Abstract. We previously demonstrated that hepatocellular carcinoma suppressor 1 (HCCS1) exerts potent anti-tumor activity. In this study, we constructed a new dual tumor-targeting oncolytic adenovirus vector, PD55-HCCS1, in which E1A was driven by the promoter of progression elevated gene-3, which is hepatoma-specific, and a CMV-HCCS1 expression cassette replaced E1B55. The PD55-HCCS1-mediated selective expression of E1A and HCCS1 in hepatoma cells and tumor-selective cytotoxicity in vitro and in vivo demonstrated the strongest inhibition of BEL-7404 cell xenografts in nude mice among a number of control Ad vectors. These data indicated the efficacy and safety of the PD55-HCCS1 system for HCC treatment.

Introduction

Hepatocellular carcinoma suppressor 1 gene (HCCS1) was discovered as a candidate tumor suppressor gene by analysis of the highest-frequency loss of heterozygosity (LOH) region on human chromosome 17p13.3 and positional cloning in hepatocellular carcinoma (HCC) (1,2). HCCS1 was demonstrated to have a higher frequency of mutations and a significantly lower level of expression in HCC samples than in non-cancerous liver tissues. Additionally, transfection of HCCS1 into tumor cells markedly reduced the efficiency of colony formation in vitro and inhibited tumor growth in vivo (1). These findings suggested that HCCS1 plays a role in tumor progression and is a novel therapeutic gene for HCC. We have found that a high level of expression of HCCS1 protein induced apoptosis in hepatoma cells and that the pro-apoptotic process occurred mainly through the mitochondrial and lysosomal pathways (unpublished data).

HCC is one of the most frequently occurring solid cancers and more than half a million people around the world succumb to it each year (3). However, traditional treatments such as surgery, radiotherapy, chemotherapy, and liver transplantation, alone or in various combinations, are not very effective. Therefore, innovative treatments are urgently needed. Cancer gene therapy has been evaluated as a novel and promising treatment modality (4-6). The candidate cancer suppressor gene HCCS1 may be applied as a potentially therapeutic gene for HCC.

To achieve sufficient anti-tumoral effects, it is critical to deliver therapeutic genes efficiently into target cancer cells (7-9). Adenovirus (Ad) vectors can rapidly infect a broad range of human cells with high efficiency and achieve high levels of transgene expression. Moreover, the Ad viral genome is genetically stable and the inserted foreign genes are generally maintained without change through successive rounds of viral replication (10). These features make Ad vectors attractive in gene therapy. For safety reasons, however, virus vectors employed in conventional cancer gene therapy are generally replication-incompetent viruses, which limit the efficacy of gene transfer by restricting the number of tumor cells to which the therapeutic gene is delivered as well as the transgene copy number per cell. To target this problem, tumor-specific replication-competent viruses have been proposed as vectors (11). We (12) previously constructed an armed therapeutic oncolytic Ad system, ZD55-gene, an E1B55 gene-deleted Ad similar to ONYX-015 (dl1520), but with a cloning site for foreign anti-tumor gene insertion. Such a gene-virotherapy, a marriage of gene therapy and oncolytic virus therapy, is expected to create synergism and deliver therapeutic effects superior to that of each of the single strategies alone, a hypothesis that has been validated in a number of cases (13,14).
ZD55, the putative p53-dependent vector, is supposed to be attenuated in somatic cells because of the deletion of E1B55, but actually its replication appears to be similar to ONYX-015 which depends not only on p53 status but also on individual cell line characteristics concerning cell cycle regulation, induction of apoptosis, and the applied MOI (15). To minimize the toxicity of replication-competent viral vectors, a strategy using tumor-selective promoter(s) to control the expression of early viral gene(s) essential for replication, such as E1A, has been used (16, 17). Progression elevated gene-3 (PEG-3) is a rodent gene identified by subtraction hybridization and it displays elevated expression following transformation by diversely acting oncogenes, DNA damage, and cancer cell progression. The promoter of PEG-3 (PEG-3p) displays robust activity in a broad spectrum of human cancer cell lines but has marginal activity in corresponding normal cells (18). The mechanism underlying this cancer specificity involves defined binding with specific promoter sequences by PEG-3, activator protein-1 (AP-1), and polyoma enhancer activator protein-3 (PEA-3), which are expressed at elevated levels in the majority of cancer cells (19). Recombinant Ads in which E1A expression was controlled by PEG-3 resulted in selective replication in rodent and human cancer cells (18). However, no evidence exists that PEG-3p showed a higher transcriptional activity in malignant versus normal liver cells.

