

Increased Efficacy and Safety in the Treatment of Experimental Liver Cancer with a Novel Adenovirus-Alphavirus Hybrid Vector

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Abstract

An improved viral vector for cancer gene therapy should be capable of infecting tumors with high efficiency, inducing specific and high-level expression of transgene in the tumor and selectively destroying tumor cells. In the design of such a vector to treat hepatocellular carcinoma, we took advantage of (a) the high infectivity of adenoviruses for hepatic cells, (b) the high level of protein expression and proapoptotic properties that characterize Semliki Forest virus (SFV) replicon, and (c) tumor selectivity provided by α -fetoprotein (AFP) promoter. We constructed a hybrid viral vector composed of a helper-dependent adenovirus containing an SFV replicon under the transcriptional control of AFP promoter and a transgene driven by SFV subgenomic promoter. Hybrid vectors containing murine interleukin-12 (*mIL-12*) genes or reporter gene *LacZ* showed very specific and high-level expression of transgenes in AFP-expressing hepatocellular carcinoma cells, both *in vitro* and in an *in vivo* hepatocellular carcinoma animal model. Infected hepatocellular carcinoma cells were selectively eliminated due to the induction of apoptosis by SFV replication. In a rat orthotopic liver tumor model, treatment of established tumors with a hybrid vector carrying *mIL-12* gene resulted in strong antitumoral activity without accompanying toxicity. This new type of hybrid vectors may provide a potent and safe tool for cancer gene therapy. (Cancer Res 2006; 66(3): 1620-9)

Introduction

Gene therapy offers a new approach to treat cancer. Transfer of genes encoding immunostimulatory cytokines, such as interleukin-12 (IL-12), to tumors has been used with remarkable success to eliminate cancer in animal models (1–4). This strategy allows to concentrate the biological action of the cytokines at the site of the neoplasm, thus increasing the antitumor effect while avoiding systemic toxicity. Despite promising preclinical data (1–4), pilot clinical trials in patients with liver cancer showed that intratumoral administration of an adenoviral vector encoding IL-12 under the control of cytomegalovirus promoter (AdCMV-IL-12) had limited efficacy with only one partial regression being observed among eight treated patients with hepatocellular carcinoma (5). This modest result was associated with low-level expression of the

transgene in the tumor mass and poor activation of antitumor immunity (5).

Thus, vectors being able to generate high levels of transgenic proteins in the tumor and of stimulating more intense antitumor responses should be developed (6, 7). However, high-expression vectors may increase the risk of toxicity; consequently, avoidance of transgene expression at extratumoral sites is an absolute requirement for the use of such gene therapy tools (6, 7). Tumor-specific promoters allow transcriptional tumor targeting (8). However, these are normally weak promoters that do not allow high-level expression of the transgenic protein in the neoplastic tissue (9–11). Thus, strategies to achieve both transcriptional targeting and high-level expression of the transgene would be necessary to treat cancer. On the other hand, when the transgene is an immunostimulatory cytokine, the antitumor effect will be enhanced if the vector is able to induce apoptosis in the neoplastic cells. It has been shown that the release of apoptotic bodies in a cytokine-rich milieu would favor the presentation of tumor antigens and the induction of antitumor immunity (12, 13). Therefore, combination of high infectivity for tumor cells, strict tumor targeting, high production of the therapeutic protein inside tumor, and induction of selective apoptosis of cancer cells are four conditions for a vector to be successful against cancer in the clinical setting. The objective of this study is to gain in efficacy against hepatocellular carcinoma by combining in a single vector high infectivity using an adenovirus delivery system, high level of transgene expression and induction of apoptosis using an alphavirus vector (14), and tumor specificity by placing the alphavirus replicon under the transcriptional control of a tumor-specific promoter.

Helper-dependent adenoviral vectors, also called high-capacity (HC-Ad) or gutless adenoviruses, are adenovirus-based vectors devoid of all adenoviral genes, thus leaving room to allocate large expression cassettes (up to 35 kb), such as that corresponding to an alphavirus replicon (15–17). Alphaviruses are RNA-enveloped viruses that contain a single positive-strand RNA molecule as genome (18). Different alphaviruses, such as Sindbis virus (19), Semliki Forest virus (SFV; ref. 14), and Venezuelan Equine encephalitis virus (20), have served for the construction of gene therapy vectors. These are based on the use of self-replicating RNA molecules derived from alphavirus genomes in which the 5' and 3' sequences necessary for replication and the *replicase* (*Rep*) gene have been maintained, whereas the genes coding for the viral structural proteins have been deleted and substituted by a transgene. Upon transfecting the vector into a cell, *Rep* will be translated and the vector RNA will be copied into a negative-strand RNA that will be used as template for the amplification of the vector RNA. *Rep* can also recognize a subgenomic promoter in the negative-strand RNA from which it will make a smaller subgenomic

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RNA that can be translated to produce the heterologous protein. Replication and expression of the transgene occur at very high levels being the first one associated with the induction of apoptosis in the infected cells (21).

We constructed a hybrid adenoviral vector that contains the sequence of a recombinant SFV replicon under the transcriptional control of α -fetoprotein (AFP) promoter (Fig. 1A-B). In this construct, the SFV replicon contained the *IL-12* gene driven by the SFV subgenomic promoter. Our aim was to allow replication of the SFV replicon, leading to high-level production of IL-12 and apoptosis of the transduced cells only in AFP-expressing hepatocellular carcinoma tumors, but not in other cell types to gain in safety and efficacy in the treatment of liver cancer.

Materials and Methods

Cell lines and tissue culture. Human hepatocellular carcinoma cell lines Hep3B, PLC/PRF/5, and HepG2; human cervix epithelial adenocarcinoma cell line HeLa; human lung carcinoma cell line A549; human tumor cell line SK-Hep-1; human 293 cell line; rat hepatocellular carcinoma cell lines McA-RH7777 and MHC1; and normal rat hepatocyte clone 9 were obtained from American Type Culture Collection (Manassas, VA). 293 cells expressing Cre recombinase (293Cre4) were obtained from Merck Research Laboratories (West Point, PA).

Animals. Seven-week-old female BALB/c nude mice and BALB/c mice were purchased from Charles Rivers Laboratories (Barcelona, Spain). Four- to 6-week-old male Buffalo rats were obtained from Harlan (Barcelona, Spain). The animals were kept under standard pathogen-free conditions and were handled according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences. All experiments were done in accordance with the local animal ethical committee.

Construction of hybrid vectors. The 5'-end sequence of SFV (1-292 nucleotides) was amplified by PCR using pBK-SFV plasmid (containing the full sequence of SFV Rep) as template (22). Primer 1 contained an *SpeI* restriction site at the 5' end (underlined) followed by 50 nucleotides of AFP promoter sequence and the first 20 nucleotides of the SFV sequence (in italics) 5'-ACTAGTTAAACAGGCATTGCCTGAAAAGAGTATAAAA-GAATTCAGCATGATTTTCCATGGCGGATGTGTGACATAC-3'. Primer 2 contained a *XhoI* restriction site (underlined) followed by 19 nucleotides from SFV sequence (in italics): 5'-CTCGAGGATATCCAAGATGAGTGTGT-3'. A DNA fragment of 351 bp was generated by PCR and cloned directly into pGEM-T-easy plasmid (Promega, Madison, WI) to generate pGEM-Te-SFV-1. The absence of PCR errors in this plasmid was confirmed by sequencing. A 347 bp fragment was released from pGEM-Te-SFV-1 by digestion with *SpeI* and *XhoI* and cloned into pGL3/AFP digested with the same enzymes to yield pGL3/AFP-SFV-1, which has a full AFP promoter (217 bp) and enhancer (785 bp) followed by the SFV 5'-end sequence (SFV-1, comprising 292 bp). An AFP-SFV-1 (1,342 bp) cassette was released from pGL3/AFP-SFV-1 by *MluI/XhoI* digestion, treated with Klenow and cloned into pBS-SK+ digested with *EcoRV*, generating pBS/AFP-SFV-1. The SV40 late polyA (262 bp) was removed from pGL3/AFP by *XbaI/BamHI* digestion, blunted with Klenow, and inserted into the *SaI* site of pBS/AFP-SFV-1 also blunted with Klenow, giving rise to pBS/AFP-SFV-1-pA. A polylinker containing both unique *ApaI* and *NruI* sites was inserted between *BamHI* and *XmaI* sites in pBS/AFP-SFV-1-pA. The 3'-end sequence of SFV comprising 7,985 bp was removed by *SpeI/EcoRV* digestion from pBK-SFV, blunted with Klenow and inserted into *EcoRV* digested pBS/AFP-SFV-1-pA, giving rise to pBS/AFP-SFV-pA.

