Discussion

This study provides the first report of two *survivin*-responsive CRAs, both demonstrating efficient cancer-specific replication and potent therapeutic effects against cancers both *in vitro* and *in vivo*.

One of the attractive features of Surv.CRAs is their ability to target a variety of cancers. Surv.CRAs demonstrated efficient propagation and induced cell death in a wide variety of tumor cells with a variety of phenotypes, including low levels of *survivin* expression. The problem of low AGTE in certain cancer types is a critical issue in adenoviral gene therapy; Surv.CRAs are no exception. Further attempts should be made to improve adenoviral infectivity. Nevertheless, this study demonstrated that Surv.CRAs propagated even in cell types with low AGTE values, a promising result for the potential of these vectors as therapeutic agents. In addition, we intentionally used HOS-MNNG cells, which express *survivin* at relatively low levels and exhibit only moderate AGTE, for *in vivo* animal studies. The anti-cancer effect of Surv.CRAs under these conditions suggests that this agent may elicit therapeutic effects in many cancer types. Moreover, recent studies have detailed promising approaches to overcome the obstacle of low AGTE, such as fiber modification (31-33); these techniques could be also be

directly and feasibly applied to Surv.CRAs (22). Fiber-modified Surv.CRA may enhance the cancer-specificity and efficacy of this therapy for a broader range of cancer types and should be explored further.

Another crucial requirement for optimal CRA is attenuation of viral replication in normal cells. Currently, one of the best available CRAs may be Tert.CRA; TERT, the major determinant of telomerase activity, is expressed at high levels in many cancer cells, but not in normal cells (34). Several recent studies have demonstrated cancer-selective replication and anti-cancer effects of Tert.CRAs (18-20). After examining the endogenous expression levels and promoter activity of TERT in a variety of cancer and normal cells, we compared the viral replication of Surv.CRAwt to that of Tert.CRAwt in both cancer and normal cells. Surv.CRAwt showed greater promise; the replication of Surv.CRAs in normal cells was more attenuated than that of the Tert.CRA, whereas Surv.CRAwt was more efficient in replicating in two independent cancer cell types, including HOS-MNNG. As HOS-MNNG expressed *survivin* and TERT at low and high levels, respectively, it is likely that Surv.CRAs are superior to Tert.CRAs in both cancer-specificity and efficiency, although the general applicability of this trend will need to be confirmed in future studies.

Previous studies have not yet explored whether deletion of the RB-binding

domain when combined with the modulation of E1A expression using a tumor-specific promoter provides additional advantages or disadvantages over either approach alone (4). In this study, Surv.CRAmt did not provide an enhanced cancer-specificity or an attenuation of viral replication in normal cells, but also do not reduce viral replication in the cancer cells examined, including both RB-deficient and RB-intact tumor cells and normal fibroblast cells. The *survivin* promoter may confer such a high level of cancer specificity that these additional viral modifications do not provide a clear additional advantage. It is also possible that both RB-dependent and *survivin*-dependent cancer specificities target cell cycle-dysregulation; therefore, the cancer-specificity of RB- and *survivin*-dependent viruses may overlap to some extent. Future studies should be conducted to modify further the expression elements of other adenoviral genes using different promoters that target cancer-specific genetic events independent of cell-cycle dysregulation, because the replication of Surv.CRAs in normal cells was greatly attenuated, but not completely abrogated.

In conclusion, this study demonstrated the therapeutic potential of *survivin*-responsive CRAs; these Surv.CRAs confer cancer-specific replication and cytotoxicity, and thus may provide an attractive therapeutic agent for the treatment of cancer.

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Figure legend

Figure 1. *Survivin* mRNA expression (A) and promoter activity (B). (A) Endogenous *survivin* mRNA was detected by PCR. The hypoxanthine guanine phosphoribosyl transferase (HPRT) gene was amplified as an internal control. *Neg*, template was omitted from the reaction as a negative control. (B) ß-gal enzyme activity was detected 48 hours after infection with Ad.Surv-LacZ, Ad.CMV-LacZ, or Ad.RSV-LacZ at an MOI of 30. Each bar represents the mean ± SEM of three independent experiments. Adenoviral gene transduction efficiency (AGTE) is presented as the percentage of X-gal-stained cells observed among the total cells at 48 hours after Ad.CMV-LacZ infection at an MOI of 30.

Figure 2. Replication and cytotoxicity of Surv.CRAs in different cell lines. (A) Representative phase-contrast and fluorescent microscopic images (in the *upper* and the *lower* panels, respectively) are shown 1, 3, 5, and 7 days after infection with Surv.CRAwt or Surv.CRAmt at an MOI of 0.1. EGFP-positive cells increased in a time-dependent manner after infection with either Surv.CRAwt or Surv.CRAmt in all cancer cells examined; in contrast, no significant increases in EGFP-positive cells were

observed in normal WI-38 cells. The rate of spreading of EGFP-positive cells and CPE correlated well with the endogenous levels of expression of *surivin* and the AGTE levels, as shown in Fig. 1. (B) A high-power phase contrast microscopic image taken seven days after infection with control Ad.ΔE1 or Surv.CRAwt demonstrated that all of the LoVo and HepG2 cells underwent CPE after infection with Surv.CRAwt only. In contrast, no CPE was observed in WI-38 cells after infection with either adenovirus.

Figure 3. Flow cytometric analysis of EGFP-positive cells. HepG2, HOS-MNNG, and WI-38 cells were infected with Surv.CRAwt, Surv.CRAmt, or Ad.ΔE1 at an MOI of 0.1 (HepG2) or 1 (HOS-MNNG and WI-38). Twenty-four hours later, cells were fixed with 4% paraformaldehyde; the percentage of EGFP-positive cells was analyzed by flow cytometry. (A) Histograms of EGFP-positive cells (M1, negative; M2, positive) and dot-plots individually representing the EGFP-intensity and the forward scatter index are shown in the upper and the lower panels, respectively. (B) The percentages of EGFP-positive cells, shown as bars, represent the mean ± SEM of three independent experiments.

Figure 4. Cytotoxic effects *in vitro*. Cells were infected with Surv.CRAwt, Surv.CRAmt,

or Ad.ΔE1 at an MOI of 0.1 in HepG2 and Hep3B cells, MOIs of 1 and 10 in HOS-MNNG cells, and an MOI of 1 in WI-38 cells. Cell viability was determined by WST-8 assay three or five days after infection. Statistical significance was defined as $*P<0.05$ and $**P<0.001$ in comparison to the no infection control.

Figure 5. Therapeutic effects of Surv.CRAs *in vivo*. (A) Tumor volume was measured after a single injection of $1X10^8$ pfu Surv.CRAwt (n=9), Surv.CRAmt (n=8), or control Ad.ΔE1 (n=8) into pre-established subcutaneous tumors of HOS-MNNG cells in nude mice. Statistical significance was defined as $*P<0.05$ in comparison to infection with the control Ad.ΔE1. (B) Representative macroscopic pictures 14 days after injection of Ad.ΔE1, Surv.CRAwt, or Surv.CRAmt. Prominent tumor necrosis was apparent in Surv.CRAwt- and Surv.CRAmt-treated masses. (C) Representative histological images at the time of sacrifice. H&E-stained sections exhibited large necrotic areas in the tumor nodules in mice treated with either Surv.CRA. In contrast, tumor nodules contained primarily viable tumor cells without large necrotic areas in the Ad.ΔE1-treated mice. Original magnification: X20 (*upper panel*) and X100 (*lower panel*). Both the macroscopic and microscopic pictures provide a more accurate assessment of the therapeutic potential of the Surv.CRAs than the simple assessment of tumor volume.

Figure 6. Comparison of Surv.CRA with Tert.CRA. (A) Endogenous TERT mRNA was detected by RT-PCR. (B) Relative activity of the *survivin* promoter and the TERT promoter to that of the CMV promoter. X-gal activity was determined 48 hours after infection with Ad.Surv-LacZ, Ad.Tert-LacZ, or Ad.CMV-LacZ at an MOI of 30. (C) The rate of viral propagation was assessed by flow cytometric analysis, expressed as the percentage of EGFP-positive cells 1, 3, 5, and 7 days after infection with either adenoviruses at an MOI of 0.03 in HepG2 cells and an MOI of 0.1 in HOS-MNNG and WI-38 cells. $*P<0.05$. Both Surv.CRAwt and Tert.CRAwt rapidly replicated between five and seven days after infection of HepG2 cells at this MOI; infected cells detached from the culture dishes at seven days post infection (#). (D) Tumor volume was measured after a single injection of $1x10^8$ pfu Surv.CRAwt (n=8), Tert.CRAwt (n=9), or control Ad.ΔE1 (n=11) into pre-established subcutaneous HOS-MNNG tumors in nude mice. Statistical significance was defined as $*P<0.05$ and $**P<0.005$ in comparison to control Ad.ΔE1.