To evoke a potent and selective destruction of HCC with minimal injury of normal cells, we initially observed the higher transcriptional activity of PEG-3p in hepatoma cells, and based on the ZD55-gene system, we constructed a dual tumor-targeting recombinant Ad whose E1A was driven by PEG-3p, and E1B55 was replaced with a HCCS1 expressing cassette. The anti-tumoral efficacy of this dual tumor-targeting gene-virotherapy system Ad PD55-HCCS1 was evaluated for HCC in vitro and in vivo.

Materials and methods

Cell lines and culture conditions. The normal human liver cell line L-02 (WT p53), and human hepatoma cell lines BEL-7404 (mutated p53), BEL-7405 (mutated p53) and QGY-7703 (low p53 expression) were purchased from Shanghai Cell Collection (P.R. China). The human embryo kidney cell line HEK293 was obtained from Microbix Biosystems, Inc. (Toronto, Ontario, Canada). The L-02, BEL-7404, QGY-7703 and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) and the BEL-7404 cell line was grown in RPMI-1640 supplemented with 10% FBS at 37°C in 5% CO2.

Control Ads. Ad.Null, Ad-CMV-HCCS1 and Ad-PEG-3p-HCCS1 are replication-incompetent Ads. ONYX-015 and ZD55-HCCS1 are conditionally replication-competent Ads, in which ONYX-015 is structurally and functionally similar to ZD55.Null. The summary of the genetic features of the Ads used in this study are shown in Table 1.

Plasmid construction. The regions of E1A promoter (342-552) of pZD55 was deleted by overlap extension PCR. Briefly, by using the pZD55 DNA as a template, the region containing nucleotides 22-342 was amplified with the primers: 1F 5’-TATACGCGTATCATGATCATGATCAGCGGCCGCACGCGTATA-3’ and 1R 5’-CAGTGATCAGCGGCCGCACGCGTATA-3’, and the region containing nucleotides 552-1359 was amplified with the primers: 2F 5’-TATACGCGTGCCGCGCGCTGATCATGATCATGATCAGCGGCCGCACGCGTATA-3’ and 1R 5’-CAGTGATCAGCGGCCGCACGCGTATA-3’. The amplified regions were used to construct the plasmids pPD55-HCCS1, the normal E1A promoter (342-552 bp) was replaced by PEG-3p, and the HCCS1 expression cassette replaced E1B55.

Luciferase assay. Cells were plated at a density of 1.0x10^5 cells/ml and transfected with pGL3-PEG-3p using Lipo-
The cells were harvested, lysed and centrifuged, and the supernatants were used as total cell lysates for Western blot analyses of E1A and HCCS1.

Western blot analyses of E1A and HCCS1. Cells were infected with Ads at a multiplicity of infection (MOI) of 10 for 48 h. Western blot analyses were performed to detect the corresponding proteins.

ZD-antisense 5'-CATCGTACCTCAGCACCTCCA-3' which are complementary to the flank region sequences of E1B55.

The virus was identified by PCR using the primers ZD-sense 5'-AGAGCCCATGGAACCCGAGA-3' and ZD-antisense 5'-CATCGTACCTCAGCACCTTCCA-3' which are complementary to the flank region sequences of E1B55.

Western blot analyses of E1A and HCCS1. Cells were infected with Ads at a multiplicity of infection (MOI) of 10 for 48 h. The cells were harvested, lysed and centrifuged, and the supernatants were used as total cell lysates for Western blot analyses. The anti-E1A mouse monoclonal antibody (M58; NeoMarkers, Fremont, CA) and anti-HCCS1 mouse monoclonal antibody (prepared by our laboratory) were used to detect the corresponding proteins.