LacZ reporter gene was obtained from pCMV β (Clontech, Mountain View, CA) by *NotI* digestion and inserted into the *BamHI* site of pBS/AFP-SFV-pA to form pBS/AFP-SFV-LacZ-pA by blunt-end ligation. A *mIL-12* cassette containing the genes coding for the p35 and p40 subunits linked by the encephalomyocarditis virus internal ribosome entry site was removed

from pBS/*mIL-12* (23) by *SpeI/XhoI* digestion, treated with Klenow, and inserted into *BamHI* in pBS/AFP-SFV-pA, also blunted with Klenow generating pBS/AFP-SFV-*mIL-12*-pA.

The 19 kb fragment from gutless adenovirus plasmid pSTK120 (16) was obtained by *ApaI* digestion and a polylinker containing *AseI* and

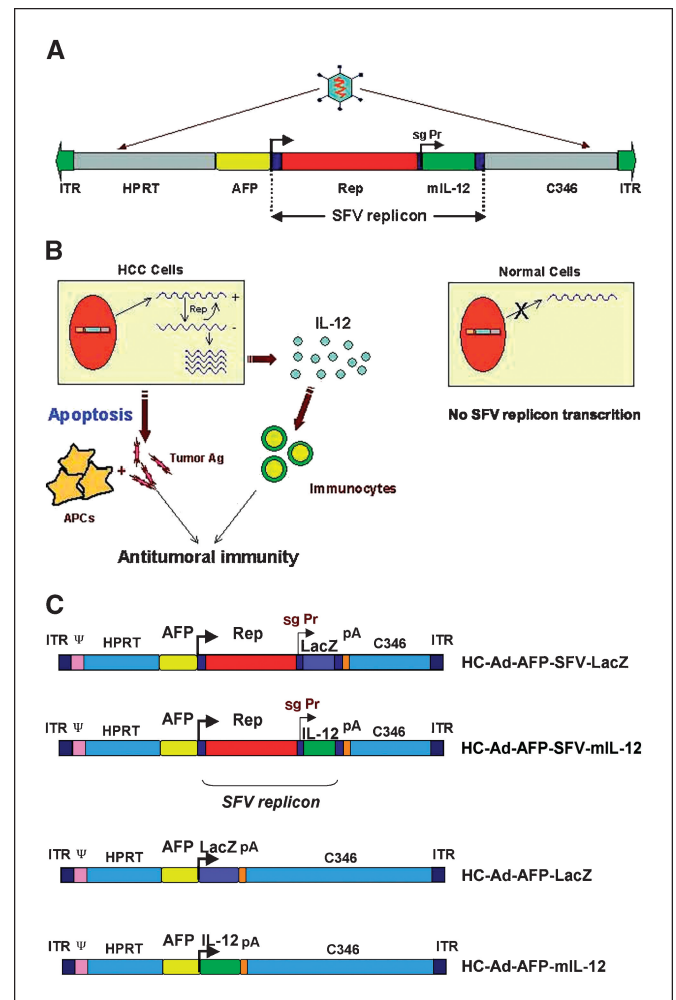


Figure 1. Diagram of gutless adenovirus-SFV hybrid vector and its potential antitumoral activity. **A**, the hybrid vector contains a gutless adenovirus sequence in which a SFV replicon has been inserted under the control of the AFP promoter/enhancer (AFP). The heterologous gene *mIL-12* is placed under the control of the SFV subgenomic promoter (sg Pr). **B**, upon infection of hepatocellular carcinoma (HCC) cells (left) with the hybrid vector, the SFV replicon mRNA can be transcribed from the tumor-specific promoter, Rep will be translated, and copy SFV RNA genome (+) into negative-strand RNA (-). Rep will also synthesize a SFV subgenomic RNA from which *mIL-12* will be expressed at high levels. The *mIL-12* secreted from infected cells will activate immunocytes at the site of infection. In addition, SFV replication will also induce apoptosis in infected cells, leading to the release of tumor antigens from apoptotic cells, which can be taken up by antigen-presenting cells (APC). Presentation of tumor antigen by antigen-presenting cells and activation of immunocytes by IL-12 will activate immune responses against the tumor. However, if this hybrid vector infects normal cells (right), the SFV replicon mRNA will not be transcribed and, therefore, neither transgene expression nor apoptosis will occur. **C**, structure of gutless adenovirus vectors. HC-Ad-AFP-SFV-LacZ and HC-Ad-AFP-SFV-mIL-12 are hybrid adenovirus vectors in which the SFV replicon sequence is under the control of the AFP promoter/enhancer and the heterologous genes *LacZ* or *mIL-12* have been placed downstream of the SFV subgenomic promoter, respectively. HC-Ad-AFP-LacZ and HC-Ad-AFP-mIL-12 are gutless adenovirus vectors that contain *LacZ* or *mIL-12* driven directly by the AFP promoter/enhancer, respectively. ITR, inverted terminal repeat; Ψ , adenovirus packaging signal; HPRT, intron region of human genomic hypoxanthine phosphoribosyltransferase as stuffer sequence; C346, cosmid C346 human genomic as stuffer sequence.