Fig. 1

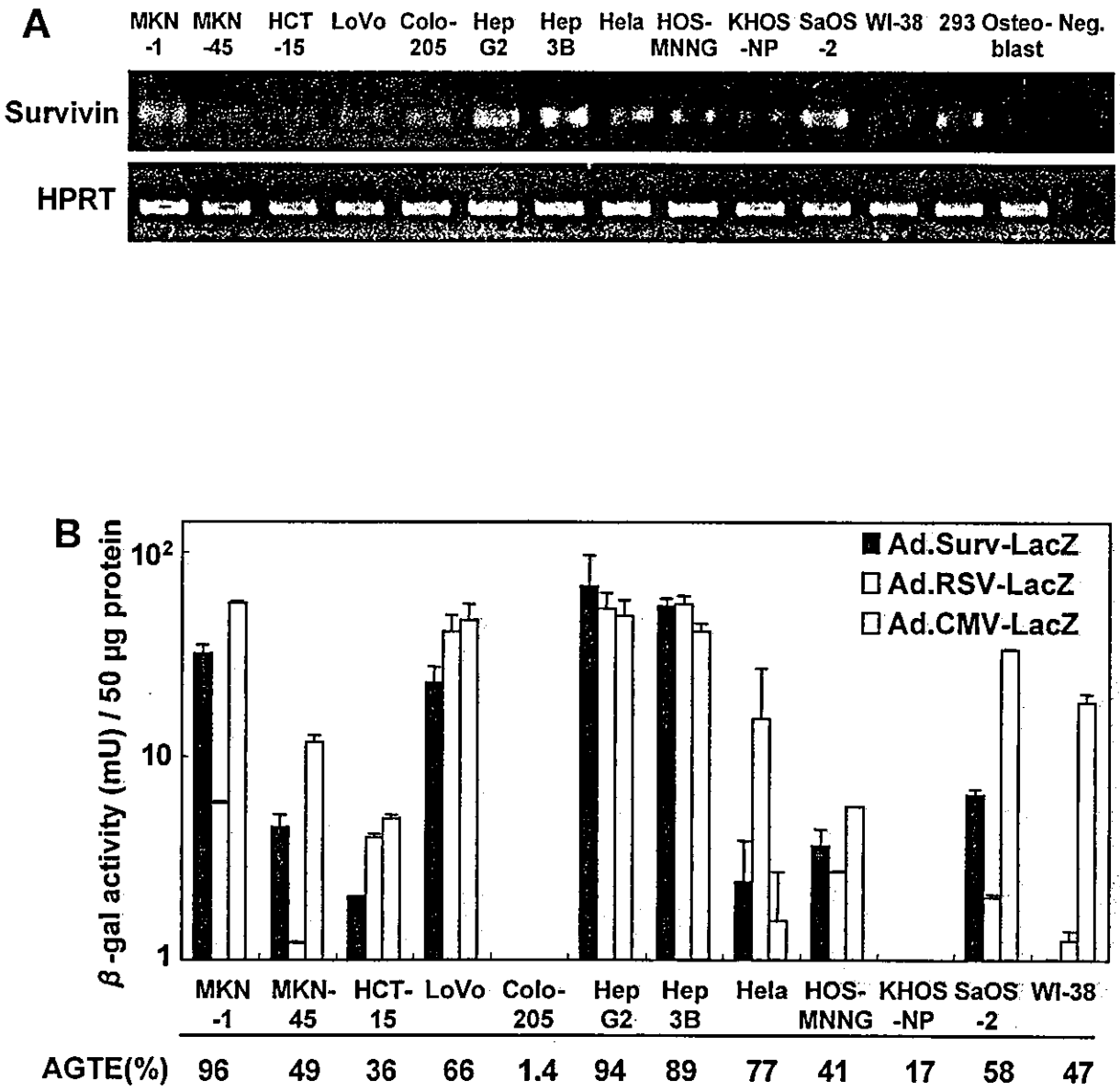

A
MKN -1
MKN -45
HCT -15
LoVo
Colo-205
Hep G2
Hep 3B
Hela
HOS-MNNG
KHOS -NP
SaOS -2
WI-38
293
Osteo-blast
Neg.
Survivin
HPRT
B
β-gal activity (mU) / 50 μg protein
10²
10
1
Ad.Surv-LacZ
Ad.RSV-LacZ
Ad.CMV-LacZ
MKN -1
MKN-45
HCT-15
LoVo
Colo-205
Hep G2
Hep 3B
Hela
HOS-MNNG
KHOS -NP
SaOS -2
WI-38
AGTE(%) 96 49 36 66 1.4 94 89 77 41 17 58 47

