyosarcoma cell can form a tumor nodule *in vivo* consisting of histologically defined rhabdomyosarcoma cells, whereas a single FGFR3-negative cell cannot form such nodules [7]. Likewise, the careful analyses in our previous study characterized FGFR3-positive rhabdomyosarcoma cells as RSCs.

Conditionally replicating adenoviruses (CRAs), also called oncolytic adenoviruses, replicate predominantly in tumor cells, which they kill via apoptosis mediated by adenoviral proteins; therefore, CRAs are promising anticancer agents [8,9]. We previously developed a method to efficiently construct diverse CRAs that can specifically target and/or efficiently treat malignant tumors using multiple factors (m-CRAs) [10]. Our m-CRA construction system expedited the process of generating, modifying, and testing diverse m-CRAs with the goal of developing an ideal m-CRA for tumor therapy; indeed, our m-CRA strategy increased the potential cancer specificity of virotherapy [10-12]. Survivin, a new member of the inhibitor of apoptosis (IAP) gene family, is expressed at high levels in cancerous but not normal tissues, and high survivin expression levels are positively correlated with poor prognosis, an accelerated rate of recurrence, and increased resistance to therapy in cancer patients [13,14]. We developed several types of survivin-responsive m-CRAs (Surv.m-CRAs) in which adenoviral E1A was regulated by the promoter of survivin; in some versions of these viruses, the p53-binding domain in E1B was deleted (*i.e.*, E1B55KD), the Rb-binding domain in E1A was deleted, or the native E1B promoter was replaced with another cancer-specific promoter [11,12]. All Surv.m-CRAs induced potent *in vitro* and *in vivo* cytotoxic effects against a variety of malignant tumors, and exhibited stronger and more cancer-selective phenotypes than telomerase reverse transcriptase (Tert)-responsive m-CRAs (Tert.m-CRAs), which are currently among the best CRAs [11,12]. Furthermore, certain types of Surv.m-CRAs significantly increased cancer specificity (*i.e.*, safety) without reduced anticancer effects [11].

CSCs are resistant to conventional chemoradiotherapies, and the therapeutic potentials of Surv.m-CRAs against CSCs have not been well examined. In order to evaluate the clinical usefulness of Surv.m-CRAs against malignant and incurable tumors, it will be necessary to perform careful comparative studies of endogenous survivin expression levels, activity of transduced survivin promoters, and relative antitumor effects on CSCs and their progeny. More generally and importantly, it has not yet been clearly elucidated whether transcriptional targeting using CRAs is a useful strategy for treating CSCs. Because FGFR3-positive RSCs are a useful model for CSCs, we examined the biological features of survivin and compared the therapeutic potentials of Surv.m-CRA against RSCs and progeny tumor cells.

Methods

Cells and cell culture

KYM-1 cell lines were purchased from Health Sciences Research Resources Bank (Tokyo, Japan). KYM-1 cells were cultured in DMEM, supplemented with 10% FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). In some experiments, KYM-1 cells were cultured in serum-free S-Clone (Eidia Co., Ltd, Tokyo, Japan) containing 10 ng/ml basic fibroblast growth factor (bFGF).

Flow-cytometric analysis and cell sorting

Cells were conjugated with anti-FGFR3 antibody (R & D, Minneapolis, MN, USA) for 30 min on ice. Cells were resuspended in the same buffer at 1.0×10^7 per ml, and then kept on ice until analysis. Flow-cytometric analysis was performed using CyAn™ ADP (Beckman Coulter, Fullerton, CA, USA). For further analyses, cells were sorted using a FACSAriaII (BD Biosciences, San Jose, CA) to isolate pure populations of FGFR3-positive and FGFR3-negative cells.

