Original Article

Cancer targeting Gene-Viro-Therapy specific for liver cancer by α-fetoprotein-controlled oncolytic adenovirus expression of SOCS3 and IL-24

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The combination of gene therapy and virotherapy for cancer treatment has received close attention and has become a trend in the field of cancer biotherapy. A strategy called ‘Cancer Targeting Gene-Viro-Therapy’ (CTGVT) or ‘Gene Armed Oncolytic Viral Therapy’ (GAOVT) has been proposed, in which an antitumor gene is inserted into an oncolytic viral vector. In our previous study, a dual-regulated oncolytic adenovirus with enhanced safety for normal cells and strict liver cancer-targeting ability, designated Ad\textsuperscript{enAFP\textsuperscript{E1A\textsuperscript{D55}}} (briefly Ad\textsuperscript{enAFP\textsuperscript{D55}}), was successfully constructed. In the current work, interleukin-24 (IL-24) and suppressor of cytokine signaling 3 (SOCS3) genes were packaged into Ad\textsuperscript{enAFP\textsuperscript{D55}}. The new constructs, Ad\textsuperscript{enAFP\textsuperscript{D55}-IL-24} and Ad\textsuperscript{enAFP\textsuperscript{D55}-SOCS3}, showed improved tumoricidal activity in hepatoma cell lines compared with the oncolytic viral vector Ad\textsuperscript{enAFP\textsuperscript{D55}}. The co-administration of Ad\textsuperscript{enAFP\textsuperscript{D55}-IL-24} and Ad\textsuperscript{enAFP\textsuperscript{D55}-SOCS3} showed much better antitumor effect than Ad\textsuperscript{enAFP\textsuperscript{D55}} alone both in vitro and in a nude mouse xenograft model. Moreover, our results also showed that blockade of the Jak/Stat3 pathway by Ad\textsuperscript{enAFP\textsuperscript{D55}} infection in HuH-7 cells could down-regulate some anti-apoptosis proteins, such as XIAP, Bcl-xL, and survivin, which might sensitize the cells to Ad\textsuperscript{enAFP\textsuperscript{D55}}-induced apoptosis. These results indicate that co-administration of Ad\textsuperscript{enAFP\textsuperscript{D55}-IL-24} and Ad\textsuperscript{enAFP\textsuperscript{D55-SOCS3}} may serve as a candidate therapeutic approach for the treatment of liver cancer.

Keywords cancer biotherapy; oncolytic adenovirus; apoptosis; suppressor of cytokine signaling 3; interleukin-24

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Introduction

To date, cancer remains one of the most refractory diseases in the world. Traditional remedies for cancer, such as surgery, chemotherapy, and radiotherapy, have inevitable drawbacks. Surgery is applicable only when the tumor can be diagnosed at an early stage, and a high rate of relapse exists among patients who receive curative resection. Chemotherapy and radiotherapy have serious side-effects for their inability to specifically target the cancer. Therefore, biotherapy has been studied extensively and may serve as an alternative therapeutic protocol for cancer treatment.

Gene therapy and virotherapy are two hot topics in the field of biotherapy. Gene therapy is a biotechnology that involves the insertion of therapeutic genes into a vector, which will deliver the genes into tissues or cells. Although the fate of gene therapy has fluctuated in the past few decades, this approach has proven to be effective in hereditary single gene-deficient diseases. Promising therapeutic effects have been achieved for patients with Leber’s congenital amaurosis [1,2], X-linked adrenoleukodystrophy, [3] and severe combined immunodeficiency [4] in recent years. Therefore, gene therapy was lauded as one piece of top 10 science news in 2009 by Science magazine, in an article titled ‘Gene Therapy Returns’. However, no great
breakthrough has taken place for cancer gene therapy. Only p53 gene therapy (Gendicine) was approved for marketing. Virotherapy developed from the idea of viral-mediated therapy of cancer. Huebner et al. treated cervical carcinoma patients with cell lysate from six wild-type adenoviruses in 1956 [5]. The first oncolytic virus was developed by Martuza et al. by using HSV-1 in 1991 [6]. One of the most important oncolytic viruses was ONYX-015 (dl 1520), which was constructed from the hybrid virus Ad2/Ad5 by the deletion of the 55 kDa protein gene of E1B in adenovirus [7]. H101, a similar product of ONYX-015, was approved as the world’s first oncolytic virus therapy for cancer treatment in China [8]. The oncolytic adenovirus ZD55 was also constructed in our lab, which was also similar to ONYX-015; however, ZD55 contained a site for cloning foreign genes into the vector for cancer therapy [9].