Fig. 2

Kamizono et al, Fig.2

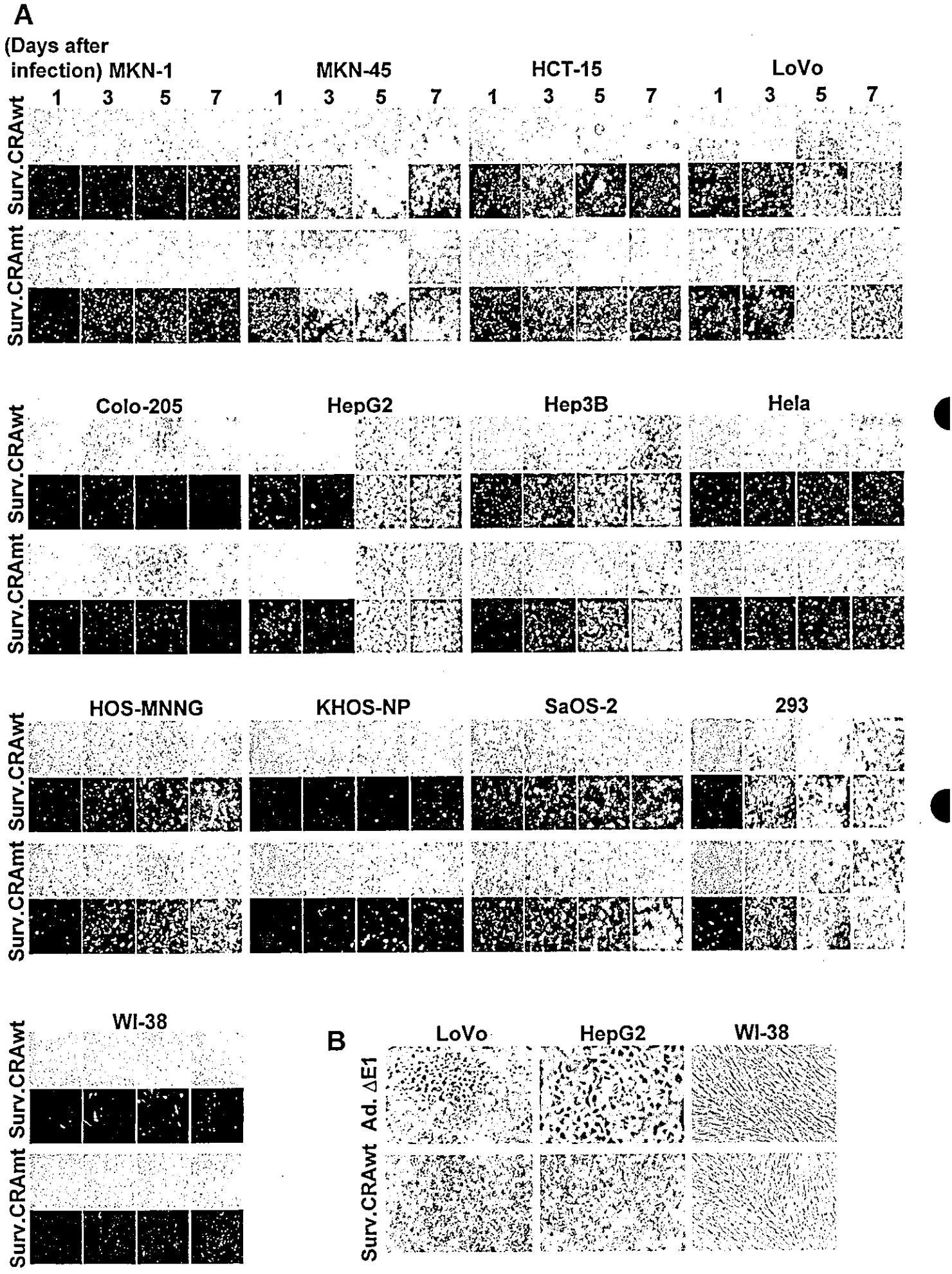


Fig. 3

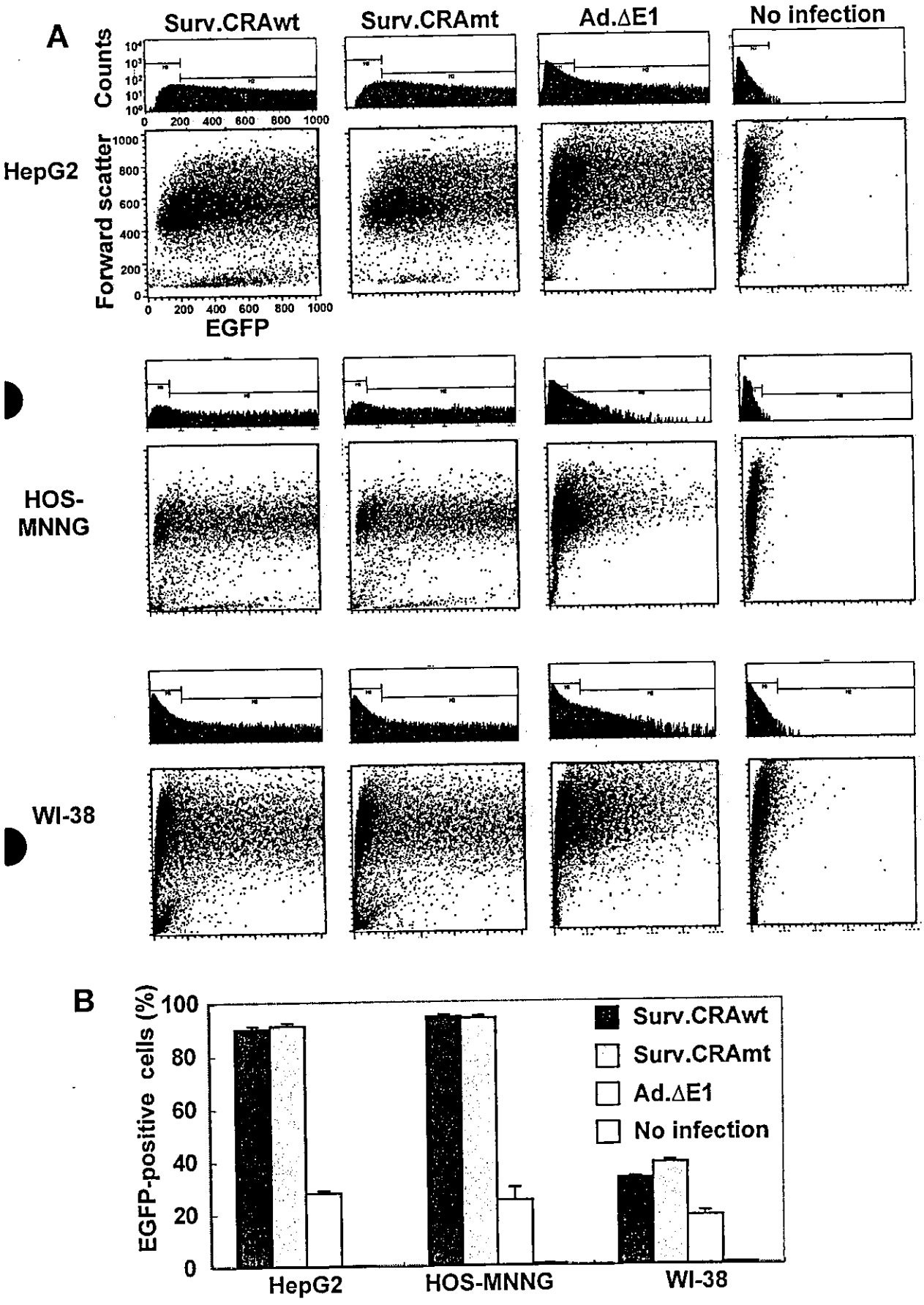

A
Surv.CRAwt
Surv.CRAmt
Ad.ΔE1
No infection
Counts
Forward scatter
EGFP
HepG2
HOS-MNNG
WI-38
B
EGFP-positive cells (%)
Surv.CRAwt
Surv.CRAmt
Ad.ΔE1
No infection
HepG2
HOS-MNNG
WI-38

Fig. 4

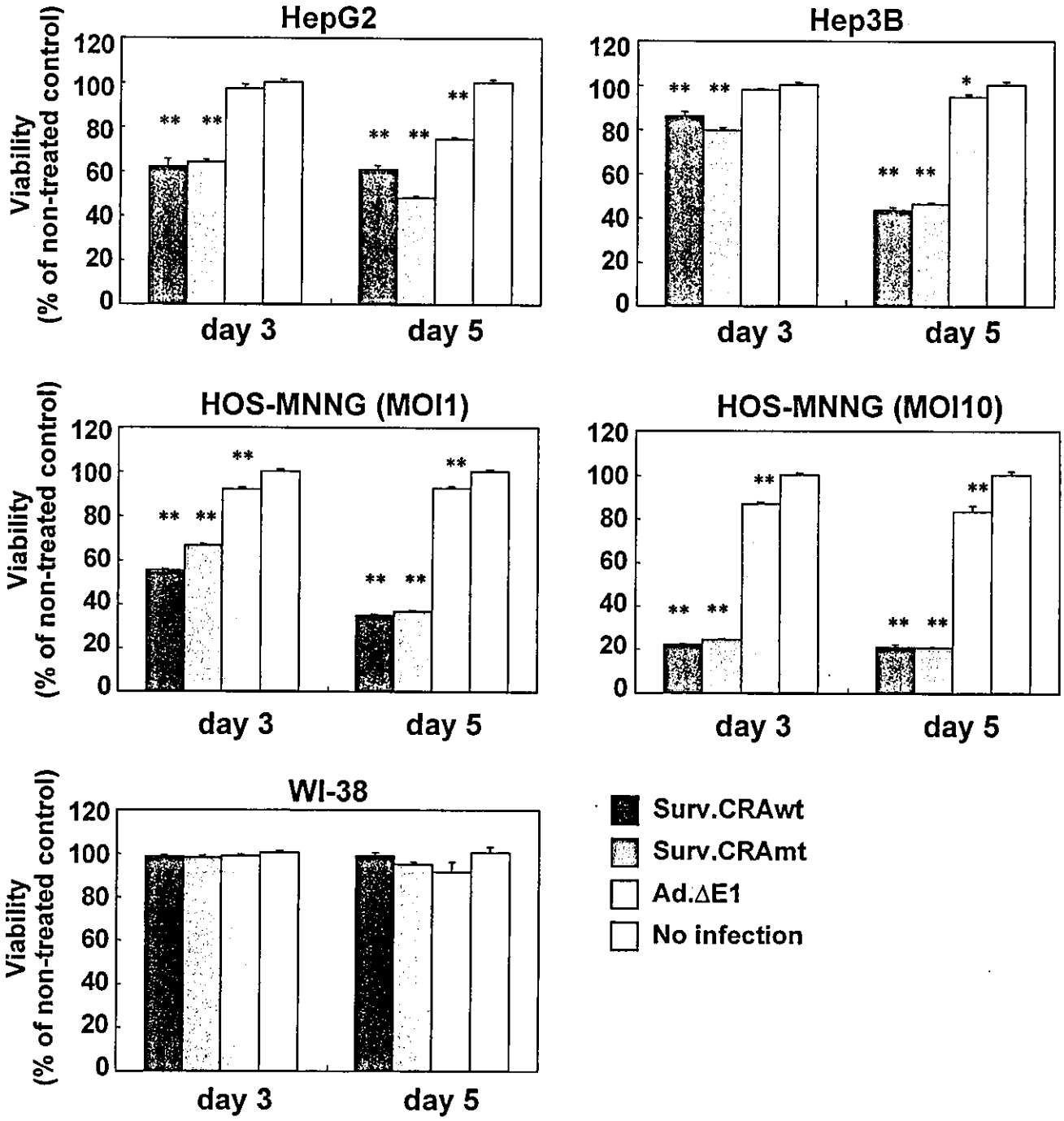

HepG2
Hep3B
HOS-MNNG (MOI1)
HOS-MNNG (MOI10)
WI-38
Viability
(% of non-treated control)
120
100
80
60
40
20
0
day 3
day 5
Surv.CRAwt
Surv.CRAmt
Ad.ΔE1
No infection