Crystal violet staining assay for evaluating cytolysis in vitro. Cells were plated in 24-well plates (2x10⁴ cells/well) 24 h before infection. Infection was performed with each virus in 500 μl serum-free growth medium at the indicated MOI (0.01, 0.1, 1 and 10) and incubation was carried out for 2 h at 37°C under 5% CO₂. The infection medium was replaced with medium containing 5% FBS. Seven days after infection, cells were fixed with formalin solution and stained with crystal violet. Twenty minutes later, the plates were washed with water to remove excess dye and then dried. Images of the plates were captured with a scanner.

Colorimetric cell viability assay in vitro. A colorimetric assay via 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT, Roche Molecular Biochemicals, Indianapolis, IN) was used to assess cell viability after viral infection. Cells were plated at 4x10³/well in 96-well plates. After incubation for 24 h, the cells were infected with 10 MOI of the viruses. Ten microliters of MTT (10 mg/ml) were added to each well at different time points (24, 48, 72 and 96 h) and the cells were incubated at 37°C for 4 h. The supernatant was discarded and dimethyl sulfoxide (DMSO), as the solubilizing solution, was added at 150 μl/well. Plates were read at 492 nm with an ELISA reader (Bio-Tek Instruments, Winooski, VT). Cell viability was calculated as:

\[ \text{Cell viability} = \frac{A_{492\text{nm}} \text{mean value of infected cells}}{A_{492\text{nm}} \text{mean value of uninfected cells}} \times 100\% \]

Anti-tumor activity in vivo. BALB/c athymic nude mice at 4-5 weeks old were obtained from the Shanghai Cancer Institute (P.R. China). Mice were inoculated subcutaneously with BEL-7404 cells (2x10⁶). After 14 days, when the tumor volume reached 100 mm³ in size, the mice were randomly divided into three groups (n=7 per group) for PD55-HCCS1, ZD55-HCCS1 and PBS, respectively. The viruses (5x10⁸ PFU) or PBS were injected intratumorally once every other day for 4 times. Since the beginning of tumor cell inoculation, tumor size was measured by a Vernier caliper every 7 days. The tumor volume (mm³) was calculated as \( (\text{length} \times \text{width}^2)/2 \). The mice were sacrificed 35 days after inoculation, and tumors were excised and weighed.

Immunohistochemical assay. Tumor tissues from the above-treated mice were fixed in 4% paraformaldehyde, processed in paraffin, sectioned with 4 μm thickness, and then dewaxed, hydrated and incubated in methanol-H₂O₂ for 20 min to remove endogenous peroxidase. Tissue sections were stained with the HCCS1 mouse monoclonal antibody (prepared by our laboratory) according to the immunohistochemical staining procedures provided by the Envision™ detection kit (Dako, Glostrup, Denmark).

Results

PEG-3p showed higher transcriptional activity in malignant versus normal liver cells. As shown in Fig. 1a, the relative luciferase activity in QGY-7703, BEL-7404 and BEL-7405 cells was 1.5-, 3.9-, and 4.7-fold of that in L-02 cells.

Table I. Summary of the genetic features of the Ads used in this study.

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<th>E1B55</th>
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<td>Ad.Null</td>
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respectively. The data indicated that the PEG-3p was able to drive the expression of exogenous genes preferentially in hepatoma cells.

**Verification of PD55-HCCS1 construction.** For safety purposes, we produced a replication-competent tumor-targeting Ad named PD55-HCCS1 by replacing the normal E1A regulatory elements with PEG-3p and inserting the HCCS1 expression cassette in the E1B55 region (Fig. 1b). A correct fragment of 3042 bp was obtained in PCR using primers ZD-sense and -antisense, which was different from the fragments of 1153 and 254 bp, respectively, for WT Ad5 and PD55.Null. In addition, a 350 bp PCR product was amplified with PEG-3p primers in PD55-HCCS1 (data not shown).

**PD55-HCCS1 mediates cancer cell-specific E1A and HCCS1 expression.** BEL-7404 (mutated p53), QGY-7703 (low p53 expression) and L-02 (WT p53) cells were infected with Ad.Null, ONYX-015, Ad-CMV-HCCS1, Ad-PEG-3p-HCCS1, ZD55-HCCS1, PD55-HCCS1 at 10 MOI; *P<0.05 and ** P<0.01.