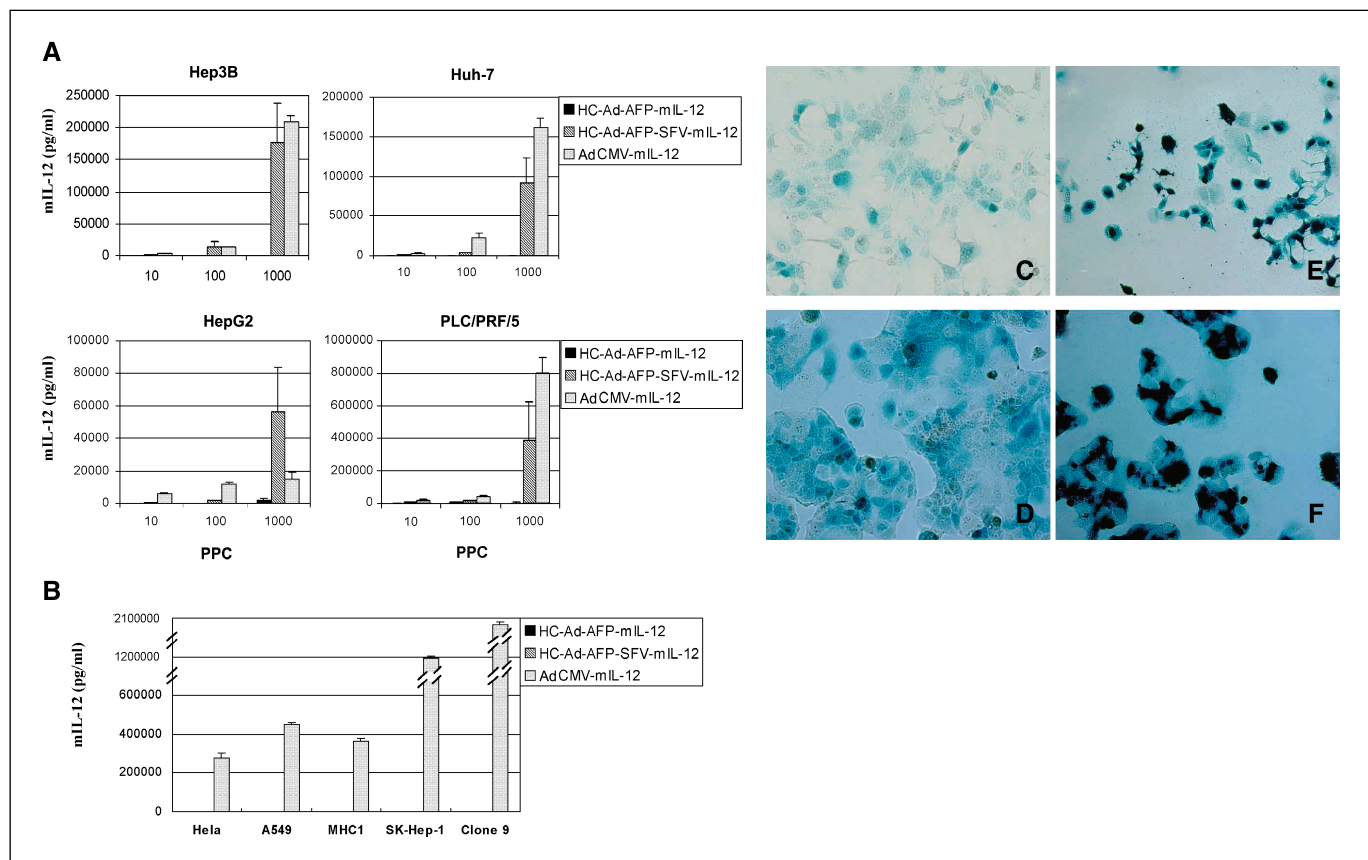


Figure 2. Specific expression of mIL-12 and β -galactosidase in hepatocellular carcinoma cells *in vitro* after infection with hybrid vectors. Four hepatocellular carcinoma cell lines (Hep3B, Huh-7, HepG2, and PLC/PRF/5) with AFP expression (A) and five hepatocellular carcinoma and non-hepatocellular carcinoma cell lines without AFP expression (HeLa, A549, MHC1, Sk-Hep-1, and clone 9; B) were infected with HC-Ad-AFP-mIL-12, HC-Ad-AFP-SFV-mIL-12, or control vector AdCMV-mIL-12 at different ppc (10, 100, and 1,000). After 2 days of infection, the supernatant was collected and the amount of mIL-12 was determined. C to F, two hepatocellular carcinoma cell lines (Hep3B and Huh-7) were infected with HC-Ad-AFP-LacZ or HC-Ad-AFP-SFV-LacZ at ppc 1,000. After 2 days of infection, the infected cells were stained with X-Gal. Microphotographs of cells infected with HC-Ad-AFP-LacZ (C and D) or HC-Ad-AFP-SFV-LacZ (E and F). C and E, Hep3B; D and F, Huh7.

SbfI sites was inserted to form pTGC3001. The AFP-SFV-LacZ cassette was removed by *ApaI* from pBS/AFP-SFV-LacZ-pA and inserted into the *ApaI* site of pTGC3001, giving rise to pTGC3011. In a similar way, the AFP-SFV-mIL-12 cassette was released from pBS/AFP-SFV-mIL-12-pA by *Bss*III digestion and inserted into the *AscI* site of pTGC3001 to generate pTGC3012.

Construction of control vectors. The AFP enhancer/promoter sequence was removed by *MluI* + *Klenow/XhoI* digestion from pGL3/AFP and inserted into pCMV β which had been previously digested with *EcoRI* + *Klenow/XhoI*. In this way, the CMV immediate early promoter was removed from pCMV β and pAFP β was generated. The AFP-LacZ cassette (5,077 bp) was subsequently removed from pAFP β by *XbaI/NarI* digestion and inserted into the *SwaI* site of pSTK120, giving rise to pTGC3013 by blunt-end ligation.

The mIL-12 cassette was removed from pBS/mIL-12 by *XhoI/SpeI* digestion and inserted into pGL3/AFP digested with *XhoI/XbaI*, which removed the luciferase gene from the latter plasmid and gave rise to pAFP-mIL-12. The AFP-mIL-12 cassette (3,760 bp) was released from pAFP-mIL-12 by *BamHI/ScaI* digestion and inserted into *SwaI*-digested pSTK120, generating pTGC3014 by blunt-end ligation.

SFV-mIL-12 vector has been described (24) and carries under the viral subgenomic promoter a mIL-12 cassette containing the genes coding for the p35 and p40 subunits linked by the encephalomyocarditis virus internal ribosome entry site. SFV-mIL-12 and SFV-LacZ recombinant viral particles were produced as previously described (25).

Rescue of gutless adenoviral vectors. Following *PmeI* digestion, phenol/chloroform extraction, and ethanol precipitation, 2 μ g of pTGC3011,

pTGC3012, pTGC3013, or pTGC3014 DNA were transfected into 293Cre4 cells, respectively. After transfection, cells were infected with helper virus AdLC8cluc. Subsequent amplification steps and large-scale preparations were done as described previously (26). All vector preparations were purified twice by CsCl equilibrium density centrifugation. The DNAs from purified vectors were analyzed by restriction digestion and showed no rearrangements. Titration of gutless adenovirus and helper virus contamination was evaluated using quantitative PCR. The titers of HC-Ad-AFP-LacZ, HC-Ad-AFP-SFV-LacZ, HC-Ad-AFP-mIL-12, and HC-Ad-AFP-SFV-mIL-12 were 1.5×10^{12} , 2.7×10^{11} , 8.9×10^{12} , and 2.1×10^{12} virus particles (vp)/mL, respectively. Helper virus contamination of these vectors was 1.2%, 4.1%, 0.6%, and 5% respectively.

Quantitative PCR. For determination of titers of gutless adenoviruses, probes and primers for quantitative PCR of LacZ and mIL-12 sequences were designed. Helper virus contamination was determined by quantitative PCR using probes and primers specific for Ad5 E4 region and wild-type Ad was determined by quantitative PCR with probes and primers specific for Ad5 E1 region. All primers were designed by TaqMan program and synthesized by Sigma-Genosys Ltd. (St. Louis, MO), and Applied Biosystems (Foster City, CA).

Transgene expression in cells infected with gutless adenoviral vectors. Cell lines were infected with each of gutless adenoviral vector at viral particles per cell (ppc) 10, 100, or 1,000, respectively. A first-generation adenovirus AdCMV-mIL-12 was used as control. Supernatants from cells infected with mIL-12 vectors and cell lysates from cells infected with LacZ vectors were collected from duplicate wells for determination

of mIL-12 and β -galactosidase levels, respectively. Cells infected with LacZ vectors were also stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). The mIL-12 level (p70) was measured with an ELISA kit (PharMingen, San Diego, CA). β -galactosidase level was measured with an ELISA kit (Roche, Basel, Switzerland). The time course of mIL-12 expression was evaluated in hepatocellular carcinoma cells after infection with HC-Ad-AFP-mIL-12, HC-Ad-AFP-SFV-mIL-12, or with control vector AdCMV-mIL-12 at ppc 1,000. Supernatants were collected daily until 5 days postinfection.

Cytotoxicity assay cell proliferation assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide incorporation. Hepatocellular carcinoma cells were infected with gutless adenoviral vectors or AdCMV-mIL-12 at ppc 1,000. Five days after infection, cell survival was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were washed once with PBS and 200 μ L of freshly prepared MTT dye solution were added per well (in 48-well plates). Cells were further cultured for 3 to 4 hours followed by addition of 500 μ L of solubilization buffer. One hundred microliters of each sample were taken for measurement of absorbance in a spectrophotometer at a wavelength of 570 nm.