Generation of adenoviruses

The following viruses were propagated and purified as described previously [15-19]: E1-deleted replication-defective adenoviruses; two types of Ads-LacZ that expressed the LacZ gene under the transcriptional control of the Rous sarcoma virus long terminal repeat (RSV promoter) (Ad.RSV-LacZ) or the survivin promoter (Ad.Surv-LacZ); two types of Ads-EGFP that expressed the enhanced green fluorescent protein (EGFP) gene under the cytomegalovirus immediate early gene enhancer/promoter (CMV promoter) (Ad.CMV-EGFP) or the cytomegalovirus enhancer and β-actin promoter (CA promoter) (Ad.CA-EGFP); Ad.dE1.3 that expressed no gene, and Ad.CA-EGFP/RGD, in which an Arg-Gly-Asp (RGD)-containing peptide was added to the HI loop of the fiber-knob domain of Ad.CA-EGFP. Surv.m-CRA with wild-type E1A downstream of the survivin promoter, E1B55KD downstream of the CMV promoter, and the EGFP gene downstream of the CMV promoter was generated as described previously and used for this study [10-12].

Adenoviral gene transduction efficiencies

The adenoviral gene transduction efficiency (AGTE) for each cell type *in vitro* was assessed by infecting cells with Ad.CMV-EGFP at several different multiplicities of infection (MOIs), detaching the cells 48 h after infection, and analyzing the percentage of EGFP-positive cells by flow cytometry [20].

Promoter activities

Promoter activities were examined as described previously with some modification [15,21]. Briefly, cells (8×10^5 cells per plate) were infected with Ad.Surv-LacZ or Ad.RSV-LacZ at an MOI of 30 for 1 h, and then incubated with fresh media. The cells were collected 48 h post-infection, and β-gal activity was measured using the β-Galactosidase Enzyme Assay System (Promega, Madison, WI, USA) as described previously [15,21]. In addition, expression levels of β-galactosidase in individual KYM-1 cells were examined by flow cytometry using the FluoReporter lacZ Flow Cytometry Kit (Molecular Probes, Leiden, The Netherlands).

Real time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis

RNA was isolated using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) or the CellAmp Direct RNA Prep Kit (Takara Bio Inc., Ootsu, Japan), and was subsequently reverse-transcribed using the PrimeScript II First Strand cDNA Synthesis Kit (Takara Bio Inc.) [22,23]. RT-PCR using QuantiFast SYBR Green PCR (Qiagen, Venlo, The Netherlands) was performed on a Rotor Gene RG-3000 (Qiagen). The relative mRNA expression levels were determined by the comparative C_t method; expression levels of individual genes were normalized against the levels of the reference gene *HPRT*, which encodes hypoxanthine guanine phosphoribosyl transferase. The following primer sets and annealing temperatures were used: survivin, 5- CCAGTGTTTCTTCTGCTTCAA-3 and 5-GAATG CTTTTTATGTTCCTCTATG-3 at 60°C; *HPRT*, 5- TGA CCTTGATTTATTTTGCATACC-3 and 5-CTCGAGCA AGACGTTCAGTC-3 at 60°C [11,12].

Cytotoxic effects *in vitro*

Cells in 96-well plates were infected with each adenovirus at an MOI of 1, and cell viability was determined after 3 and 5 days using the WST-8 assay (Dojindo Laboratories, Mashiki, Japan) as described previously [11,12,24].

Therapeutic effects *in vivo* in animal experiments

KYM-1 cells (1×10^6 cells), which had been cultured in serum-minus media containing S-Clone and 10 ng/ml bFGF, were mixed with Matrigel (BD Biosciences) and subcutaneously inoculated into 5-week-old BALB/c nude mice. After a tumor nodule reached 6–10 mm in diameter, the mice were randomly divided into three groups. On day 0, a mouse in each group was given a single intratumoral injection of 150 μL of buffer (10 mmol/L Tris–HCl pH 7.4, 1 mmol/L $MgCl_2$, 10% glycerol, and 20 μg/mL hexadimethrine bromide) containing 1×10^9