Despite the progresses that have been made in gene therapy and virotherapy, the efficacies of these approaches for cancer treatment remain suboptimal when used singly. The ‘Cancer Targeting Gene-Viro-Therapy (CTGVT)’ strategy was developed by Liu in 2001. This strategy was one of the first reports of the idea of inserting of an antitumor gene into an oncolytic adenoviral vector for cancer therapy [10]. Hermiston later suggested the use of the armed therapeutic virus for the treatment of human tumors in 2002 [11], and in 2003, Zhang et al. showed that the cytokine deaminase (CD) gene-armed oncolytic virus ZD55 has potent antitumor effects in vitro and in vivo [9]. Since the oncolytic virus can replicate with several hundred folds in cancer cells, the inserted genes can also be amplified at the same magnitude. Because CTGVT or Gene Armed Oncolytic Viral Therapy (GAOVT) combines the advantage of gene therapy and virotherapy, this approach has now become a major interest in cancer therapy research.

In our previous study, a dual-regulated oncolytic adenovirus, named Ad•enAFP•E1A•E1B (Δ55) (briefly Ad•enAFP•D55), was constructed. This adenovirus showed improved safety for normal cells and replicated selectively in α-fetoprotein (AFP)-positive liver cancer cells [12]. In the current work, interleukin-24 (IL-24) and suppressor of cytokine signaling 3 (SOCS3) genes were individually inserted into Ad•enAFP•D55, named as Ad•enAFP•D55-(IL-24) and Ad•enAFP•D55-(SOCS3), respectively. IL-24/melanoma differentiation-associated gene 7 (Mda-7) was identified by subtraction hybridization in human melanoma H0-1 cells treated with interferon-β and mezerein [13]. This gene was thought to be a ‘magic bullet’ for cancers with multiple functions and might be developed as a cancer therapeutic [14,15]. The promoter of the SOCS3 gene is frequently methylated in its CpG island in hepatocellular carcinoma cells, which leads to SOCS3 silencing. SOCS3 silencing has been shown to ubiquitously activate the Janus kinase (Jak)/signal transducer and activator of transcription (Stat) pathway [16,17]. Restoration of SOCS3 by an oncolytic adenovirus induced potent antitumor activities in hepatocellular carcinoma [18].

Materials and Methods

Cell cultures

The HepG2, Hep3B (human hepatocellular carcinoma, human HCC), and MRC-5 (normal lung fibroblast cells) cell lines were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The human HCC cell line HuH-7 was obtained from the RIKEN Cell Bank (Ibaraki, Japan). The human hepatoma cell line PLC/PRF/5 was kindly provided by Dr Qijun Qian (Second Military Medical University, Shanghai, China). The HEK293 cell line was purchased from Microbix Biosystems Inc. (Toronto, Ontario, Canada). These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO BRL, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL). All of the cell lines were cultured at 37°C in a humidified air atmosphere with 5% CO2.