Detection of SFV Rep by immunofluorescence. Cells were seeded on glass coverslips in six-well plates (1×10^5 per well) and infected with HC-Ad-AFP-mIL-12, or HC-Ad-AFP-SFV-mIL-12 at ppc 1,000. Two days after infection, coverslips were rinsed twice with PBS, and cells were fixed with methanol at -20°C for 6 minutes. Coverslips were incubated with primary antibody [monoclonal antibody (mAb) antireplicase, kindly provided by Dr. W. Bodemer, Veterinary Medicine and Primate Husbandry, German Primate Center, Goettingen, Germany] diluted 1:10 at room temperature for 30 minutes followed by incubation with the secondary antibody (FITC-conjugated rabbit anti-mouse, Sigma) diluted 1:250 for 30 minutes. Finally, coverslips were mounted on glass slides using Vecta shield with 4',6-diamidino-2-phenylindole (DAPI) to stain cell nuclei.

Detection of SFV RNA by Northern blot. Cells were seeded in six-well plates (1×10^5 per well) and infected with HC-Ad-AFP-mIL-12, HC-Ad-AFP-SFV-mIL-12 at ppc 1,000, or mock infected. Two days after infection, total RNA was extracted by using RNeasy minikit (Qiagen, Hilden, Germany) and analyzed by Northern blot. Three micrograms of total RNA were size fractionated on a 1.2% agarose/formaldehyde gel, transferred to a nitrocellulose membrane (Hybond-N+, Amersham), and hybridized to a ^{32}P -labeled oligonucleotide specific for SFV subgenomic promoter (5'-GAATTCTGTGTATTAACGCAC-3') or to a ^{32}P -labeled probe specific for human β -actin (5'-CTGGTGCCTGGGGCG-3'). RNA extracted from baby hamster kidney (BHK) cells infected with SFV-IL-12 at 24 hours postinfection was used as positive control.

Induction of hepatocellular carcinoma xenograft and study of *in vivo* gene transfer efficiency. Huh-7 cells (2×10^6) were injected s.c. into the right flank of BALB/c nude mice. When tumor nodules reached 6 to 8 mm in diameter at 4 weeks after inoculation, 1×10^{10} vp of HC-Ad-AFP-LacZ ($n = 4$) or HC-Ad-AFP-SFV-LacZ ($n = 4$) were injected intratumorally. Mice were sacrificed at day 3 postinoculation to obtain tumor and liver samples. Frozen tissues were sectioned for staining with X-Gal and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL, Roche). To study specificity of hybrid vectors *in vivo*, normal BALB/c mice were injected i.v. with HC-Ad-AFP-LacZ ($n = 4$), HC-Ad-AFP-SFV-LacZ ($n = 4$), or AdCMV-LacZ at 1×10^{10} vp/mouse. After 3 days, animals were sacrificed and main organs were collected for LacZ expression analysis.

Induction of orthotopic hepatocellular carcinoma and *in vivo* gene therapy. McA-RH7777 cells (5×10^5) were inoculated into the left liver lobe of Buffalo rats. A single tumor nodule (7-10 mm in diameter) was observed in each animal 10 days after inoculation of tumor cells. Tumors were treated with 2×10^{11} vp of either HC-Ad-AFP-mIL-12, HC-Ad-AFP-SFV-mIL-12, or with 10^8 IU of control vectors SFV-mIL-12 or SFV-LacZ. Two and 4 weeks after treatment, animals were anesthetized and underwent laparotomy to observe the evolution of the tumor. Survival was checked daily in all animals. Tumor size was determined by measuring the length and width of each nodule and applying the formula: Tumor volume = (length) \times (width) $^2 \times 0.5236$.

Results

Characterization of hybrid vectors *in vitro*. Four gutless adenoviral vectors have been constructed as shown in Fig. 1C. To examine the specificity of transgene expression in the recombinant vectors, four human hepatocellular carcinoma cell lines (Hep3B, HepG2, Huh-7, and PLC/PRF/5) producing high levels of AFP (27) and five cell lines (HeLa, A549, SK-Hep-1, MHC1, and clone 9) that do not express AFP were infected with HC-Ad-AFP-mIL-12, HC-Ad-AFP-SFV-mIL-12, or AdCMV-mIL-12 as a positive control. No mIL-12 expression could be detected in human hepatocellular carcinoma cells infected with HC-Ad-AFP-mIL-12 at ppc 10 or 100; a very low level of mIL-12 expression was observed only at ppc 1,000 (Fig. 2A). In contrast, infection of these cell lines with HC-Ad-AFP-SFV-mIL-12 at ppc 10, 100, or 1,000 resulted in mIL-12 expression in a dose-dependent manner (Fig. 2A). The level of mIL-12 expression in cells infected with ppc 10 of HC-Ad-AFP-SFV-mIL-12 was comparable with the level obtained in cells infected with HC-Ad-AFP-mIL-12 at ppc 1,000. Moreover, the mIL-12 level obtained from hepatocellular carcinoma cells infected with HC-Ad-AFP-SFV-mIL-12 was comparable with the one obtained with control vector AdCMV-mIL-12. However, infection of cells that do not express AFP with HC-Ad-AFP-mIL-12 or HC-Ad-AFP-SFV-mIL-12 did not result in detectable mIL-12 production even when the highest ppc (1,000) was used (Fig. 2B). In these cells, however, control vector AdCMV-mIL-12 induced production of high amounts of mIL-12. Similar data were observed by quantification of β -galactosidase expression in hepatocellular carcinoma cells infected with HC-Ad-AFP-LacZ or HC-Ad-AFP-SFV-LacZ vectors (Supplementary Fig. S1). Representative microphotographs of hepatocellular carcinoma cells infected with these vectors followed by staining with X-Gal are shown in Fig. 2C-F. Infection of hepatocellular carcinoma cells with HC-Ad-AFP-LacZ resulted in a low percentage of infected cells with weak staining (Fig. 2C and D). In contrast, infection of hepatocellular carcinoma cells with HC-Ad-AFP-SFV-LacZ produced a high percentage of strongly stained cells (Fig. 2E and F). Importantly, HC-Ad-AFP-LacZ and HC-Ad-AFP-SFV-LacZ did not induce transgene expression in non-hepatocellular carcinoma cells (data not shown). These observations indicate that a hybrid vector carrying an SFV replicon under the control of the AFP promoter/enhancer can induce strong transgene expression specifically in AFP-expressing tumor cells.

Kinetics of mIL-12 expression in hepatocellular carcinoma cells *in vitro*. To study the kinetics of mIL-12 production after infection of hepatocellular carcinoma cells with the hybrid vector, human hepatocellular carcinoma cell lines (Hep3B and Huh-7) were infected with HC-Ad-AFP-mIL-12 or HC-Ad-AFP-SFV-mIL-12. A steady increase in mIL-12 expression from days 1 to 4 postinfection was observed in cells infected with HC-Ad-AFP-SFV-mIL-12 (Supplementary Fig. S2). However, at day 5 postinfection, the mIL-12 level was stabilized or slightly reduced. In cells infected with HC-Ad-AFP-mIL-12, the level of expression was much lower although a slight increase of mIL-12 production was observed along time in culture (Supplementary Fig. S2).

Induction of cell death after infection of hepatocellular carcinoma cells with hybrid vectors *in vitro*. It has been reported that replication of SFV vectors induces apoptotic cell death in most cells of vertebrate origin (21). To check if the hybrid vector also induced apoptosis in hepatocellular carcinoma cells, Hep3B and McA-RH7777 cells, which express AFP, were infected

with HC-Ad-AFP-SFV-mIL-12 or HC-Ad-AFP-SFV-LacZ vectors. As shown in Fig. 3A, survival at day 5 postinfection was <20% in cells infected with either vector. However, infection of the same cells with HC-Ad-AFP-mIL-12, HC-Ad-AFP-LacZ, or with control vector AdCMV-mIL-12 did not affect cell survival.

