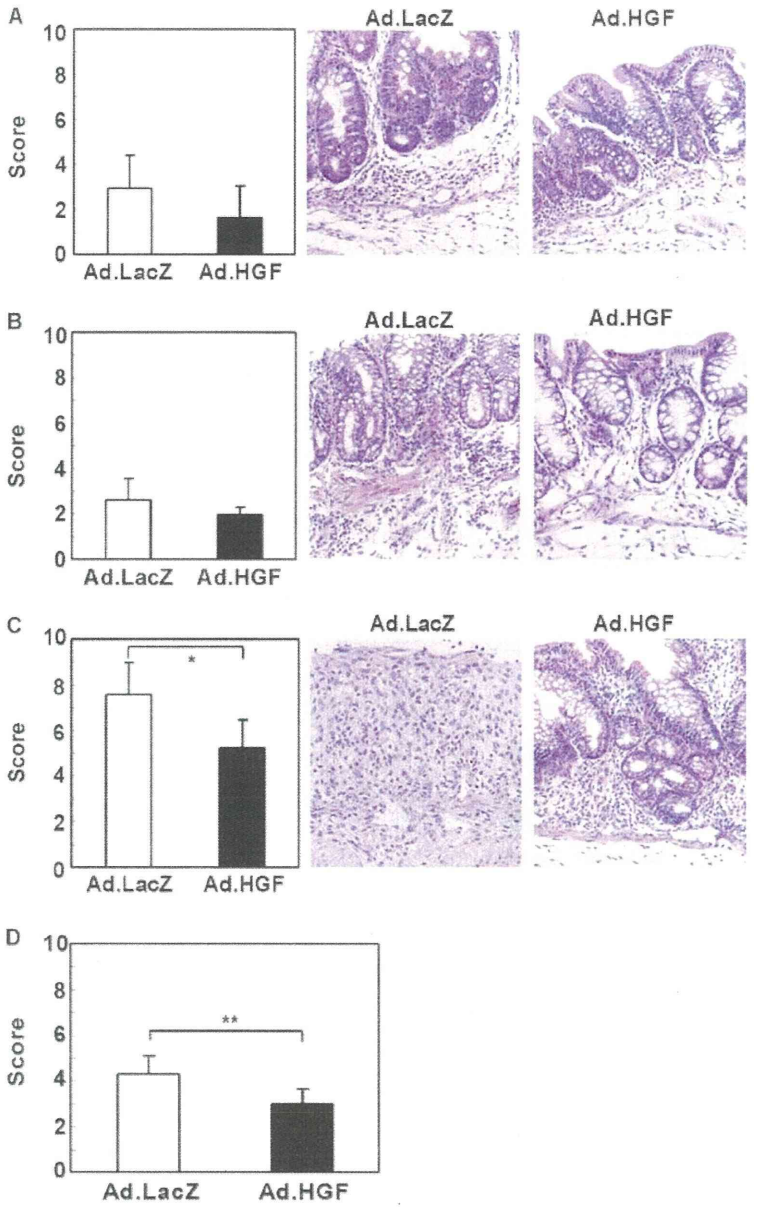

Figure 4. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) decreased colon inflammation in dextran sodium sulfate (DSS)-induced colitis. (A) Cecum, (B) proximal, (C) distal, and (D) total colon samples from the anal ring were used for histological evaluation. Colonic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