Fig. 5

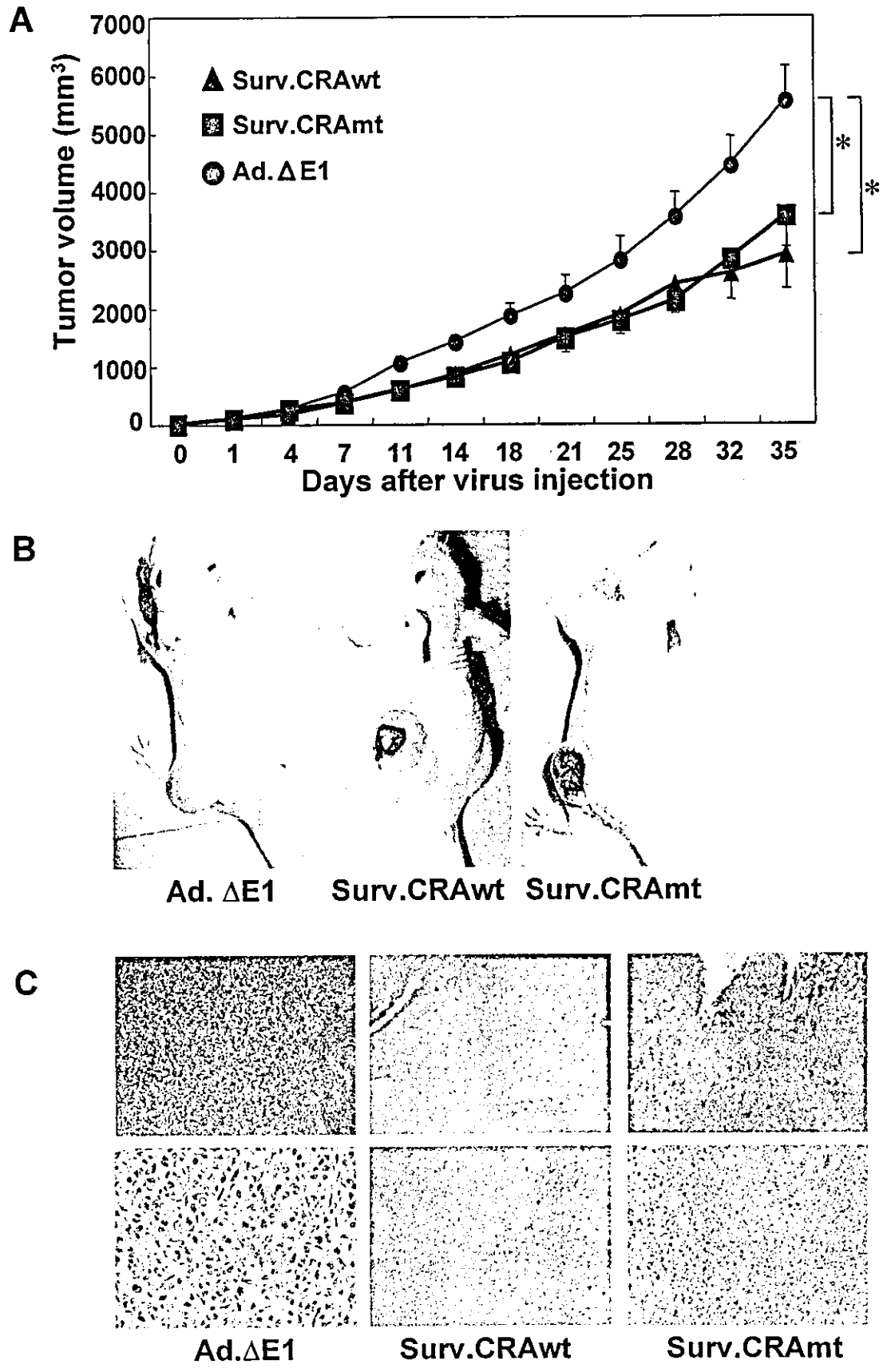
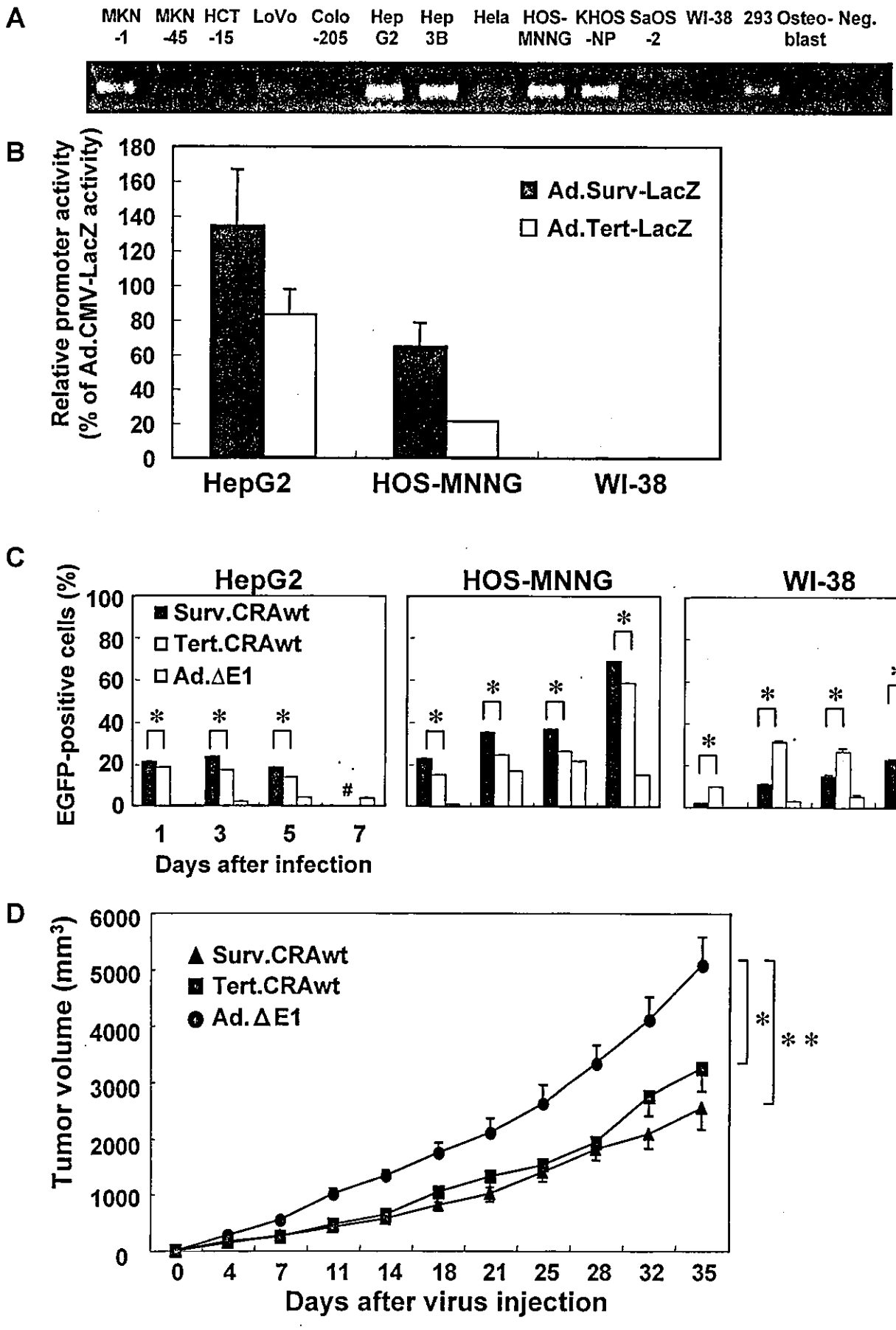

A
7000
6000
5000
4000
3000
2000
1000
0
Tumor volume (mm3)
Surv.CRAwt
Surv.CRAmt
Ad.ΔE1
*
*
0 1 4 7 11 14 18 21 25 28 32 35
Days after virus injection
B
Ad. ΔE1
Surv.CRAwt
Surv.CRAmt
C
Ad.ΔE1
Surv.CRAwt
Surv.CRAmt

Fig. 6


A
MKN -1
MKN -45
HCT -15
LoVo
Colo -205
Hep G2
Hep 3B
Hela
HOS-MNNG
KHOS -NP
SaOS -2
WI-38
293
Osteo-blast
Neg.
B
Relative promoter activity (% of Ad.CMV-LacZ activity)
180
160
140
120
100
80
60
40
20
0
Ad.Surv-LacZ
Ad.Tert-LacZ
HepG2
HOS-MNNG
WI-38
C
HepG2
HOS-MNNG
WI-38
EGFP-positive cells (%)
100
80
60
40
20
0
Surv.CRAwt
Tert.CRAwt
Ad.ΔE1
*
#
1
3
5
7
Days after infection
D
Tumor volume (mm³)
6000
5000
4000
3000
2000
1000
0
Surv.CRAwt
Tert.CRAwt
Ad.ΔE1
*
**
0
4
7
11
14
18
21
25
28
32
35
Days after virus injection

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Adenoviral gene transduction of hepatocyte growth factor elicits inhibitory effects for hepatoma

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Abstract. Hepatocyte growth factor (HGF) gene therapy may have potential for treating chronic hepatitis (CH) and liver cirrhosis (LC). However, the lack of an HGF gene therapy study on hepatomas that are often associated with CH or LC, together with the stimulatory effects of HGF on many types of cancer, may hamper its application. This study explored the effects of adenoviral HGF gene transduction and their mechanisms on two types of hepatoma cells (hepatoblastoma and hepatocellular carcinoma) in *in vitro* experiments. Both types of hepatomas were revealed to have higher adenoviral gene transduction efficiencies and more efficient expressions of the HGF transgene, which successfully activated the HGF receptor/c-Met in an autocrine fashion, than those of other types of cancer. Notably, not only HGF, but also adenoviral infection, inhibited DNA synthesis, whereas only HGF but not adenoviral infection exerted a potent apoptotic effect. Moreover, adenoviral HGF gene transduction additively exerted inhibitory effects on cisplatin-treated hepatomas. In conclusion, inhibitory and apoptotic effects of adenoviral HGF gene transduction in hepatomas in contrast to potent mitogenic and antiapoptotic effects of HGF for hepatocytes are not only of biological interest, but also pose clinical benefits for adenoviral HGF gene therapy for CH and LC.