Expression of SFV Rep in hepatocellular carcinoma cells infected with hybrid vectors *in vitro*. Expression of SFV Rep was examined in human hepatocellular carcinoma (Hep3B and Huh-7) and in AFP-nonexpressing (A549, SK-Hep-1, MHC1, and clone 9) cells infected with HC-Ad-AFP vectors by immunofluorescence using a specific mAb. Only human hepatocellular carcinoma cells infected with HC-Ad-AFP-SFV-mIL-12 displayed a strong cytoplasmic staining for Rep (Fig. 3D). In contrast, human hepatocellular carcinoma cells infected with HC-Ad-AFP-mIL-12 or AFP-nonexpressing cells infected with either vector did not show any staining (Fig. 3B, F, and H). Similar data was observed when human hepatocellular carcinoma cells were infected with HC-Ad-AFP-SFV-LacZ and HC-Ad-AFP-LacZ (data not shown).

Replication of SFV RNA in hepatocellular carcinoma cells infected with hybrid vectors *in vitro*. Replication of SFV RNA was examined in human hepatocellular carcinoma cells (Hep3B) and in AFP-nonexpressing cells (clone 9 or SK-Hep-1) infected with

HC-Ad-AFP-SFV-mIL-12, HC-Ad-AFP-mIL-12, or mock infected. Only human hepatocellular carcinoma cells infected with hybrid vector HC-Ad-AFP-SFV-mIL-12 showed two specific bands that corresponded to the size of genomic and subgenomic RNAs derived from SFV-IL-12 (Fig. 3J, lane 2). These bands were also detected in BHK cells infected with SFV-IL-12, used as a positive control (Fig. 3J, lane 1). No SFV RNAs were detected in human hepatocellular carcinoma cells infected with HC-Ad-AFP-mIL-12 or in AFP-nonexpressing cells infected with either vector.

Gene transfer efficiency and induction of apoptosis in human hepatocellular carcinoma tumors by hybrid vectors. To study the transduction efficiency of the hybrid vectors *in vivo*, we used a model of human hepatocellular carcinoma based on s.c. implantation of Huh-7 cells in BALB/c nude mice. When tumors reached 6 to 8 mm in diameter, animals were injected intratumorally with 1×10^{10} vp of HC-Ad-AFP-SFV-LacZ or HC-Ad-AFP-LacZ as control. As shown in Fig. 4A, there was a weak transgene expression in tumor sections in animals receiving HC-Ad-AFP-LacZ. In contrast, very strong expression of LacZ was found in tumor sections from animals that had received HC-Ad-AFP-SFV-LacZ (Fig. 4B). No transgene expression was observed in liver sections from animals that had received either

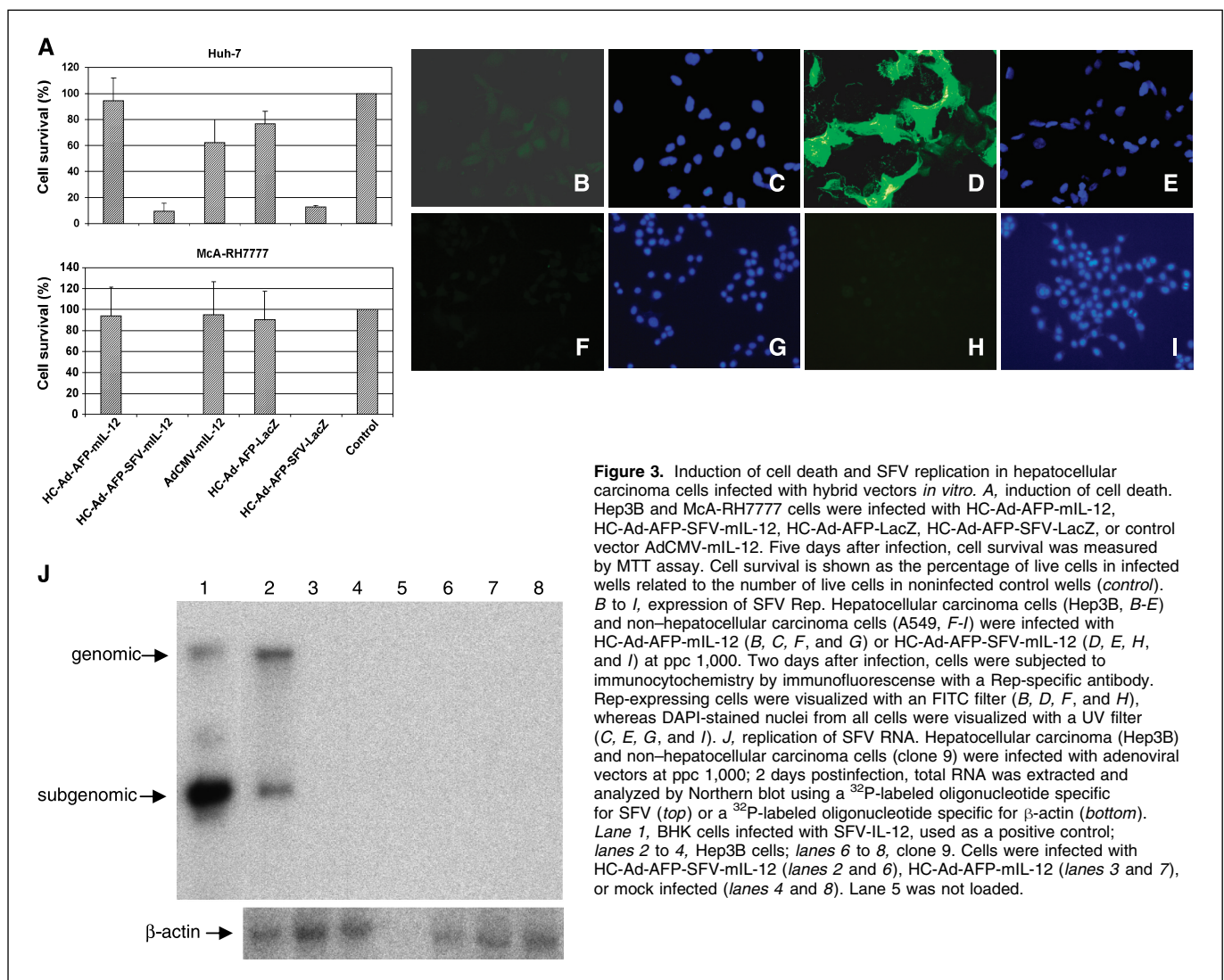


Figure 3. Induction of cell death and SFV replication in hepatocellular carcinoma cells infected with hybrid vectors *in vitro*. **A**, induction of cell death. Hep3B and McA-RH7777 cells were infected with HC-Ad-AFP-mIL-12, HC-Ad-AFP-SFV-mIL-12, HC-Ad-AFP-LacZ, HC-Ad-AFP-SFV-LacZ, or control vector AdCMV-mIL-12. Five days after infection, cell survival was measured by MTT assay. Cell survival is shown as the percentage of live cells in infected wells related to the number of live cells in noninfected control wells (control). **B** to **I**, expression of SFV Rep. Hepatocellular carcinoma cells (Hep3B, **B-E**) and non-hepatocellular carcinoma cells (A549, **F-I**) were infected with HC-Ad-AFP-mIL-12 (**B, C, F, and G**) or HC-Ad-AFP-SFV-mIL-12 (**D, E, H, and I**) at ppc 1,000. Two days after infection, cells were subjected to immunocytochemistry by immunofluorescence with a Rep-specific antibody. Rep-expressing cells were visualized with a FITC filter (**B, D, F, and H**), whereas DAPI-stained nuclei from all cells were visualized with a UV filter (**C, E, G, and I**). **J**, replication of SFV RNA. Hepatocellular carcinoma (Hep3B) and non-hepatocellular carcinoma cells (clone 9) were infected with adenoviral vectors at ppc 1,000; 2 days postinfection, total RNA was extracted and analyzed by Northern blot using a 32 P-labeled oligonucleotide specific for SFV (top) or a 32 P-labeled oligonucleotide specific for β -actin (bottom). Lane 1, BHK cells infected with SFV-IL-12, used as a positive control; lanes 2 to 4, Hep3B cells; lanes 6 to 8, clone 9. Cells were infected with HC-Ad-AFP-SFV-mIL-12 (lanes 2 and 6), HC-Ad-AFP-mIL-12 (lanes 3 and 7), or mock infected (lanes 4 and 8). Lane 5 was not loaded.