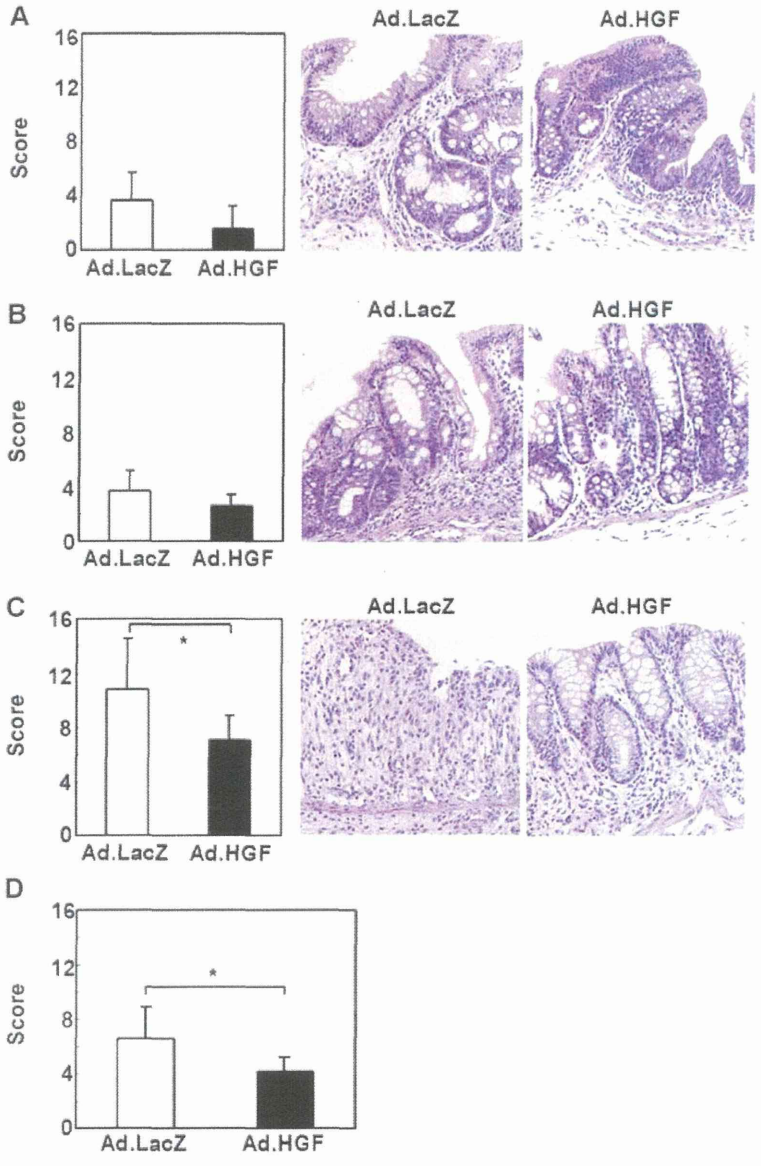

Figure 5. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) prevented crypt destruction in dextran sodium sulfate (DSS)-induced colitis. (A) Cecum, (B) proximal, (C) distal, and (D) total colon samples from the anal ring were used for histological evaluation. Colonic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

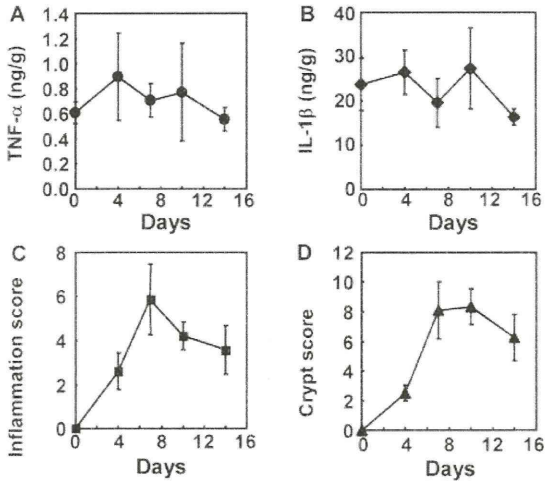

Figure 6. Expression of tumor necrosis factor (TNF)-α and interleukin (IL)-1β, and inflammation and crypt scores, in dextran sodium sulfate (DSS)-induced colitis. Twenty mice were given distilled drinking water containing 5% DSS for 7 days and 1% DSS for 7 days, *ad libitum*. Five mice were sacrificed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