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Abbreviations: ADV, adenoviral vector; AGTE, adenoviral gene transduction efficiency; MOI, multiplicity of infection; RSV, Rous sarcoma virus long terminal repeat; X-gal, o-nitrophenyl-ß-D-galactophyranoside; ELISA, enzyme-linked immunosorbent assay

Key words: adenoviral vector, hepatocyte growth factor, hepatoma, apoptosis, DNA synthesis, cisplatin

Introduction

Hepatocyte growth factor (HGF), originally identified (1-4) and cloned (5,6) as a potent mitogen for hepatocytes, is a multifunctional cytokine that exhibits mitogenic, motogenic, morphologic, angiogeneic, antiapoptotic and organotrophic effects on a variety of tissues (7). We recently showed that HGF exerts a potent antiapoptotic effect on hepatocytes and antifibrotic effects, and can be used to treat acute hepatitis, including fulminant hepatic failure (8,9), and chronic hepatitis (CH) and liver cirrhosis (LC) in animals (10-12). Thus, these beneficial effects, together with the inducible effect for liver regeneration (13), suggest that HGF gene therapy may be a promising treatment for CH and LC.

However, certain types of growth factors may play important roles in the carcinogenesis, tumor growth, and angiogenesis of cancer. Accumulating data suggest that HGF may enhance the growth and metastases of a majority of cancers, probably due not only to direct stimulation of cancer cell growth, but to the enhancement of angiogenesis (14,15). In fact, the HGF antagonist has been shown to inhibit cancer cell growth, metastasis, and angiogenesis of some cancers in animals (16,17). In contrast, other studies suggested that HGF might exert an inhibitory effect on certain types of cancers (18-23) although their overall features and mechanisms remain obscure.

In particular, the roles and effects of HGF on hepatomas remain largely controversial. In transgenic mouse (Tg) studies,

two distinct types of HGF Tg, in which HGF was over-expressed under the transcriptional control of either the hepatocyte-specific albumin promoter (Alb-HGF-Tg) (24) or the ubiquitously active mouse metallothionein gene promoter (MT-HGF-Tg) (25,26), demonstrated completely opposite results concerning hepatocarcinogenesis. Alb-HGF-Tg never formed a hepatocellular carcinoma (HCC), and furthermore, analyses using two kinds of double Tg; Alb-HGF/MT-TGF-α Tg (27) and Alb-HGF/Alb-*c-myc*-Tg (28), showed that the overexpression of HGF in hepatocytes inhibited both TGF-α and c-myc-induced hepatocarcinogenesis. In contrast, MT-HGF-Tg formed cancers in several organs, including HCC in the liver (25,26). Thus, rather than elucidating this issue, these Tg studies have led to confusion, probably due to the artificial factors in the studies. On the other hand, *in vitro* studies have recently shown that recombinant HGF has an inhibitory effect on the human hepatoblastoma cell line, HepG2 (29), but no study has as yet explored the effects of HGF on human hepatocellular carcinoma (HCC). More importantly, there has not only been no direct studies of HGF gene therapy for hepatoma, but also no biological studies of adenoviral HGF gene transduction in hepatomas, including no investigation of the effects of adenoviral infection itself or the effects of exogenous HGF gene expression in an artificial autocrine fashion.

HCC, which is the major type of hepatoma found in adult patients, is usually associated with CH or LC. Alternatively, CH and LC have a high risk of hepatocarcinogenesis (30-32), and some patients with CH and/or LC may potentially have latent HCC at an undetectably small size. Thus, although HGF gene therapy may have potential for treating CH and LC, its clinical application may be prohibited due to the uncertain effects of HGF gene therapy for hepatomas. In this regard, the present study biologically explored the effect of adenoviral HGF gene transduction in hepatoma cells and its mechanism.

Materials and methods

Human cell lines. The human cell lines Hep3B (HCC), HepG2 (hepatoblastoma), HeLa (cervical carcinoma), MKN-28 (gastric carcinoma), colo-205 (colon carcinoma), HOS-NP (osteosarcoma), A549 (lung carcinoma) and 293, were cultured in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin and 10% fetal calf serum.

Recombinant adenoviral vectors (Ad) and adenoviral gene transduction efficiencies (AGTEs). Replication-defective Ads, Ad.RSV-HGF and Ad.RSV-LacZ, which encode the human HGF and ß-galactosidase gene, respectively, downstream of the transcriptional control of the Rous sarcoma virus long terminal repeat (RSV) promoter were constructed, prepared, and titered as described previously (33-35). AGTEs were assessed by 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) staining after Ad.RSV-LacZ infection, as described previously (36-38).

HGF enzyme-linked immunosorbent assay (ELISA). HeLa, Hep3B and HepG2 cells at 1000, 1000 and 2000 cells/well in 96-well plates, respectively, were infected with Ad.HGF, and the supernatant was collected 48 or 96 h later. The human HGF levels in the supernatant were measured by ELISA according to the manufacturer's protocol (R&D Systems, Inc., Minneapolis, MN).

C-Met phosphorylation. C-Met phosphorylation in the cells was detected by the immunoprecipitation method (39). Briefly, the cells were lysed in 500 µl RIPA buffer (1% Triton X, 150 mM NaCl, 50 mM Tris-HCl pH 7.6, 10% glycerol, 1 mM Vanadate, 1 mM phenylmethylsulfonyl fluoride) with protease inhibitors after incubation with the serum-free media for 24 h and subsequently with new serum-free media containing recombinant HGF (R&D Systems) or the supernatant from Ad.RSV-HGF-infected HepG2 cells for 10 min. After centrifugation, the supernatant was incubated with 0.5 ng/ml anti-c-Met monoclonal antibody (C-12, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 4 h, and sequentially incubated with 10 µl Protein G Sepharose beads for 3 h. The proteins bound to beads were dissolved in sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Phosphorylated c-Met was immunoblotted with anti-phosphotyrosine antibody (PY20, Transduction Laboratories, Lexington, KY).

WST-8 cell viability assay. The cells were infected with each Ad in the same manner as in the HGF ELISA experiment. Cell viability was determined by WST-8 assay (Dojindo Laboratories Co., Mashiki, Japan) at 2, 4, 6 or 8 days after Ad infection according to the manufacturer's protocol (36,37).

To explore the effect of HGF in cisplatin (cis-Diammine-dichloroplatinum (II))-treated hepatoma cells, cisplatin was added to the media in the HepG2 and HeLa cells (125 µg/ml) and in the Hep3B cells (250 µg/ml) at 24 h after Ad-infection.

Bromo-2'-deoxyuridine (BrdU) uptake assay. The cells were infected with each Ad in the same manner as in the HGF ELISA experiments. Four days later, the cells were incubated with 10 µM of BrdU for 4 h and harvested. The percentage of cells that had BrdU uptake was measured by ELISA according to the manufacturer's protocol (BrdU Labeling and Detection Kit III, Roche Diagnostics GmbH, Mannheim, Germany).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labelling (TUNEL) assay. Hep3B or HeLa ($1x10^5$) cells, or HepG2 ($2x10^5$) cells were infected with each Ad, and apoptotic cells were detected at 2, 4, 6 or 8 days later using an ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Intergen Company, New York, USA) according to the manufacturer's protocols.

Detection of active form of caspase-3. The cells were lysed in hypotonic buffer (25 mM HEPES pH 7.5, 5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride) with protease inhibitors at 2, 3 or 4 days after adenoviral infection at the multiplicity of infection (MOI) of 10. Protein (30 µg) was subjected to SDS-PAGE, and Western blotting was performed using anti-caspase-3 antibody (BD Pharmingen, San Diego, CA), peroxidase-conjugated anti-rabbit IgG and

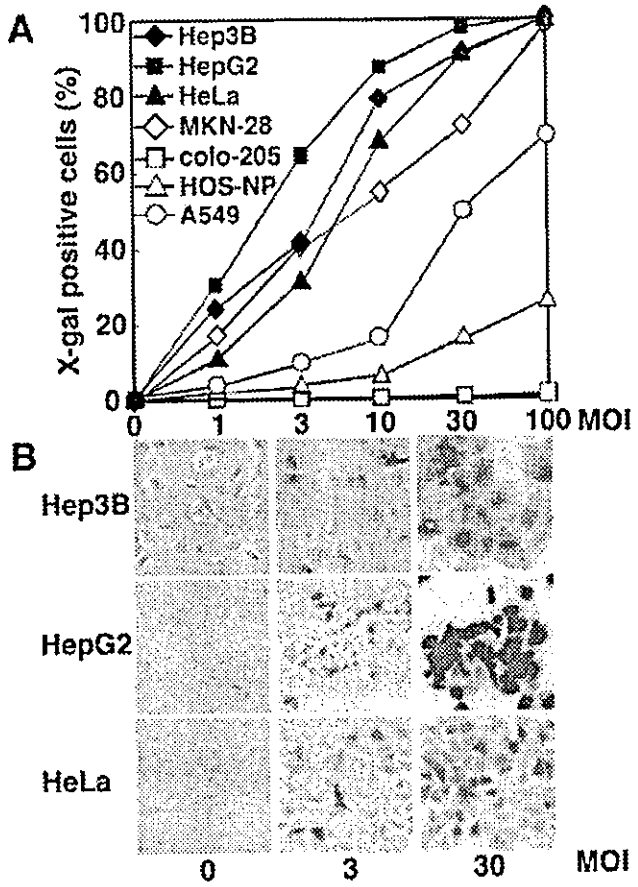

Figure 1. AGTEs. Cells were infected with Ad.RSV-LacZ at indicated MOIs and stained with X-gal. (A), The AGTEs in Hep3B and HepG2 were as high as those in HeLa cells, and higher than those in other types of cells. (B), The representative pictures of X-gal-stained cells.