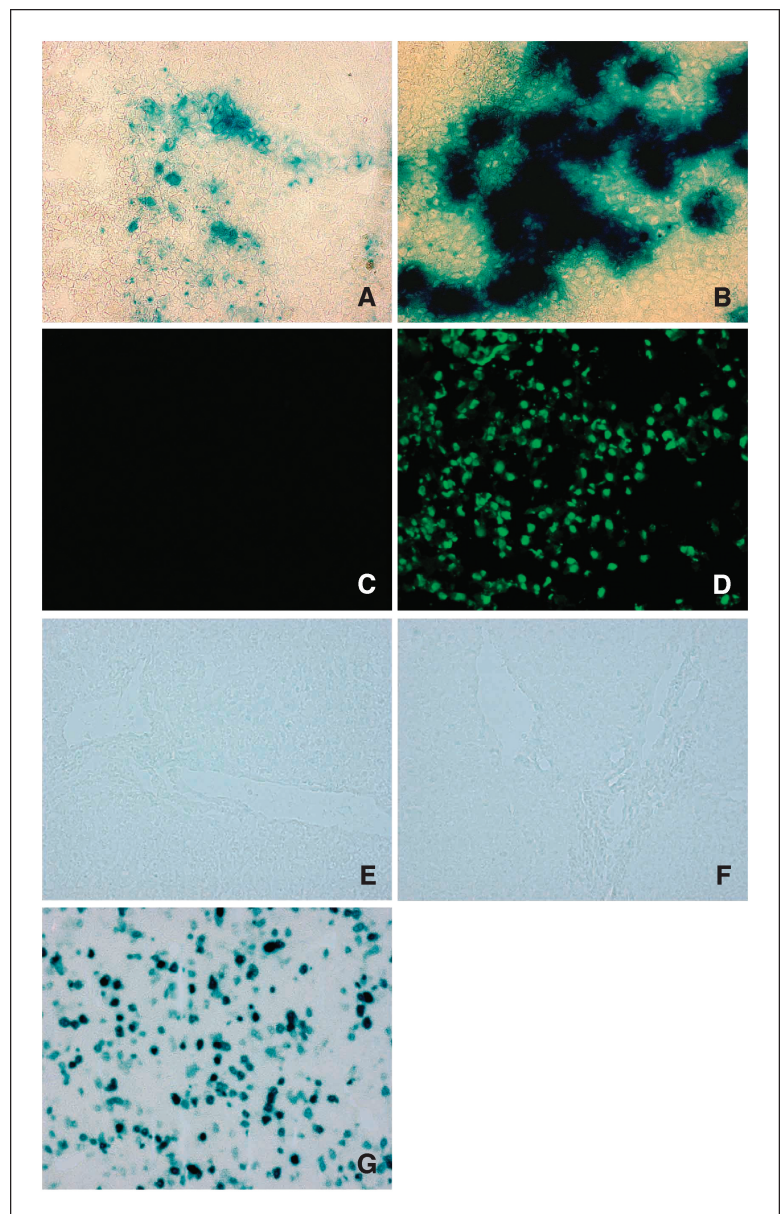
HC-Ad-AFP-LacZ or HC-Ad-AFP-SFV-LacZ (data not shown). Sections of the tumor nodules treated with the adenoviral vectors were also analyzed by TUNEL to study whether hybrid vectors could cause apoptotic cell death. No signal of apoptosis was observed in samples from animals given HC-Ad-AFP-LacZ (Fig. 4C). In contrast, there was a dramatic induction of cell apoptosis in tumors from animals receiving HC-Ad-AFP-SFV-LacZ (Fig. 4D). These data indicate that hybrid vectors do not only specifically induce gene expression in tumor cells at high level but also selectively cause cell death.

Specificity of hybrid vectors *in vivo*. To further show the specificity of hybrid vectors, 10^{10} vp of HC-Ad-AFP-LacZ, HC-Ad-AFP-SFV-LacZ, or control vector AdCMV-LacZ were administered i.v. to BALB/c mice. As shown in Fig. 4E-F, neither HC-Ad-AFP-LacZ nor HC-Ad-AFP-SFV-LacZ vectors were able to induce any detectable transgene expression in liver tissue. However, in animals receiving AdCMV-LacZ, a high proportion of β -galactosidase-

positive cells was observed in liver sections (Fig. 4G). These data further confirm that transgene expression from hybrid vectors is specifically confined to tumor cells.

Antitumoral efficacy and toxicity of hybrid vectors in orthotopic hepatocellular carcinoma in Buffalo rats. To investigate the antitumoral efficacy of the hybrid vector carrying mIL-12, orthotopic hepatocellular carcinomas were established in Buffalo rats by implantation of rat McA-RH7777 cells into the liver. This model was chosen because McA-RH7777 cells have been reported to express AFP (28). Our data showed that animals receiving HC-Ad-AFP-mIL-12 had a reduced tumor size (Fig. 5A) compared with control animals that experienced a steady increase of tumor size along the experiment. However, treatment with HC-Ad-AFP-SFV-mIL-12 resulted in complete tumor regression in 4 of 12 treated rats and partial tumor regression in the rest of animals (Fig. 5A). In animals receiving SFV-mIL-12, a complete tumor regression was observed in 50% of treated animals (Fig. 5A). The

Figure 4. Gene transfer efficiency and induction of apoptosis by hybrid vectors in Huh-7 tumors. Human Huh-7 tumors established in nude mice were treated by intratumoral injection of HC-Ad-AFP-LacZ ($n = 4$) or HC-Ad-AFP-SFV-LacZ ($n = 4$) at 1×10^{10} vp/animal. Three days after virus administration, mice were sacrificed and tumor and liver sections were analyzed for transgene expression by X-Gal staining and for cell apoptosis by TUNEL. A and C, microphotographs from tumors receiving HC-Ad-AFP-LacZ. B and D, microphotographs from tumors receiving HC-Ad-AFP-SFV-LacZ. A and B, stained by X-Gal; C and D, stained by TUNEL. Original magnification, $\times 200$. E to G, specificity of hybrid vectors *in vivo*. BALB/c mice were administered i.v. with HC-Ad-AFP-LacZ ($n = 4$), HC-Ad-AFP-SFV-LacZ ($n = 4$), or AdCMV-LacZ at 1×10^{10} vp/animal. Liver sections were stained with X-Gal. E to G, representative photomicrographs from liver sections of animals receiving HC-Ad-AFP-LacZ, HC-Ad-AFP-SFV-LacZ, or AdCMV-LacZ, respectively. Original magnification, $\times 200$.