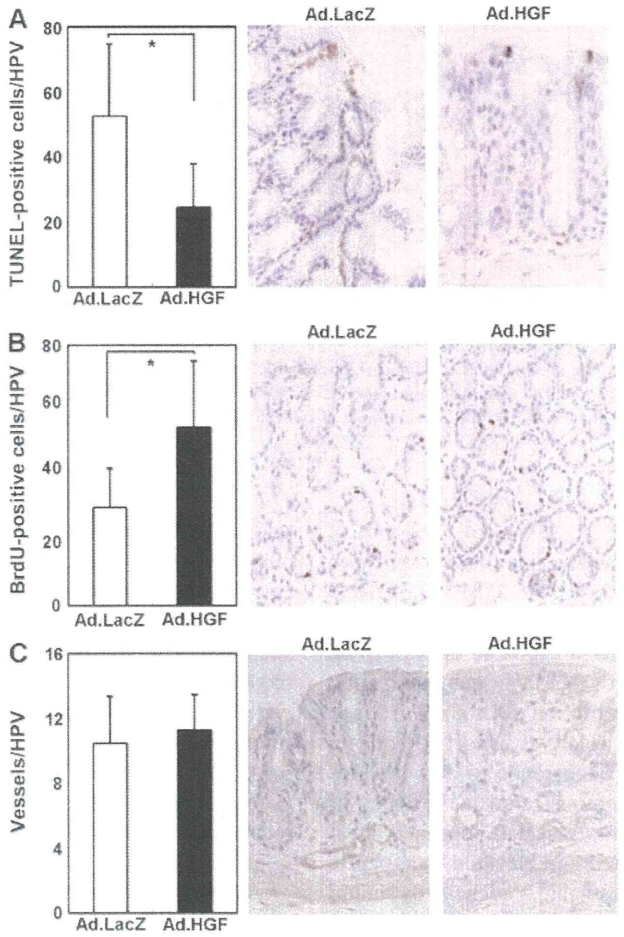

Figure 7. Adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) prevented apoptosis and stimulated intestinal epithelial regeneration in dextran sodium sulfate (DSS)-induced colitis. Colon tissues were stained by immunohistochemistry (representative histopathological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

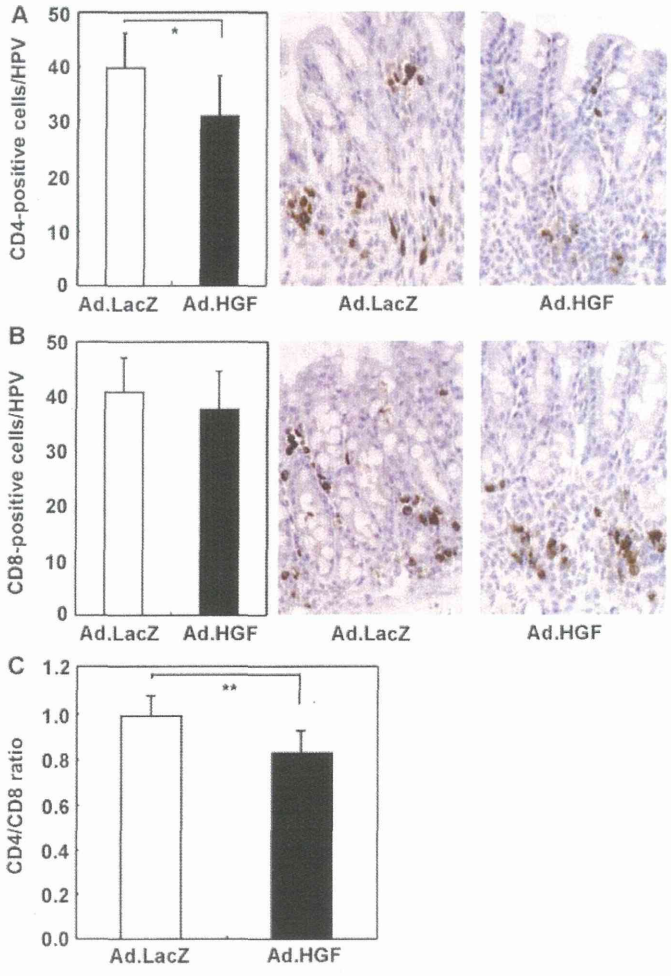

Figure 8. Effects of adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) on inflammatory cells in dextran sodium sulfate (DSS)-induced colitis. (A) CD4 immunostaining of the distal colon. (B) CD8 immunostaining of the distal colon. The two images on the right are representative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

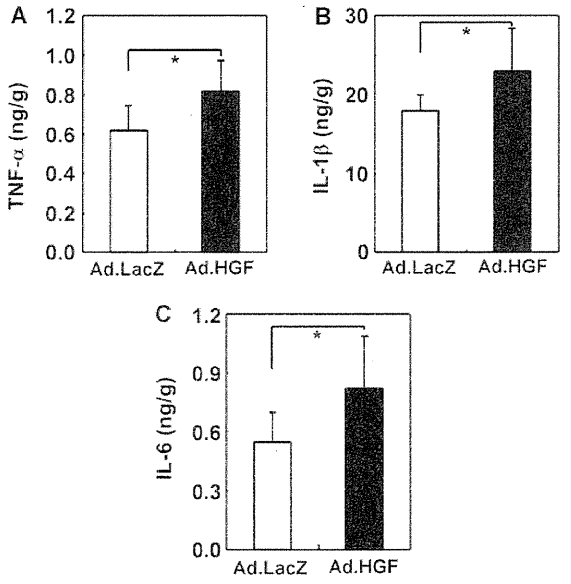

Figure 9. Effects of adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) on inflammatory cytokines in dextran sodium sulfate (DSS)-induced colitis. On day 5 of DSS administration, the expression of inflammatory cytokines was evaluated by enzyme-linked immunosorbent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