SuperSignal® West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL).

Statistical analysis. All results are expressed as mean ± standard deviation. Statistical comparison were made using Student's t-test.

Results

High AGTE and HGF expression in hepatoma cells. The AGTEs in the Hep3B and HepG2 cells were as high as those in the HeLa cells, and higher than those in other types of human carcinoma cell lines at each MOI (Fig. 1). Because of this, the HeLa cells were chosen as a control for subsequent experiments.

The exogenous human HGF levels in the supernatant were much higher at 48 h than at 96 h after the Ad.RSV-HGF infection in the Hep3B, HepG2 and HeLa cells (Table I). The HGF levels largely differed among these 3 cell lines, and the levels in the HepG2 and Hep3B cells were remarkably higher than those in the HeLa cells at each point. In addition, the HGF levels in the supernatant from the Ad.RSV-HGF-infected MKN-28 cells were much lower than those of the HeLa cells (data not shown). Thus, hepatoma may be a good target for adenoviral HGF gene therapy in terms of the efficient secretion of exogenous HGF in the autocrine mode as well as a high AGTE.

Table I. HGF levels after adenoviral HGF gene transduction.

		HGF levels (ng/ml)	
Cell lines	MOIs	48 h	96 h
Hep3B	0	0.0	0.0
	3	<0.1	0.1
	10	0.1	6.0
	30	1.1	21.7
HepG2	0	0.0	0.0
	3	<0.1	2.1
	10	0.4	83.4
	30	5.9	>4125.0
HeLa	0	0.0	0.0
	3	<0.1	<0.1
	10	<0.1	0.1
	30	0.1	2.7

The supernatant was collected 48 or 96 h after infection with Ad.RSV-HGF at indicated MOIs. The human HGF levels in the supernatant was measured by ELISA.

Exogenous HGF in the autocrine fashion activates HGF receptor/c-Met. Immunoprecipitation and Western blot analysis showed that the Hep3B, HepG2, and HeLa cells were all abundant in the c-Met, which was similarly phosphorylated (i.e., activated) in an HGF dose-dependent manner (Fig. 2A).

HGF natively acts on target cells in a paracrine fashion in the body. Namely, a single chain prepro HGF is secreted from mesenchymal cells and then converted by a specific protease to biologically active mature HGF, which binds to c-Met and confers a biological function in hepatocytes (7). To explore whether transduced HGF in hepatoma cells may function in an artificial autocrine fashion, we initially tried to directly detect c-Met phosphorylation in the Ad.RSV-HGF-infected HepG2 and Hep3B cells. However, several attempts did not result in successful detection because it was difficult to detect short term c-Met phosphorylation under biologically complex circumstances in which persistent HGF expression might lead to repeated cycles of activation, downregulation and the revival of c-Met. Therefore, we indirectly explored the above issue as follows. The supernatant was collected from Ad.RSV-HGF-infected HepG2 cells, and the HGF concentration in it was measured. Subsequently, supernatant or recombinant HGF at the same concentration was added to intact HepG2 (Fig. 2B) or Hep3B cells (data not shown), and the c-Met phosphorylation was explored. As a result, c-Met activation was found to occur in both types of hepatomas in a dose-dependent manner similar to the cases of additions of recombinant HGF, indicating that exogenous HGF protein from the transgene might be efficiently converted to the active form by proteases natively present in the serum, and finally verifying that exogenous HGF correctly activated c-Met.

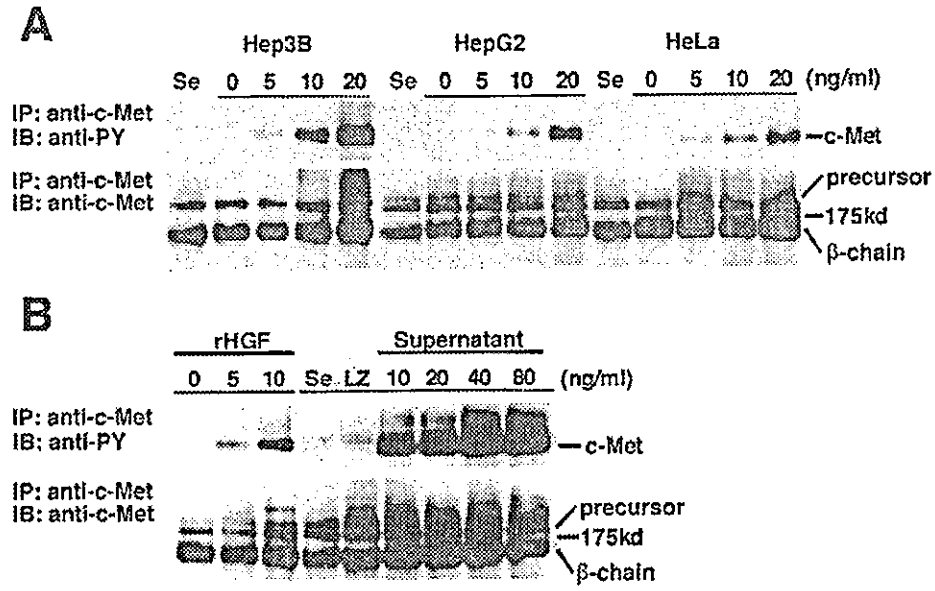

Figure 2. HGF receptor/c-Met and its activation. (A), Hep3B, HepG2 and HeLa cells were abundant in the c-Met, which was similarly phosphorylated in HGF dose-dependent manner. (B), The c-Met on intact HepG2 cells was similarly phosphorylated by additions of the supernatant from Ad.RSV-HGF-infected HepG2 cells. IP, immunoprecipitation. IB, immunoblot. rHGF, recombinant HGF.

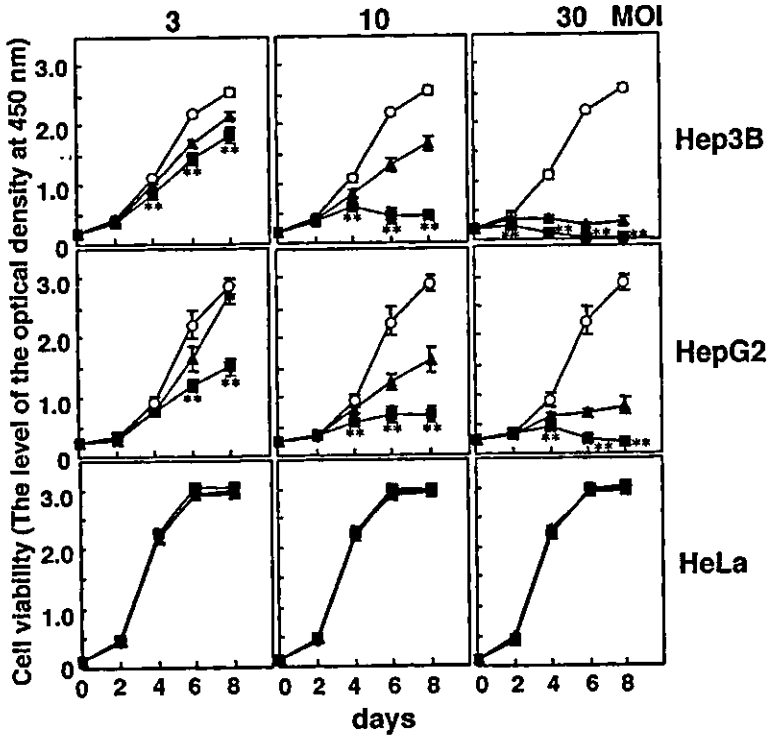

Figure 3. Inhibitory effects of adenoviral HGF gene transduction. Cell viability was determined by WST-8 assay 2, 4, 6 or 8 days after infection with each Ad at indicated MOIs. Although Ad.RSV-LacZ infection as well as Ad.RSV-HGF infection revealed some inhibitory effects for the HepG2 and Hep3B cells, but not for the HeLa cells, the degree of this inhibitory effect was more prominent in the Ad.RSV-HGF-infected cells (square) than the Ad.RSV-LacZ-infected ones (triangle). *P<0.05; **P<0.01 (Ad.RSV-HGF versus Ad.RSV-LacZ groups on each day at each MOI). No treatment control, circle. N=8, each point in each group.