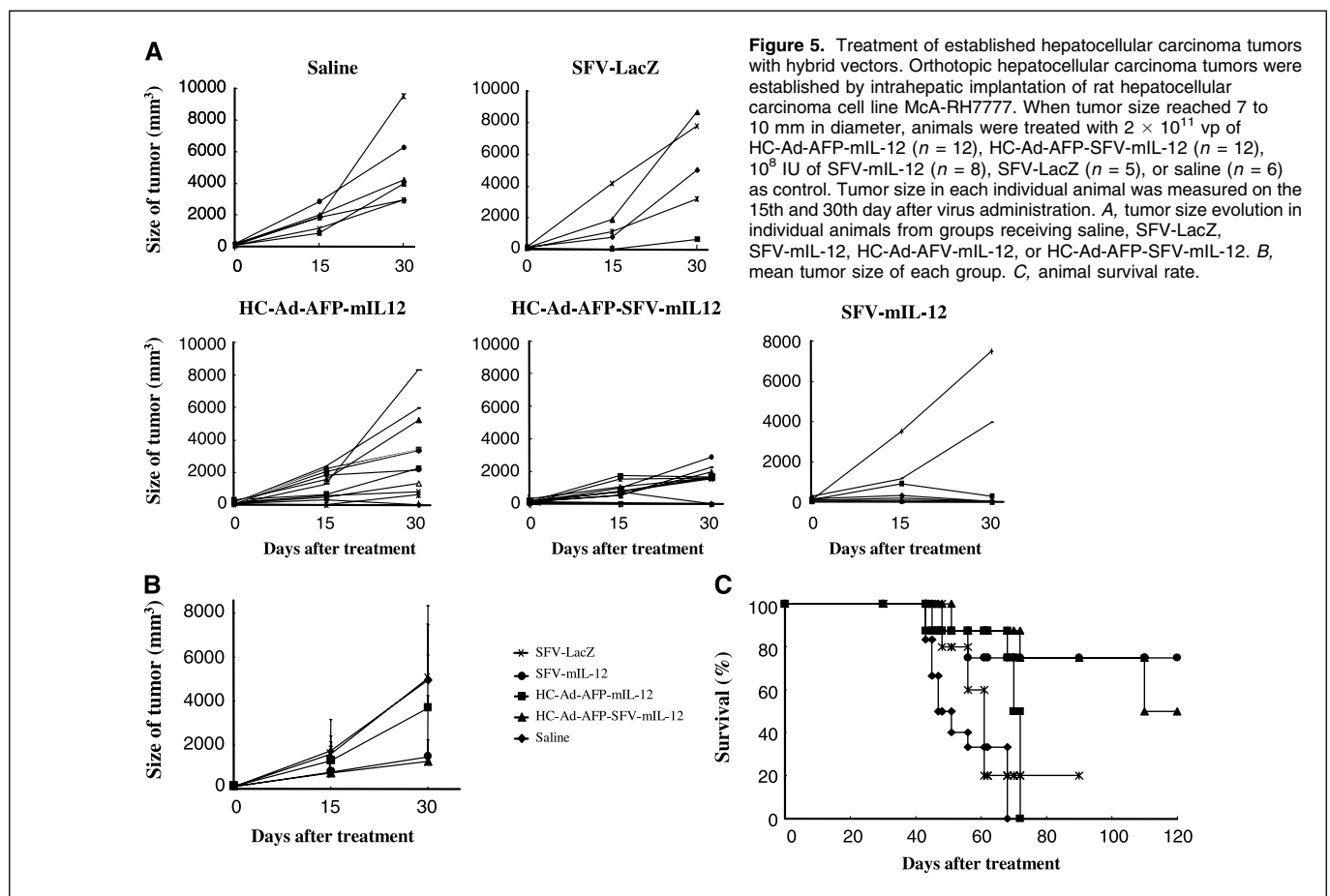
mean value for tumor size in this group was comparable with the one in animals treated with HC-Ad-AFP-SFV-mIL-12 (Fig. 5B). In contrast, animals treated with SFV-LacZ had similar tumor growth as that observed in control animals. Importantly, treatment with HC-Ad-AFP-SFV-mIL-12 induced long-term survival in 50% of treated animals, whereas treatment with HC-Ad-AFP-mIL-12 or SFV-LacZ only increased survival slightly (Fig. 5C).

To evaluate the possible toxicity associated with the administration of vectors *in vivo*, we analyzed serum levels of transaminases [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] and GGT in tumor-bearing animals receiving either 2×10^{11} vp of hybrid vectors or 10^8 IU of SFV-mIL-12 at days 4 and 8 after therapy. As shown in Fig. 6A to C, rats treated with HC-Ad-AFP-mIL-12 ($n = 12$) or HC-Ad-AFP-SFV-mIL-12 ($n = 12$) showed similar levels of transaminases and GGT compared with control animals receiving saline ($n = 6$). However, the levels of transaminases and GGT in animals treated with SFV-mIL-12 were significantly higher than those of other groups ($P < 0.05$). In accordance with these results, no IL-12 could be detected in serum from rats receiving either HC-Ad-AFP-mIL-12 or HC-Ad-AFP-SFV-mIL-12 vectors, in contrast with animals that had received SFV-mIL-12 where low levels of IL-12 were detected at day 4 (data not shown). Furthermore, histologic examination of liver sections showed a normal structure without inflammation in those animals receiving either saline, HC-Ad-AFP-mIL-12, or HC-Ad-AFP-SFV-mIL-12 vectors (Fig. 6D-F). However, moderate inflammation with mononuclear cell infiltration was observed in liver sections from rats treated with SFV-mIL-12 (Fig. 6G). These data suggest that the hybrid HC-

Ad-AFP-SFV-mIL-12 vector does not cause liver toxicity, whereas the SFV-mIL-12 vector can induce liver damage.

Discussion

To combine safety and efficacy in cancer gene therapy, it is necessary for the therapeutic transgenic protein to be expressed at high level only in the treated tumor. Although specific transgene expression in tumor cells can be accomplished by using tumor-specific promoters, high levels of gene expression are usually hard to reach with these rather weak promoters (8–11). In this study, we have developed a novel strategy to potentially increase transgene expression specifically in hepatocellular carcinoma by placing a recombinant alphavirus replicon under the transcriptional control of a tumor-specific promoter. We have selected the AFP promoter and enhancer because AFP is expressed in a high proportion of primary liver tumors (11, 29, 30). The idea was that once the promoter initiates the transcription of the SFV replicon, this self-replicating RNA may induce high production of subgenomic RNA and transgenic protein. The AFP-SFV replicon containing the *mIL-12* or *LacZ* genes downstream of the subgenomic promoter was introduced into a gutless adenovirus. This type of vector was selected for two reasons: (a) it can accommodate large expression cassettes and (b) it exhibits strong tropism for liver cells and high infectivity of liver-derived tumor cells. Thus, the rationale behind the development of the HC-Ad-SFV hybrid vector was the possibility of combining the following in a single vector: (a) high infectivity, by using an adenoviral scaffold; (b) high level of transgene expression