Construction, purification, and titration of recombinant adenovirus

The adenovirus shuttle plasmid penAFP-D55, in which E1A was driven by the simian virus 40 (SV40) enhancer/ AFP composite promoter (briefly enAFP) and E1B-55K was deleted, was previously constructed and preserved in our laboratory [12]. The IL-24 expression cassette (with the hCMV promoter and SV40 polyadenylation signal) was released from the previously constructed plasmid pCA13-IL-24 by digestion with Bg/II and inserted into the Bg/II site of penAFP-D55 to generate the recombinant plasmid penAFP-D55-IL-24. The SOCS3 cDNA was a kind gift from Professor Cheng Qian (Zhejiang Sci-Tech University, Hangzhou, China) [18]. The plasmid penAFP-D55-SOCS3 was constructed using a similar method. Recombinant oncolytic adenoviruses, including Ad•enAFP•D55, Ad•enAFP•D55-(IL-24), and Ad•enAFP•D55-SOCS3, were generated by standard homologous recombination techniques involving transfection of the adenoviral shuttle plasmid (penAFP-D55, penAFP-D55-IL-24, and penAFP-D55-SOCS3, respectively) and the adenovirus packaging plasmid pBHGE3 (Microbix Biosystems) in HEK293 cells with Effectene Transfection Reagent (Qiagen, Hilden, Germany). Large-scale purification of the adenoviruses was performed by ultracentrifugation with cesium chloride, and the purifications were subject to dialysis. The viral titers were...
determined by the tissue culture infectious dose 50 (TCID<sub>50</sub>) method in HEK293 cells.

**Cytotoxic assay**

Different cell lines were grown to subconfluence and infected with adenoviruses at various multiplicities of infection (MOI). Five days after infection, the cells were exposed to 2% crystal violet in 20% methanol for 15 min, washed with distilled water (dH<sub>2</sub>O), and photographed.

**Cell viability by the colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

Cells were seeded in 96-well plates at a density of 6 × 10<sup>3</sup> cells per well. When the cells reached subconfluence, they were infected with adenoviruses at the indicated MOI. Five days post-infection, MTT solution (20 μl; 5 mg/ml) was added to each well, and the cells were further incubated at 37°C for 4 h. The supernatant was replaced with dimethyl sulfoxide (DMSO) to dissolve the solid product. The absorbance at wave length 570 nm was measured with a Multiskan MK3 microplate reader (Thermo Scientific, Waltham, USA). The ratio of the absorbance of treated cells relative to that of the untreated cells was calculated.

**Quantitative analysis of synergy**

The pharmacological interaction between Ad•enAFP•D55-(IL-24) and Ad•enAFP•D55-(SOCS3) was determined by the Chou-Talalay multiple drug effect analysis [19]. A parameter called the combination index (CI) was calculated using the CalcuSyn software (Biosoft, Waltham, USA). At CI = 1, the interaction is considered additive. At CI < 1, synergy is indicated, and at CI > 1, antagonism is indicated.

**Flow cytometric analysis**

Both adherent and floating HuH-7 cells were harvested at 72 h after mock infection, with Ad•enAFP•D55, Ad•enAFP•D55-(IL-24), Ad•enAFP•D55-(SOCS3), or with their combination at an MOI of 10. For analysis of mitochondrial membrane potential (ΔΨ<sub>m</sub>), cells harvested from experimental samples were stained with the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarboxyanine iodide (JC-1) fluorescent probe staining kit (Beyotime, Haimen, China) according to the manufacturer’s instructions. The proportion of cells with JC-1 aggregates or monomers was analyzed with an FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, USA).

**Western blot analysis**

Whole-cell lysates were separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride filter membranes (Amersham, Piscataway, USA). Membranes were blocked in 5% non-fat dry milk, hybridized to various primary antibodies, detected by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, revealed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA), and visualized with the LAS-4000 System Configured for Multifunctional Analysis (Fujifilm Life Science, Tokyo, Japan). The primary antibodies used were: anti-E1B 55 kDa (Oncogene, La Jolla, USA; 1:500), anti-IL-24 (GenHunter, Nashville, USA; 1:2000), anti-SOCS3 (Abcam, Cambridge, UK; 1:1000), anti-GAPDH (CWBio, Beijing, China; 1:500), anti-Stat3(S727) (Bioworld, Minneapolis, USA; 1:500), anti-Stat3, anti-Stat3(Y705), anti-XIAP, anti-Bcl-xL, anti-survivin, anti-cleaved caspase 3 (CST, Danvers, USA; 1:1000), anti-E1A, anti-PARP, and anti-caspase 3 (Santa Cruz Biotechnology, Santa Cruz, USA; 1:200). All HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (1:2000).