Ad.RSV-HGF infection exerts inhibitory effects for hepatoma cells. The WST-8 assay showed that the number of viable Hep3B and HepG2 cells was significantly smaller in the Ad.RSV-HGF-infected cells than in the Ad.RSV-LacZ-infected cells at each MOI (Fig. 3). In addition, the infection of Ad.RSV-LacZ revealed some inhibitory effects for the HepG2 and Hep3B cells in an Ad dose-dependent manner, but not for the control HeLa cells, although the degree of this inhibitory effect was milder than that by Ad.RSV-HGF. In contrast, there was no difference in the number of viable HeLa cells among the Ad.RSV-HGF-infected, Ad.RSV-LacZ-infected cells, or no Ad-infected cells at any points. Thus,

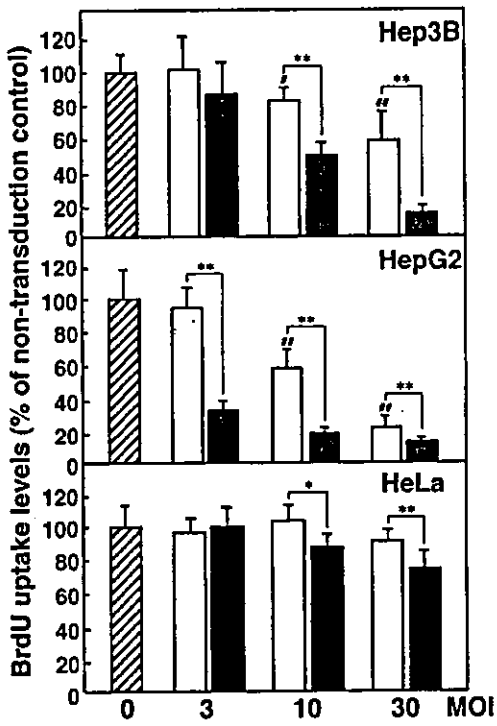

Figure 4. Inhibition of DNA synthesis. BrdU uptake assay was done 4 days after each Ad infection at indicated MOIs. Although the BrdU uptake levels were reduced by Ad.RSV-LacZ infection as well as Ad.RSV-HGF infection in HepG2 and Hep3B cells, the degree of this reduction was more prominent in the Ad.RSV-HGF-infected cells than the Ad.RSV-LacZ-infected ones. The BrdU uptake levels in HeLa cells were mildly reduced by Ad.RSV-HGF infection. *P<0.05; **P<0.01 (Ad.RSV-HGF groups versus Ad.RSV-LacZ groups at each MOI). #P<0.05; ##P<0.01 (Ad.RSV-LacZ versus non-transduced control). N=8, each point in each group.

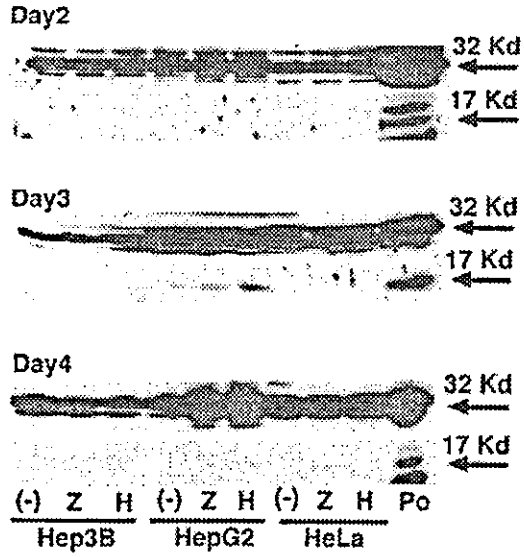

Figure 5. Caspase-3 activation. Pro-caspase-3 (32 kDa) and active caspase-3 (17 kDa) were detected by Western blotting of cells 2, 3, 4 days after no Ad (-), Ad.RSV-LacZ (Z) or Ad.RSV-HGF (H) infection at MOI of 10. Positive control (Po); cisplatin-treated HepG2 cells. More prominent activation of caspase-3 was seen in HepG2 cells 3 days after Ad.RSV-HGF infection than after Ad.RSV-LacZ or no Ad infection.

both types of hepatomas were remarkably sensitive not only to HGF, but also to the adenoviral infection itself, both of which independently and additively exerted inhibitory effects.

Ad.RSV-HGF infection inhibits DNA syntheses of hepatoma cells. There are two possible causes of inhibitory effects; one is the inhibition of cell growth, and the other is the induction of cell death. To assess the former, DNA replication was analyzed by the BrdU uptake assay (Fig. 4). The BrdU uptake levels in the Hep3B and HepG2 cells were both significantly reduced by not only Ad.RSV-HGF, but also by Ad.RSV-LacZ infection in an Ad dose-dependent manner. Statistical differences between the Ad.RSV-HGF and Ad.RSV-LacZ groups at each MOI further indicated that HGF itself inhibited cell growth in both types of hepatomas. The BrdU uptake levels in the HeLa cells were reduced only by Ad.RSV-HGF infection at a high MOI, but not by the adenovirus infection itself. Thus, not only HGF, but also the adenoviral infection itself, independently and additively inhibits cell growth in both types of hepatomas.

HGF but not adenoviral infection exerts apoptotic effect for hepatoma cells. To investigate the apoptotic effect of HGF on hepatomas, the activation of caspase-3, which leads to the activation of important apoptosis-related proteins such as caspase-activated DNase (40) and Acinus (41), was investigated by Western blotting (Fig. 5). Ad.RSV-HGF infection in the HepG2 cells led to a more prominent appearance of the active form of caspase-3 (17 kDa) than that of Ad.LacZ or no Ad infection. In contrast, a significant activation of caspase-3 was not detected in the Hep3B or HeLa cells after Ad.RSV-HGF, Ad.RSV-LacZ or no Ad infection, despite several trials. In addition, the activation of caspase-3 was not detected in any samples using the CPP32/Caspase-3 Colorimetric Protease Assay Kit (data not shown). Thus, despite substantial technical difficulty with this experimental system, including the issue of abundant pro-caspase-3 in comparison to a limited amount of active caspase-3, the detection of a more prominent band of active caspase-3 in the Ad.RSV-HGF-infected HepG2 cells suggests that exogenous HGF in the autocrine fashion may activate apoptotic signals in hepatoma cells.

We next performed TUNEL assays, which provided clearer and more definitive results (Fig. 6). TUNEL-positive Hep3B and HepG2 cells were significantly increased after the Ad.RSV-HGF infection, in contrast to no increase after the Ad.RSV-LacZ infection. On the other hand, TUNEL-positive HeLa cells were not seen after the Ad.RSV-HGF or Ad.RSV-LacZ infection. These findings indicate that HGF in an autocrine fashion may directly exert a potent apoptotic effect for both types of hepatomas; however, adenoviral infection itself does not induce apoptosis, in contrast to its inhibitory effect on cell growth.

Ad.RSV-HGF infection additively exerts inhibitory effect for cisplatin-treated hepatoma cells. To explore whether adenoviral HGF gene therapy is potentially applicable to hepatoma patients who undergo conventional carcinostatic treatments, we investigated the effect of Ad.RSV-HGF infection on cisplatin-treated hepatoma cells (Fig. 7). We chose cisplatin because cisplatin is one of the representative

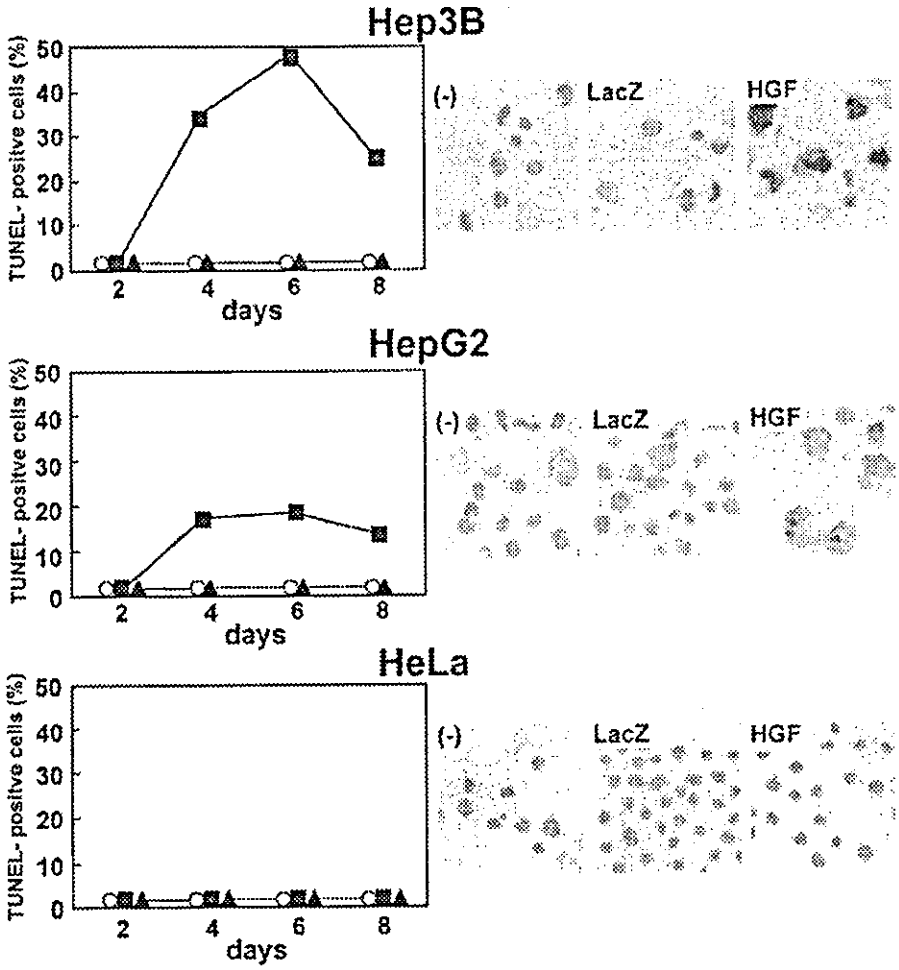

Figure 6. TUNEL assay. TUNEL-positive cells were significantly increased after Ad.RSV-HGF infection in Hep3B and HepG2 cells, but not in HeLa cells. In contrast, TULEL-positive cells were not seen after Ad.RSV-LacZ infection or control (no Ad infection). The circle, triangle and square symbols indicate no Ad-, Ad.RSV-LacZ- or Ad.RSV-HGF-treated groups, respectively. The representative pictures of TUNEL-positive cells 6 days after each Ad infection are shown in the right panel.