HCCS1 protein expression resulting from Ad infection differed for the four transgenes containing Ads. In L-02 cells, infection with ZD55-HCCS1 and Ad-CMV-HCCS1 resulted in a robust HCCS1 production, whereas infection with Ad-PEG-3p-HCCS1 or PD55-HCCS1 resulted in barely detectable (Ad-PEG-3p-HCCS1) or very low (PD55-HCCS1) levels of HCCS1 production (Fig. 2a). In hepatoma cell lines, infection with Ad-CMV-HCCS1, Ad-PEG-3p-HCCS1, ZD55-HCCS1, or PD55-HCCS1 produced a significant HCCS1 expression and the two replication-competent vectors resulted in higher HCCS1 expression levels than the former two replication-incompetent vectors (Fig. 2a). Taken together, these findings documented that PEG-3p facilitated hepatoma cell-selective Ad replication and HCCS1 expression.

**Selective anti-tumor activity and safety of PD55-HCCS1 for HCC in vitro.** The cytopathic effects (CPE) of PD55-HCCS1 on BEL-7404, QGY-7703 and L-02 were investigated with crystal violet staining assay. Compared to Ad.Null, ONYX-015, Ad-CMV-HCCS1, Ad-PEG-3p-HCCS1 and ZD55-HCCS1, PD55-HCCS1 showed an approximate cytotoxicity in the L-02 cells. However, PD55-HCCS1 infection resulted in an ~10-100-fold higher anti-tumor activity than ONYX-015, Ad-CMV-HCCS1 or Ad-PEG-3p-HCCS1 infection, and an ~1-10-fold higher anti-tumor activity than ZD55-HCCS1 infection (Fig. 2b).
The selective inhibition of liver cancer cells by PD55-HCCS1 was further corroborated by MTT colorimetric assay (Fig. 2c). PD55-HCCS1 showed a similar cytotoxicity in L-02 cells as Ad-CMV-HCCS1 (P>0.05), a significantly less cytotoxicity than ZD55-HCCS1 (P<0.01), and a higher cytotoxicity than Ad.Null, ONYX-015 or Ad-PEG-3p-HCCS1 (P<0.01). In contrast, PD55-HCCS1, at 96 h after viral infection, demonstrated more cytotoxicity in BEL-7404 or QGY-7703 cells than any of the five control Ads (P<0.05).

Antitumoral efficacy of PD55-HCCS1 in nude mice. Since PD55-HCCS1 had demonstrated dual tumor-targeting characteristics in vitro which suggested its higher safety profile for treating hepatoma, experiments were performed on nude mice to identify whether PD55-HCCS1 was able to specifically inhibit tumor growth in vivo. In our previous experiments (data not shown), ZD55-HCCS1 has been confirmed to be the most effective therapeutic Ad in vivo and in vitro against SW-620 and BEL-7404, among all types of Ads mentioned above (not including the new recombinant PD55-HCCS1). Therefore, ZD55-HCCS1 may be used as a good positive control in the PD55-HCCS1 experiment in vivo.

As shown in Fig. 3a, a significant inhibition of tumor growth was observed in the mice treated with PD55-HCCS1 or ZD55-HCCS1 compared with those treated with PBS (P<0.01). The suppression efficacy of PD55-HCCS1 on tumor growth was also found to be greater than that of ZD55-HCCS1 (P<0.05). The mice were sacrificed 35 days after inoculation. As shown in Fig. 3b and c, the mean wet weight of tumors that received injection of PD55-HCCS1 was less than those receiving an injection of ZD55-HCCS1 (P<0.05) or PBS (P<0.01). The suppression rate of the tumor weight from PD55-HCCS1 reached (77.41±11.65)%.

Expression of HCCS1 was demonstrated by immuno-histochemical staining in BEL-7404 cells in mice treated with ZD55-HCCS1 or PD55-HCCS1 which presented as concentrated positive staining of cytoplasm (Fig. 3d). The expression level of HCCS1 in cells treated with PD55-HCCS1 was comparable to or even higher than those treated with ZD55-HCCS1. The results indicated an effective expression of HCCS1 protein from the two recombinant Ad infections in xenograft in vivo.