**Animal experiments**

Four-week-old female athymic nude mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China). All procedures were performed in accordance with the National Institute of Health (NIH, Bethesda, USA) Guide for the Care and Use of Laboratory Animals. For the treatment of pre-established tumors, 5 × 10<sup>6</sup> HuH-7 cells in 150 μl DMEM were subcutaneously injected into the right flank of each mouse. When the tumors reached 90–120 mm<sup>3</sup>, the mice were randomly divided into five groups (eight mice per group) and treated by intratumor injection of Ad•enAFP•D55, Ad•enAFP•D55-(SOCS3), Ad•enAFP•D55-(IL-24), or Ad•enAFP•D55-(SOCS3) plus Ad•enAFP•D55-(IL-24) at 2 × 10<sup>9</sup> plaque-forming units (PFUs) per animal or PBS as a control. A daily dose of 6.67 × 10<sup>8</sup> PFUs viruses suspended in 100 μl of PBS or 100 μl PBS alone was administrated intratumorally for 3 days. Tumor volume (mm<sup>3</sup>) was measured weekly by a Vernier caliper and calculated as follows: Tumor volume (mm<sup>3</sup>) = (length × width<sup>2</sup>) / 2.

**Histopathology and immunohistochemical analysis**

Liver or tumor tissues were fixed in 4% formaldehyde, dehydrated with an ethanol gradient, embedded in paraffin, and cut in 4 μm sections. For histopathological analysis, the paraffin sections of livers or tumors were stained with hematoxylin and eosin (H&E). For immunohistochemical analysis, tissue sections were dewaxed and rehydrated according to a standard protocol. The sections were washed with PBS, treated with 3% H<sub>2</sub>O<sub>2</sub>, and blocked with blocking solution. These steps were followed by overnight incubation with the primary antibody at the proper dilution.
The following procedures were performed with the avidin–biotin–peroxidase complex reagent (Vector Laboratories, Burlingame, USA) and detected with DAB (3,3′-diaminobenzidine) solution containing 0.006% hydrogen peroxide. Hematoxylin was used as a counterstain. All sections were examined with bright field microscopy.

**TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay**

Apoptotic cells on glass coverslips or in tumor tissue sections were assessed using an *in situ* apoptosis detection kit (Trevigen, Gaithersburg, USA) according to the manufacturer’s instructions. Cells on glass coverslips were counterstained with methyl green, and tumor tissue sections were counterstained with hematoxylin.

**Statistical analysis**

Statistical significance was assessed with the Student’s *t*-test and one-way analysis of variance (ANOVA) by GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA). The results were considered statistically significant when *P* < 0.05.

**Results**

**Construction and characterization of the therapeutic gene-armed dual-regulated oncolytic adenoviruses**

The DNA sketch map of the adenoviruses used in this work is shown in Fig. 1(A). The IL-24 or SOCS3 gene expression cassette was inserted in the backbone of Ad•enAFP•D55, a dual-regulated oncolytic adenovirus with AFP-targeting ability. To characterize the viruses, the expression of E1A and E1B-55K was examined in HuH-7 cells infected with the viruses by western blot analysis, using wild-type adenovirus-infected sample as a positive control. E1A expression was detected in all virus-infected samples, except for the mock-infected sample, whereas E1B-55K expression was only detected in the wild-type adenovirus-infected sample. The exogenous IL-24 or SOCS3 expression was also validated by western blot analysis [Fig. 1(B)].