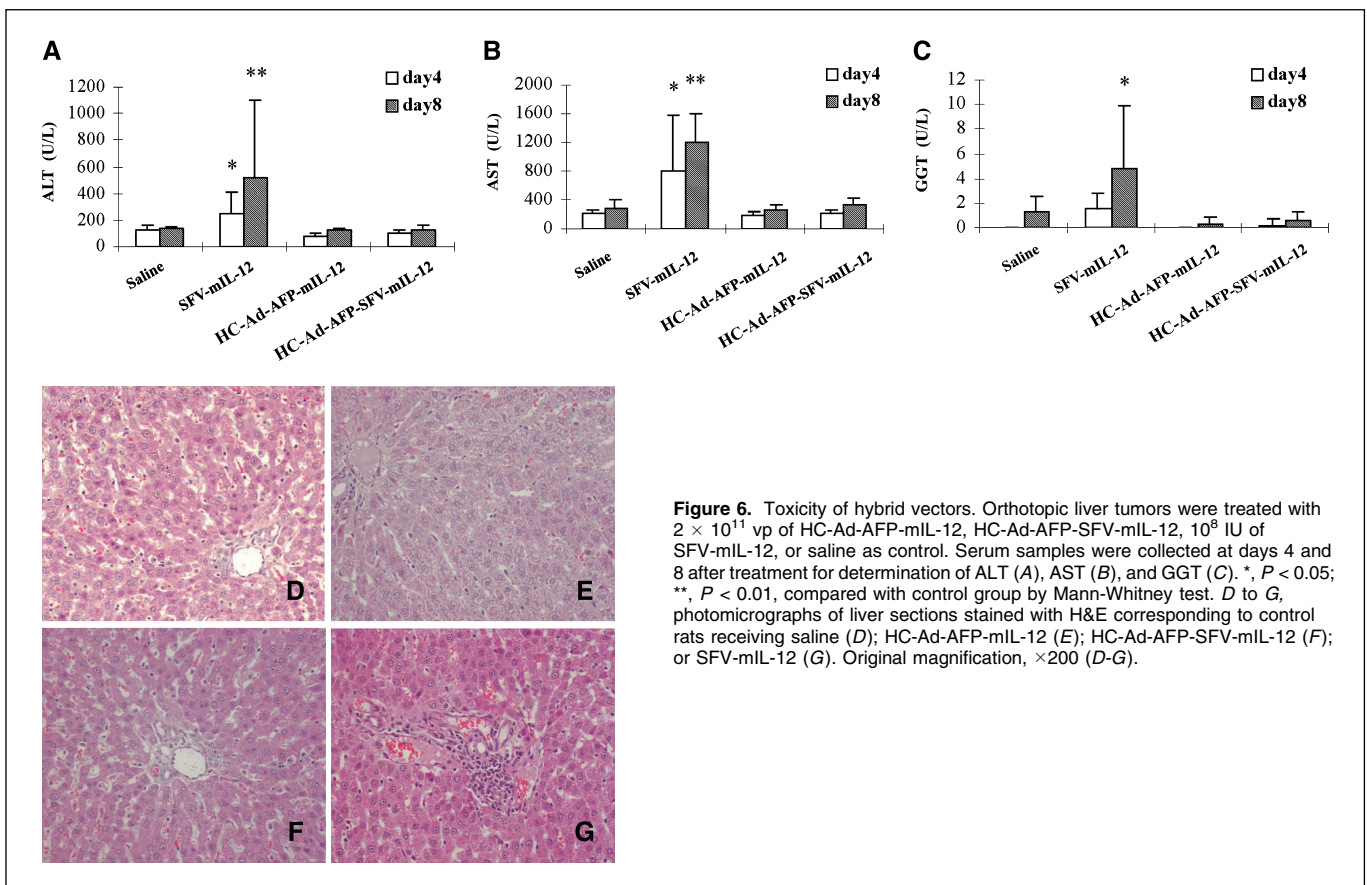


and induction of apoptosis by using a SFV replicon; and (c) tumor specificity by using a tumor-specific promoter driving the transcription of the self-replicating SFV vector.

Data presented here show that both reporter gene *LacZ* and therapeutic gene *mIL-12* could be specifically expressed at very high levels in human hepatocellular carcinoma tumor cells after infection with hybrid HC-Ad-SFV vectors. The observed expression levels were comparable with those induced by the strong universal CMV promoter. In contrast, very low level of transgenic protein could be observed in hepatocellular carcinoma tumor cells after infection with conventional HC-Ad vectors in which transgenes were placed directly under the transcriptional control of the AFP promoter and enhancer. Most importantly, our data showed that in AFP-nonexpressing cells, no transgene expression could be detected after infection with either hybrid or conventional AFP vectors either *in vitro* or *in vivo*. In accordance with these results, SFV replicase was also being expressed specifically in hepatocellular carcinoma cells infected with hybrid vectors. The detection of SFV subgenomic RNA specifically in hepatocellular carcinoma cells infected with HC-Ad-AFP-SFV vector provided additional evidence of SFV RNA replication in these cells. These data indicated that despite the use of a weak promoter, the SFV replicon is able to induce high level of transgene expression without affecting the tissue specificity of the selected promoter. In addition, we have observed that the transgene expression provided by the hybrid vector increased steadily in hepatocellular carcinoma cells after infection during 4 days, reaching a plateau at day 5, probably due to apoptotic cell death of the infected cells. In contrast, the expression

level induced by conventional HC-Ad vectors was always much lower than with the hybrid vector increasing slightly during the period of study.

Alphavirus replication induces apoptosis in most cells from vertebrate origin by mechanisms that are not yet completely understood (21, 31). Because induction of cell death correlates with a high level of SFV RNA replication, it was of great interest to check if apoptosis was also being induced in cells infected with hybrid vectors. Several observations led to the conclusion that this was the case. First, it was observed that some hepatocellular carcinoma tumor cells showed a cytopathic effect at 2 days after infection with hybrid vectors, whereas cells infected with control vectors seemed to be normal. Furthermore, we found that at day 5 postinfection, cell survival was <20% in hepatocellular carcinoma cells infected with the hybrid HC-Ad-SFV vectors, whereas there was no reduction in viability in cells transduced with control vectors. A third proof of evidence came from TUNEL studies done in xenografted Huh-7 tumors infected with HC-Ad-AFP-SFV-LacZ vector, where many cells experienced apoptosis after *in vivo* infection. The ability of hybrid vectors to induce tumor cell apoptosis may contribute to their antitumor efficiency through the release of apoptotic bodies and facilitation of presentation of tumor antigens to the immune system (12, 13, 32). In fact, the apoptotic property of alphavirus vectors has been used by itself to mediate antitumoral responses by treating tumors with repetitive high doses of SFV vectors expressing reporter genes (33). However, in the present study, as well as in previous publications (24), it was shown that at the doses that were used, the cytopathic effect induced



by the replication of SFV was not enough by itself to induce a good antitumoral response; the expression of antitumoral cytokines, such as IL-12, is also necessary to generate a good therapeutic effect. This could probably be due to the fact that when no cytokines are expressed from the vector, a very high transduction efficiency is needed in the tumor to promote a therapeutic response.

Our *in vivo* studies in an animal model of s.c. hepatocellular carcinoma tumor further showed that the transduction efficiency of the hybrid vector containing LacZ was much higher than that of conventional HC-Ad-AFP-LacZ vector. Importantly, transgene expression was not observed in liver from animals receiving either intratumoral or systemic administration of the hybrid vector. The potency and toxicity of HC-Ad-AFP-SFV-mIL-12 was evaluated in an orthotopic rat hepatocellular carcinoma model. The use of this vector resulted in complete tumor regression in 33% of tumor-bearing rats and induced long-term survival in 50% of animals. In contrast, all animals treated with HC-Ad-AFP-mIL-12 (devoid of SFV replicon) were dead by day 70 after treatment. Interestingly, when we used the SFV-mIL-12 vector directly, outside the adenoviral scaffold and, therefore, without the transcriptional restriction provided by the AFP promoter, we found an efficacy comparable with the one obtained with HC-Ad-AFP-SFV-mIL-12. Although the induction of apoptosis per se could be responsible for the antitumoral effect induced by HC-Ad-AFP-SFV-mIL-12 and SFV-mIL-12, the fact that SFV-LacZ did not induce any significant therapeutic effect suggests that expression of IL-12 is also necessary to achieve a good antitumoral response.

When animals were assayed for toxicity, we observed that although there was no change in serum transaminases nor in liver histology in rats that received HC-Ad-AFP-SFV-mIL-12, a significant increase of liver enzymes and a liver inflammatory reaction was found in animals treated with SFV-mIL-12. This toxicity may result from the escape of viral particles to the general circulation, leading to infection of the liver and other organs with production of mIL-12 at high levels outside the treated lesion and induction of apoptosis in transduced cells. More detailed toxicologic studies will

be needed to verify whether liver toxicity associated with SFV vectors could limit their clinical application. These observations reveal that the hybrid HC-Ad-AFP-SFV vector displays potent antitumor effects together with a high safety profile, being superior to other nontargeted vectors and indicating that it may represent a useful tool for clinical applications.

Hepatocellular carcinoma is usually accompanied by liver cirrhosis and it has been observed that AFP can be expressed in some cases in cirrhotic liver (34). Theoretically, this could compromise the specificity of the hybrid HC-Ad-AFP-SFV vector. However, results from our group using positron emission tomography to detect transgene expression in a clinical trial conducted to treat hepatocellular carcinoma with an adenoviral vector encoding HSV-tk showed that after intratumoral injection, the transgene is only expressed in the tumor nodule with complete sparing of the surrounding cirrhotic liver tissue (35). This finding could be explained by the fact that the injected vector escaping to the general circulation failed to infect the cirrhotic liver due to the physical barrier formed by fibrotic tissue between vascular lumen and hepatocytes. Some reports have also shown that cirrhotic liver was difficult to be transduced by adenoviral vectors given by intravascular route in an experimental animal model (36). As well as AFP promoter/enhancer is used for hepatocellular carcinoma, other promoters, such as prostate-specific antigen for prostate cancer (37) or E2F and TERT for various types of tumors (38, 39), could be used instead of AFP, adapting this new strategy to different types of tumors.

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