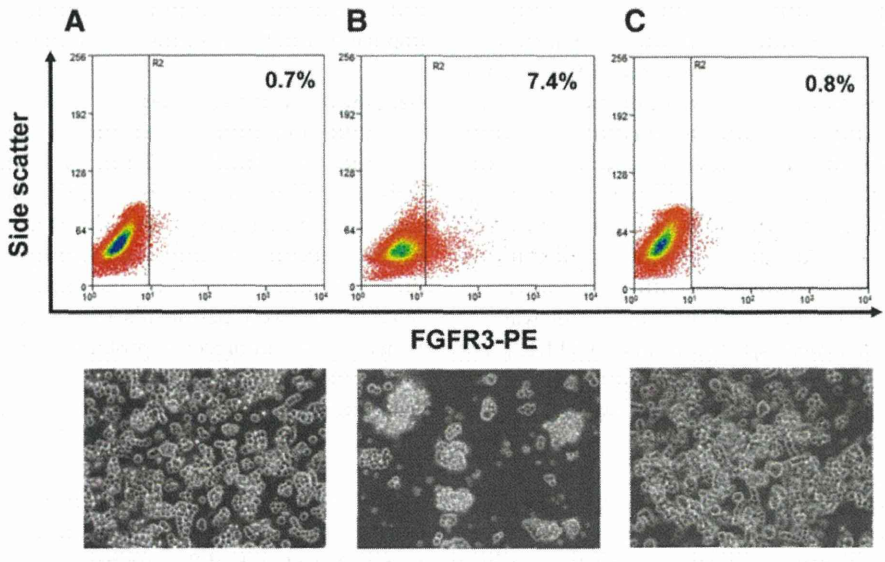

Figure 1 The percentage of FGFR3-positive cells determined by flow-cytometric analysis (upper), and microscopic image of KYM-1 cells (200× magnification; lower) in each culture condition. KYM-1 cells were cultured in serum-plus media **(A)**, then cultured for 3 weeks in serum-minus media containing 10 ng/ml bFGF **(B)**, and subsequently cultured in the serum-plus media for 1 week **(C)**. The percentages of FGFR3-positive cells and spheres were significantly increased by the serum-minus culture and decreased by serum-plus culture.

plaque-forming units (pfu) of Surv.m-CRA (n = 6), Ad. dE1.3 (n = 7), or phosphate-buffered saline (PBS) (n = 8). Subsequently, tumor size was measured twice a week, and tumor volume was calculated according to the following formula: volume = long axis × (short axis)^2 × 0.5.

For histopathologic analysis, tumors were fixed in 10% buffered formalin, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin.

All animal studies were performed in accordance with National Institutes of Health guidelines and with the approval of the Division of Laboratory Animal Science, Natural Science Center for Research and Education, Kagoshima University. All reasonable efforts were made to minimize suffering.

Statistical analysis

Data were represented as the means ± standard errors (s.e.). Statistical significance was determined using Student's *t* test. $P < 0.05$ was defined as statistically significant.

Results

RSCs can be maintained, expanded, and differentiated *in vitro*

We characterized FGFR3-positive KYM-1 cells as RSCs in a previous study [7]. Therefore, in this study, we first reproduced two distinct culture conditions that successfully induced FGFR3-positive RSC-enriched and FGFR3-negative RSC-exiguous cell fractions *in vitro*. Flow-cytometric analysis revealed that FGFR3-positive cells accounted for only 0.7% of the population when cells were cultured in regular serum-plus media (Figure 1A). However, the percentage of FGFR3-positive cells significantly increased, to 7.4% of the population, after 3-week culture in serum-minus media (Figure 1B). The population of FGFR3-positive cells was drastically decreased to 0.8%, *i.e.*, almost to the original level, only 1 week after the RSC-enriched cell fractions were returned to culture in regular serum-plus media (Figure 1C). Sphere formation, which represented clonogenic growth of the floating cells and was therefore a useful indication of enrichment of CSCs, was observed