**Cytotoxicity assay and synergy analysis**

To investigate the cytotoxicity of the newly constructed adenoviruses, several human hepatocellular cancer cell lines (HuH-7, HepG2, and PLC/PRF/5) and a normal human lung fibroblast cell line (MRC-5) were infected with Ad•enAFP•D55, a dual-regulated oncolytic adenovirus with AFP-targeting ability. To characterize the viruses, the expression of E1A and E1B-55K was examined in HuH-7 cells infected with the viruses by western blot analysis, using wild-type adenovirus-infected sample as a positive control. E1A expression was detected in all virus-infected samples, except for the mock-infected sample, whereas E1B-55K expression was only detected in the wild-type adenovirus-infected sample. The exogenous IL-24 or SOCS3 expression was also validated by western blot analysis [Fig. 1(B)].

Figure 1 Characterization of the newly constructed adenoviruses (A) Schematic diagram of AdWT, Ad•enAFP•D55, and Ad•enAFP•D55-(gene). In Ad•enAFP•D55-(gene), the native E1A promoter was replaced by an enAFP composite promoter and E1B-55K was replaced by the expression cassette of the exogenous genes driven by the hCMV promoter. ITR, inverted terminal repeat; ψ, packaging signal. (B) Expression of E1A, E1B-55K, SOCS3, and IL-24. HuH-7 cells were infected with Ad•enAFP•D55, Ad•enAFP•D55-(SOCS3), Ad•enAFP•D55-(IL-24), or the combination of Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) at an MOI of 10, and AdWT was used as a positive control. After 48 h, proteins in the cell lysates were detected by western blot analysis. GAPDH was used to normalize the amount of proteins loaded.
cancer cells, while no inhibitory effect was observed in MRC-5 cells with any treatment [Fig. 2(A)].

The cytotoxicity of the different adenoviruses was further evaluated by the MTT assay. Similar results were obtained compared with those from the crystal violet assay. The inhibitory effect on tumor cell proliferation was greater and more pronounced in the cancer cells treated with Ad•enAFP•D55, Ad•enAFP•D55-(SOCS3), Ad•enAFP•D55-(IL-24), and the combination of Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) at the indicated MOIs. Five days later, the cells were fixed and stained with crystal violet. (B) All cells as above were infected with the indicated MOI (MOI = 1 or 10) of Ad•enAFP•D55, Ad•enAFP•D55-(SOCS3), Ad•enAFP•D55-(IL-24), and the combination of Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) or were treated with mock infection. Five days post-infection, cell viability was determined by the MTT assay. The results are presented as the mean ± SD (n = 4, *P < 0.05; **P < 0.01; ***P < 0.001; NS indicates P > 0.05, not significant) and are expressed as the percentage relative to mock-treated control cells. (C) Dose-effect curves for HuH-7 cells after treatment with different combinations of Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) at a fixed ratio (1:1). Cell viability was determined by the MTT assay. Uninfected cells were considered to be 100% viable. The results are presented as the mean ± SD (n = 4). (D) Combinational index determined by the median-effect analysis program was calculated by the CalcuSyn software. Fa, fraction-affected; CI, combinational index. * indicates synergism (CI < 1).

cancer cells, while no inhibitory effect was observed in MRC-5 cells with any treatment [Fig. 2(A)].

The cytotoxicity of the different adenoviruses was further evaluated by the MTT assay. Similar results were obtained compared with those from the crystal violet assay. The inhibitory effect on tumor cell proliferation was greater and more pronounced in the cancer cells treated with Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) co-infection than with either virus alone under most circumstances. Little inhibitory effect was detected in normal cells [Fig. 2(B)].