carcinostatics for hepatoma patients, especially in the case of transcatheter arterial embolization (TAE) therapy (42,43). WST-8 analysis showed that the Ad.RSV-HGF infection exerted additional inhibitory effects on both Hep3B and HepG2 cells independently and additively to those of cisplatin. Ad.RSV-LacZ infection exerted a milder but apparent inhibitory effect on cisplatin-treated hepatoma cells in an Ad dose-dependent manner. Thus, the effects of HGF, Ad and cisplatin on hepatomas were all inhibitory but independent, and, therefore, the Ad.RSV-HGF infection exerted the most prominent inhibitory effects for hepatoma cells as the result of the additive effects of all 3 factors. In contrast, neither Ad.RSV-HGF nor Ad.RSV-LacZ infection changed the effect of cisplatin in HeLa cells.

Discussion

The present study demonstrated not only that adenoviral HGF gene transduction significantly exhibited inhibitory effects on both HCC and hepatoblastoma, but also that such inhibitory effects were due to both the inhibition of cell growth and the induction of apoptosis.

The opposing activities of HGF, i.e., its antimitotic and mitotic effects and its apoptotic and antiapoptotic effects on hepatoma cells and hepatocytes are of biological interest. In terms of effects on cell growth, the following explanations were suggested by recent studies using HepG2 cells. The sustain duration of p21/waf1 induction might be a determinant of HGF-induced inhibitory effects (44), and integrin-mediated signals from the extracellular matrix could modulate HGF-mediated signals (45). The levels of ERK activity might determine the opposing proliferation responses; HGF-induced growth inhibition was caused by the cell cycle arrest, which resulted from hypophosphorylated pRB via high-intensity ERK signals (29). Although HepG2 cells were solely used for these investigations, some of the findings might be applicable to the different phenotypic effects of HGF between hepatoma cells and hepatocytes; hypothetically, the different responsiveness of these molecules might at least in part determine the differences in HGF-induced signaling between them.

In terms of apoptosis and HGF, a recent study interestingly showed the direct interaction of c-Met and the death receptor Fas in hepatocytes; such interaction prevented Fas self-aggregation and Fas ligand binding, thus inhibiting Fas activation and apoptosis (46). Together with the frequent observation of altered Fas expressions and systems in hepatoma cells (47,48), hypothetically, an altered Fas system in

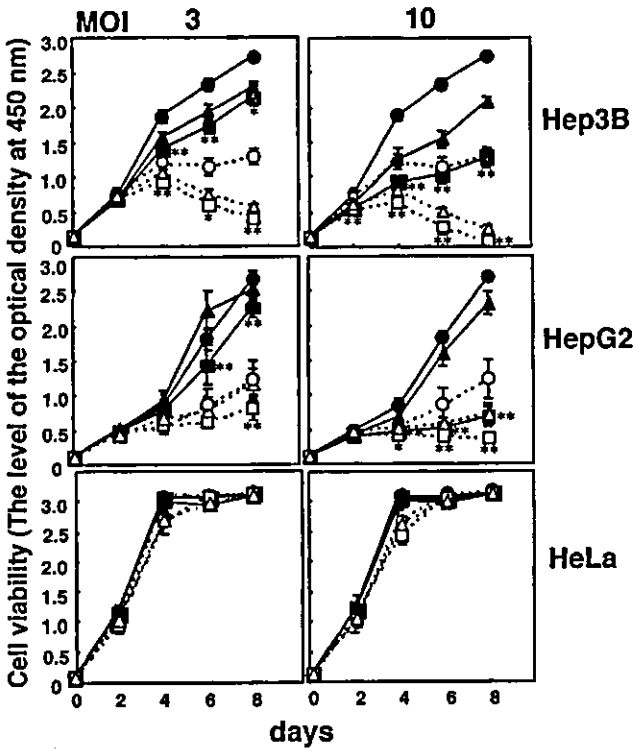

Figure 7. Additive inhibitory effects of Ad.RSV-HGF for cisplatin-treated hepatoma cells. Cell viability after infection with each Ad with or without cisplatin was determined by WST-8 assay. Although Ad.RSV-LacZ infection as well as Ad.RSV-HGF infection revealed additional inhibitory effects for HepG2 and Hep3B cells, but not for HeLa cells, additively to those of cisplatin, the degrees of the inhibitory effects were more prominent in the Ad.RSV-HGF-infected cells than those in the Ad.RSV-LacZ-infected ones. The open or closed symbols are indicated as the groups treated with or without cisplatin, respectively, while the circle, triangle and square symbols indicate no Ad-, Ad.RSV-LacZ- or Ad.RSV-HGF-treated groups, respectively. *P<0.05, **P<0.01 (Ad.RSV-LacZ groups versus Ad.RSV-HGF groups on each day at each MOI). N=8, each point in each group.

hepatomas might mean that the sequestration of Fas is lost by the c-Met and HGF-induced antiapoptotic effect in hepatomas. Although the identification of the molecules that were involved in these features were not the focus of the present study, overall elucidation in future studies may not only be of biological interest, but clinically useful for developing more effective HGF gene therapy.

On the other hand, the present study revealed clinically important information. The beneficial data obtained here may allow for the clinical application of adenoviral HGF gene therapy for CH and LC. First, high AGTE and the efficient expressions of exogenous HGF in an autocrine fashion in hepatomas may suggest that hepatomas are a good target site for gene transduction in the case of adenoviral HGF gene therapy for associated CH or LC; adenovirus-associated hepatotoxicity may be reduced. Second, the inhibitory effects of adenoviral HGF gene transduction for hepatomas, which resulted from not only the HGF-induced and Ad-induced inhibition of cell growth but also the HGF-induced apoptotic effects, are obviously encouraging for treating hepatoma itself. Another encouraging result was that Ad-induced and HGF-induced inhibitory effects were found to additively enhance (rather than diminish) cisplatin-induced cytotoxicity against hepatoma. Several mechanisms of the cisplatin-induced inhibitory effects have been reported, including cell cycle arrest by activating the CDK kinase inhibitor (49), and the induction of apoptosis by upregulating Fas and the Fas ligand (50,51). On the other hand, previous studies demonstrated that recombinant Ad induced cell cycle dysregulation by causing the inappropriate expression of cyclin proteins and down-regulation of E2F-1 independent of the Rb and p53 status (52,53). The present results suggest that each signal transduction pathway induced by cisplatin, adenovirus or HGF, including the unknown pathway mentioned above, may not directly cross-talk with each other. Thus, the additive and independent inhibitory effects of Ad, HGF and cisplatin against hepatomas may be clinically beneficial because HGF gene therapy may be not only applicable to, but also therapeutic for, hepatoma patients undergoing treatment with cisplatin.

To further verify such clinical implication, we tried to establish clinically-relevant animal models of orthotopic HCC and hepatoblastoma by transplanting Hep3B and HepG2 cells into severe combined immunodeficiency mice, however, high mortality and uncertain tumor formation were obstacles to further therapeutic experiments. On the other hand, the size of subcutaneously implanted tumor was slightly but insignificantly smaller in Ad.HGF-treated mice than that in Ad.LacZ-treated ones in our preliminary experiments (data not shown). However, we did not pursue these results because subcutaneous tumor models have sometimes led to rather misleading results due to the lack of tissue characteristics or native microenvironments (36,37). Likewise, current HCC-Tg models, in which multiple nodules of HCC appear without CH or LC on various schedules, are neither clinically relevant nor useful for assessing the clinical usefulness of gene therapy strategy (25). In this context, the clinically relevant animal model of HCC with LC or CH should be newly generated and the clinical usefulness of HGF gene therapy should be carefully investigated in future studies.

In conclusion, adenoviral HGF gene transduction in human hepatoblastomas and HCC inhibited their growth and induced apoptosis, as well as additively enhancing the cisplatin-induced inhibitory effect. These effects may be beneficial for HGF gene therapy for CH and LC associated with hepatomas.

Acknowledgements

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