Discussion

HCCS1 has been identified as a novel candidate for tumor suppressor genes in our previous study (1). After transfection in human hepatocarcinoma cells, HCCS1 exhibited significant inhibition of cell proliferation in vitro and ex vivo. To confirm the anti-tumor efficacy of the HCCS1 gene, we have demonstrated in our recent study as well as this study that the HCCS1 gene, delivered by Ad-CMV-HCCS1 and ZD55-HCCS1, which were replication-incompetent and -competent Ads, respectively, and where HCCS1 expression was driven by the CMV promoter, provided sufficient inhibition of tumor cell growth in vitro (Fig. 2b and c) and in vivo (data not shown). These data further established the potent activity of HCCS1 in cancer gene therapy. In order to enhance the safety of cancer...
gene therapy by utilizing HCCS1 as the therapeutic gene. Ad-PEG-3p-HCCS1 was produced where HCCS1 was driven by PEG-3p, a tumor-selective promoter, and this vector showed less cytotoxicity than Ad-CMV-HCCS1 in L-02 cells (Fig. 2c). Based on the above findings and in order to enhance the tumor-selective and therapeutic effect, PD55-HCCS1, which was a recombinant conditionally replication-competent Ad, was generated. Its E1A was driven by PEG-3p and the CMV-HCCS1 expression cassette was inserted in the region of the deleted E1B55. Since PD55-HCCS1 replicated better in cancer versus normal cells and preferentially expressed HCCS1 protein in tumor cells, it demonstrated a strong selectively inhibitory activity in a number of HCC cell lines tested and the selective inhibition was superior to ONYX-015, Ad-CMV-HCCS1, Ad-PEG-3p-HCCS1 and ZD55-HCCS1. The in vivo experiment showed that PD55-HCCS1 produced a marked tumor growth suppression greater than that of ZD55-HCCS1, which may be explained by the result of immunohistochemical staining, in that the expression level of HCCS1 in cells treated with PD55-HCCS1 was comparable to or even higher than those treated with ZD55-HCCS1.

The cytotoxicity of Ad-CMV-HCCS1 was similar to Ad.Null in L-02 (Fig. 2b and c) but Ad-CMV-HCCS1 had a higher cytotoxicity than Ad.Null in HCC cells. It suggested that HCCS1 as a novel tumor suppressor gene candidate alone has tumor-selective cytotoxicity to some extent. The precise mechanism is still unclear and more studies on normal cell lines from different origins are needed to verify whether it is an extensive phenomenon. We find that PEG-3p has a higher transcriptional activity in malignant versus normal liver cells by luciferase assay. Since early activation of AP-1 probably contributes to the acquisition of a transformed phenotype during hepatocarcinogenesis (20), it is possible that the up-regulation or activation of AP-1 plays a role in robust PEG-3p transcriptional activity in HCC cells.

Most tumor suppressor genes efficiently trigger apoptosis in human cancer cells via death receptors or the mitochondrial pathway. In contrast, conditionally replication-competent Ad can kill a variety of human cancer cells via a process morphologically distinct from apoptosis which is characterized by chromosome condensation and nuclear shrinkage and fragmentation (21). These two types of anticaner elements using different cytotoxic machineries have been shown to induce a synergistic effect (12,13). Therefore, HCCS1 as a tumor suppressor gene is suitable for use in combination with replication-competent Ad to establish a novel compound cancer therapeutic approach.

In summary, this study has established the anti-tumor activity of HCCS1, a tumor suppressor gene candidate, in vitro and in vivo. This novel dual tumor-targeting Ad PD55-HCCS1 gene-virotherapy system is capable of selective replication in lysis of, and efficient expression of HCCS1 protein in malignant liver cells, and is a potentially effective and safe therapeutic approach for HCC.

Acknowledgements

This study was supported by the grants from the Key Programs of the National Natural Science Foundation of P.R. China (No. 30330350).

References