To address the interplay of Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24), HuH-7 cells were infected with the two viruses separately or in combination at various MOIs in a fixed ratio (1:1). As shown in the dose–response curves, the half-maximal inhibitory concentration (IC50) for Ad•enAFP•D55-(SOCS3) alone and Ad•enAFP•D55-(IL-24) alone was 1.21 MOI and 0.97 MOI, respectively, but after combination the IC50 for each virus was reduced to 0.53 MOI [Fig. 2(C)]. A synergistic effect was induced by using the two viruses in combination at 2.5 MOI and 5 MOI, demonstrated by a CI of 0.34 and 0.47, respectively [Fig. 2(D)].

**Effects of combined use of Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) on apoptosis**

TUNEL staining was performed on HuH-7 cells with different treatments. Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) infection separately, or in
combination, caused brown staining in the nucleus, which was a hallmark of apoptosis [Fig. 3(A)]. A drop in mitochondrial membrane potential ($\Delta \Psi _m$) is considered as an early event during the process of apoptosis, which can be measured by JC-1 staining and is reflected as an increase in JC-1 monomers. To further assess apoptosis in a quantitative manner, fluorescence-activated cell-sorting (FACS) analysis was performed to measure the apoptosis induction by combined use of Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) in the HuH-7 cells. As shown in Fig. 3(B), 63.54% of HuH-7 cells lost $\Delta \Psi _m$ with Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) co-infection, while this proportion of cells after Ad•enAFP•D55-(SOCS3) or Ad•enAFP•D55-(IL-24) single infection was 27.47% and 30.77%, respectively. Three independent experiments were performed, and cells with JC-1 monomers were calculated [Fig. 3(C)]. The data indicated that the apoptotic effect of combined infection with Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) was more significant than infection with either Ad•enAFP•D55-(SOCS3) or Ad•enAFP•D55-(IL-24) alone. To address the mechanisms underlying the apoptosis-inducing phenomena, the key proteins involved in apoptosis were detected by western blot analysis in the HuH-7 cells. The phosphorylation of Stat3 on tyrosine 705 or serine 727 was reduced in Ad•enAFP•D55-(SOCS3) or combination treated samples, which blocked the Jak/Stat3 pathway and resulted in down-regulation of Bcl-xL, survivin, and XIAP. The expression level of pro-caspase 3 was decreased and cleaved caspase 3 was elevated in samples infected with Ad•enAFP•D55-(SOCS3) or Ad•enAFP•D55-(IL-24) separately or in combination [Fig. 3(D)].

Figure 3 Apoptosis induction by combined Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) (A) HuH-7 cells were infected with Ad•enAFP•D55, Ad•enAFP•D55-(SOCS3), Ad•enAFP•D55-(IL-24), and the combination of Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) at an MOI of 10. Two days post-infection, TUNEL staining was performed. Arrows indicate representative cells with apoptotic features. Scale bar = 50 $\mu$m. (B) HuH-7 cells were infected with Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) separately or in combination at an MOI of 10, using Ad•enAFP•D55 as a control. After treatment for 72 h, the cells were harvested for JC-1 staining. The percentage of apoptotic cells with a drop in $\Delta \Psi _m$ was determined by flow cytometry. (C) Three independent experiments in (B) were performed and the data are presented as the mean ± SD ($n=3$; ** $P<0.01$, *** $P<0.001$). (D) HuH-7 cells with the indicated virus treatment at an MOI of 10 for 3 days were harvested for western blot analysis. GAPDH was used as a loading control.
Antitumor effect of the combined use of Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) in an animal tumor model

The efficacy of tumor growth inhibition of the combined gene-viral therapy was studied in vivo. A tumor model in nude mice bearing human hepatocellular carcinoma was established by implantation of HuH-7 cells in the right flank of the animal. At the end of the experiment, the tumor volume of the PBS-, Ad•enAFP•D55-, Ad•enAFP•D55-(SOCS3)-, Ad•enAFP•D55-(IL-24)-treated and combination-treated groups were 2926.3 ± 634.5, 1824.9 ± 82.1, 1242.2 ± 473.4, 1161.8 ± 74.9, and 310.8 ± 109.8 mm³, respectively [Fig. 4(A)]. The tumor growth was inhibited in either group treated with viruses compared with the negative control group treated with PBS. Combined treatment with Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) led to the most potent inhibition of tumor masses in all treated animals. With regard to the lifespan, the survival rate of the PBS- and Ad•enAFP•D55-treated groups were 2/8 and 3/8, respectively. However, all the mice in the Ad•enAFP•D55-(SOCS3)-, Ad•enAFP•D55-(IL-24)-treated or combination-treated groups were alive [Fig. 4(B)].