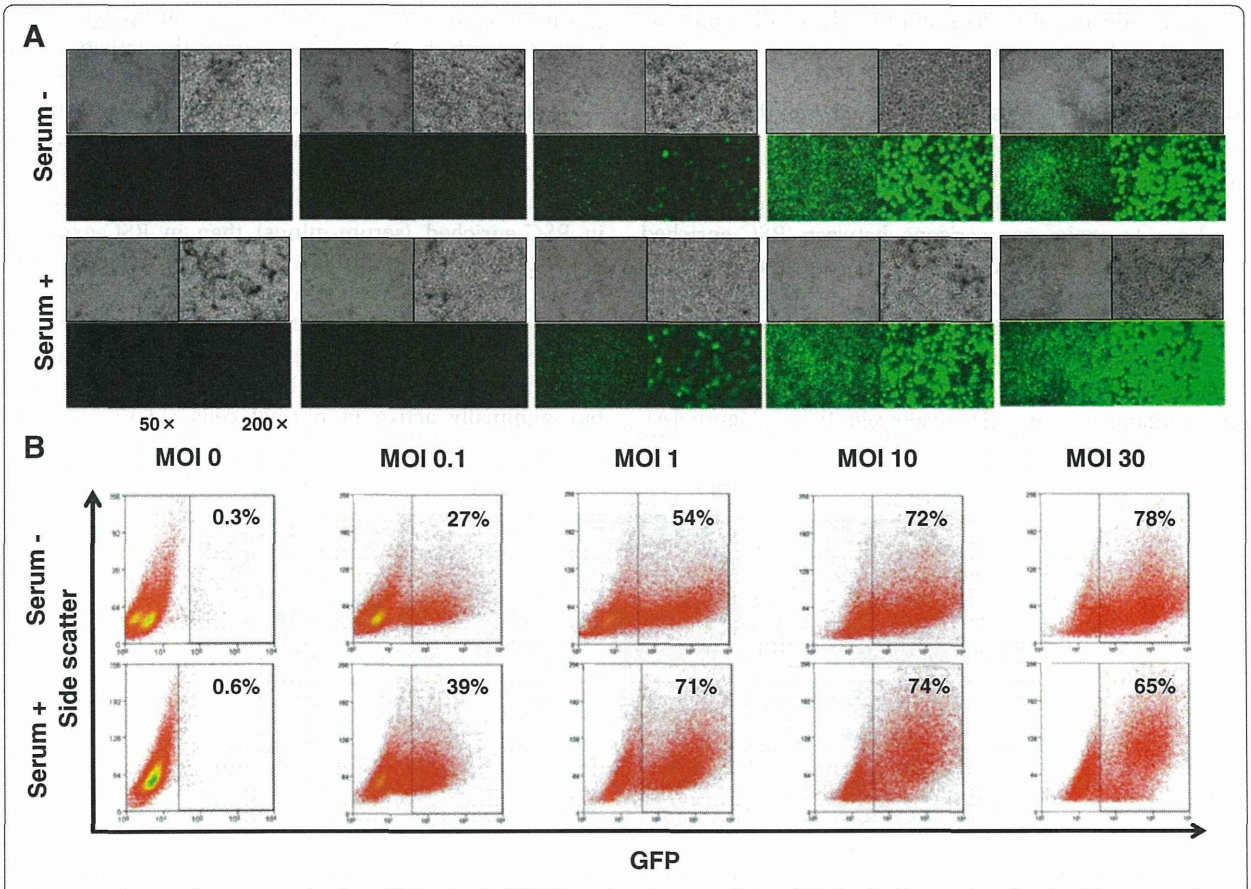

Figure 2 Adenoviral gene transduction efficiencies (AGTEs) in each culture condition. Cells that had been cultured under either serum-minus or serum-plus conditions were infected for 48 h with Ad.CMV-EGFP at MOI of 0 (none), 0.1, 1, 10, or 30. Subsequently, fluorescence-microscopic analysis **(A)** and flow-cytometric analysis **(B)** were performed to assess EGFP-expressing cells. **(A)** Representative images of phase-contrast (upper) and the fluorescence (lower) microscopy are shown at 50× (left) and 200× (right) magnification. No difference in AGTEs between the two groups was apparent.

solely in the serum-minus condition. Thus, we established two distinct *in vitro* conditions that allow accurate assessments of survivin and Surv.m-CRAs in RSCs and their progeny.

AGTEs in KYM-1 cells

KYM-1 cells, cultured in either serum-minus or serum-plus media, were infected with Ad.CMV-EGFP at various MOIs and analyzed by fluorescence microscopy or flow cytometry to assess AGTEs (Figure 2). AGTEs in KYM-1 cells were higher than those in other previously examined cell types [11,12]; however, the difference in AGTEs between RSC-enriched and RSC-exiguous cells was not significant, or at least was not drastic. We further compared AGTEs between infections with Ad.CA-EGFP and Ad.CA-EGFP/RGD, because a number of previous studies demonstrated that modification of the fiber knob with the RGD peptide increased AGTEs in some cell types [19]. Fiber modification did not drastically increase or change AGTEs in KYM-1 cells, either in RSC-enriched or RSC-exiguous fractions (Figure 3). Based on these results, we decided to use replication-defective adenoviral vectors and m-CRAs with wild-type fibers for subsequent experiments.