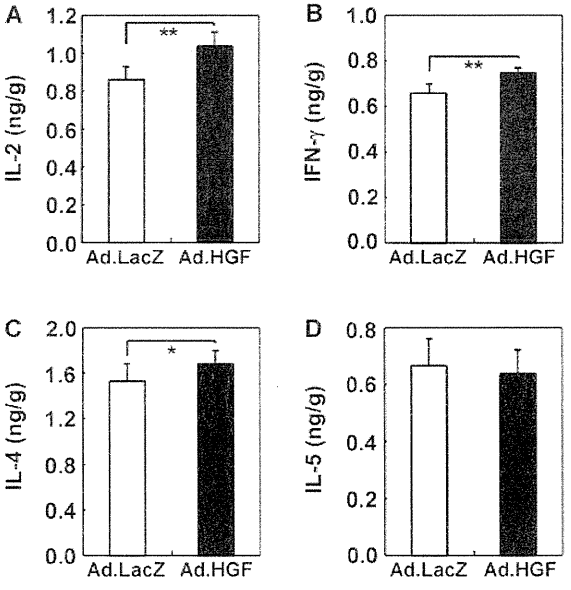

Figure 10. Effects of adenoviral human hepatocyte growth factor (hHGF) intramuscular gene transduction (IMGT) on Th1 and Th2 cytokines in dextran sodium sulfate (DSS)-induced colitis. The expression of the Th1 [(A) interleukin (IL)-2 and (B) interferon (IFN)-γ] and Th2 [(C) IL-4 and (D) IL-5] cytokines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

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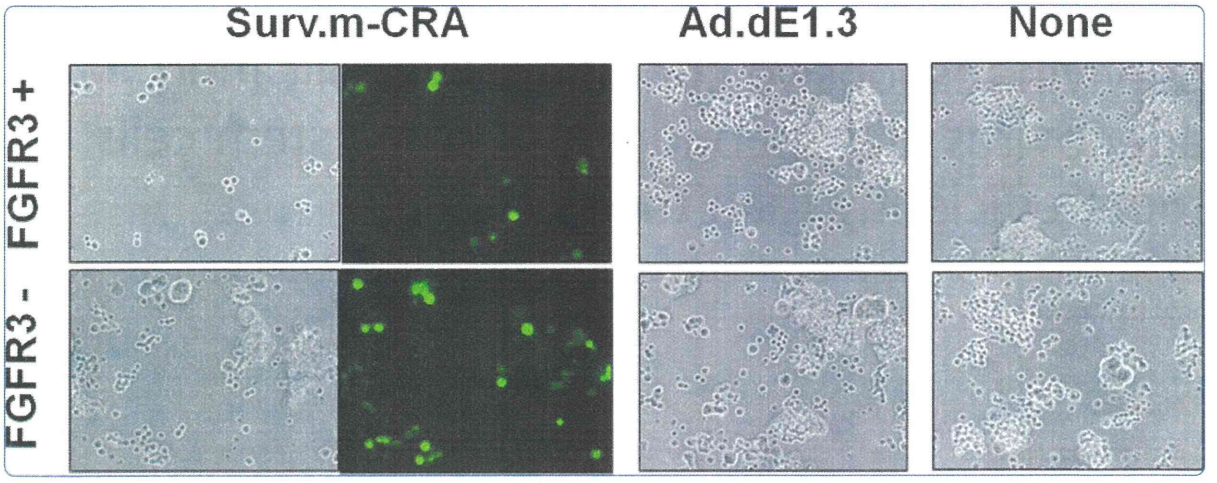

Survivin-responsive conditionally replicating adenovirus kills rhabdomyosarcoma stem cells more efficiently than their progeny

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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

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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].