Seven days after the first injection, tumor specimens were collected to perform histopathology, immunohistochemistry, and TUNEL staining. H&E staining of tumor tissue slides indicated that necrotic areas in the combined treatment group were much larger than that in any of the other treated groups [Fig. 4(C)]. However, H&E staining of the liver tissues seemed quite normal for all groups, which suggests that no liver toxicity was caused by the treatment (Supplementary Fig. 1). The adenoviral capsid protein hexon and the therapeutic gene product SOCS3 and IL-24 were detected by immunohistochemistry in tumor tissue slides. TUNEL staining indicated that the combined treatment induced the most dramatic apoptosis in the tumor tissue, which is consistent with the result obtained from the in vitro studies with the cell line [Fig. 4(C)].

Discussion

To generate CTGVT with a higher targeting ability and antitumor effect, many modifications were made in our laboratory. One promising modification is called Cancer Targeting Gene-Viro-Therapy for Tissue Specific Cancer (CTGVT-TSC). According to our definition, the CTGVT-TSC should meet two requirements: (i) use of an oncolytic construct containing a tissue-specific cancer promoter to drive the viral replication-essential genes (E1A for adenovirus) and armed with a cancer suppressor gene that is deficient or down-regulated in this specific tissue-derived cancer; (ii) use of an additional construct containing insertion of a stronger antitumor gene into a similar vector, to be used in combination with the above construct. In the current work, an enAFP composite promoter with liver cancer-specific transcriptional activity was used to drive the adenoviral E1A expression. The SOCS3 gene, a cancer suppressor gene ubiquitously silenced in human hepatocellular carcinoma by promoter methylation [16], was used to arm the oncolytic adenoviral vector and form the construct.
Ad•enAFP•D55-(SOCS3). The potent antitumor gene IL-24 was also applied to arm the same vector and form another construct Ad•enAFP•D55-(IL-24). Therefore, co-administration of the Ad•enAFP•D55-(SOCS3) and Ad•enAFP•D55-(IL-24) was a novel Cancer Targeting Gene-Viro-Therapy specific for liver cancer (CTGV-T-LC).

The CTGV-TSC approach takes advantage of the Cancer Targeting Dual Gene-Viro-Therapy (CTGV-DG) strategy, in which two genes with compensatory or synergetic effects are used. In this work, Ad•enAFP•D55-(SOCS3) down-regulated anti-apoptosis proteins, such as Bcl-xL, survivin, and XIAP, through dephosphorylation of Stat3 by SOCS3 overexpression [Fig. 3(D)]. Because inhibitors of apoptosis (IAP) are usually constitutively expressed in HCC cell lines [20] and IL-24 induces apoptosis of diverse cancer cell lines through Jak/Stat-independent pathways [21], SOCS3 overexpression may sensitize cells to IL-24-induced apoptosis in liver cancer.

However, it is notable that our oncolytic adenoviral constructs developed in this work are replication selective, but not infection selective. Previous study demonstrated that intravenous injection resulted in adenovirus accumulation in the liver [22]. The coagulation factor X directly binds to the Ad5 hexon leading to liver infection, which compromises the adenovirus’ potential efficacy following intravenous delivery [23]. In order to achieve both ‘replication-selective’ and ‘infection-selective’ for cancer, our laboratory is developing nanoparticles with special ligand to target cancer cells, and the novel adenoviral constructs are packaged into such nanoparticles.

Supplementary Data
Supplementary data are available at ABBS online.

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