Higher expression levels of *survivin* mRNA in the FGFR3-positive KYM-1 cell

To assess whether the expression levels of survivin mRNA were changed in RSCs, we performed qRT-PCR analyses to make comparisons between RSC-enriched (serum-minus) and RSC-exiguous (serum-plus) cell fractions and between sorted FGFR3-positive and FGFR3-negative cells. The RSC-enriched cell fraction exhibited a slight increase in survivin mRNA level; however, the difference between the RSC-enriched and RSC-exiguous cell fractions was not statistically significant (Figure 4A). We next examined survivin mRNA levels in sorted FGFR3-postive and FGFR3-negative cells, because such an examination in purified FGFR3-positive cells should make their characteristic phenotypes more apparent. In accordance with that speculation, survivin mRNA levels in sorted FGFR3-positive KYM-1 cells were significantly higher than in FGFR3-negative cells (Figure 4B). These results suggest that survivin is more highly expressed in FGFR3-positive RSCs than in FGFR3-negative progeny.

Higher activities of survivin promoter in FGFR3-positive RSCs

We examined the activities of the survivin and control RSV promoters using replication-defective adenoviral vectors with the LacZ gene downstream of each of these promoters. Flow-cytometric analysis revealed that almost 100% of cells expressed LacZ after Ad.RSV-LacZ infection, and that less than 0.6% of cells were nonspecifically positive after no infection or infection with Ad.dE1.3 (lacking the transgene); thus, the experimental conditions were appropriate (Figure 4C). The percentages of cells expressing LacZ under control of the survivin promoter were 8.3 and 5.6% under the RSC-enriched (serum-minus) and RSC-exiguous (serum-plus) conditions, respectively. Next, we examined the activity of the survivin promoter relative to that of the RSV promoter, a representative control promoter with constitutively strong activity, by measuring β-galactosidase activities after infection with Ad.Surv-LacZ (Figure 4D and E). The survivin promoter activity was significantly higher in RSC-enriched (serum-minus) than in RSC-exiguous (serum-plus) conditions, and also significantly higher in sorted FGFR3-positive than in FGFR3-negative cells. This result, together with the same tendency in endogenous survivin gene expression, suggests that the survivin promoter, which is highly active in tumor cells but minimally active in normal cells [11,12], is more

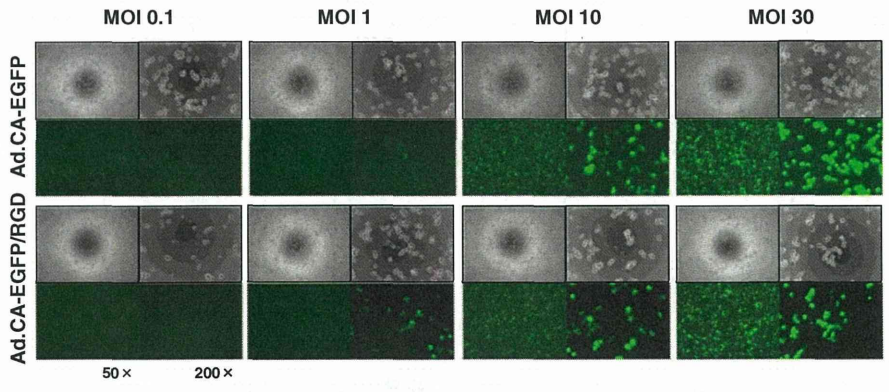

Figure 3 AGTEs of adenoviral vectors with wild or modified fiber. KYM-1 cells were infected with either Ad.CA-EGFP or Ad.CA-EGFP/RGD, which have wild-type or modified fiber (RGD-peptide added to the fiber knob), respectively, at an MOI of 0.1, 1, 10, or 30, and then observed under phase-contrast and fluorescence microscopy 48 h later. Representative phase-contrast (upper) and fluorescence (lower) images are shown at 50× (left) and 200× (right) magnification. There was no apparent difference in AGTEs between the two groups.