Telomerase-specific oncolytic virotherapy for human hepatocellular carcinoma

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AIM: To evaluate the therapeutic efficiency of replicative adenovirus CNHK300 targeted in telomerase-positive hepatocellular carcinoma.

METHODS: CNHK300, ONYX-015 (55 kDa protein deleted adenovirus) and wtAd5 (wild type adenovirus 5) were compared, and virus proliferation assay, cell viability assay, Western blot and fluorescence microscopy were used to evaluate the proliferation and cytolysis selectivity of CNHK300.

RESULTS: The replicative multiples in Hep3B and HepG2 after 48 h of CNHK300 proliferation were 40 625 and 65 326 fold, respectively, similar to that of wtAd5. However, CNHK300 exhibited attenuated replicative ability in normal fibroblast cell line BJ. CNHK300 could lyse hepatocellular carcinoma cells at a low multiplicity of infection (MOI), but could not affect growth of normal cells even at a high MOI.

CONCLUSION: CNHK300 is a cancer-selective replication-competent adenovirus which can cause oncolysis of liver cancer cells as well as wtAd5 (wild type adenovirus 5), but had severely attenuated replicative and cytolytic ability in normal cells. This novel strategy of cancer treatment offers a promising treatment platform.

Key words: Gene therapy; Virus therapy; Replicative adenovirus; Hepatocellular carcinoma

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INTRODUCTION

Replication-defective adenoviruses have been successfully established to deliver genes for cancer gene therapy. But, progress in clinical practice has been hampered by lack of cell specific infectivity and poor viral distribution within the tumor mass[1-2]. To confer specificity of infection and increase viral spread in the tumor mass, replication-competent adenovirus is being developed as a promising cancer therapeutic agent. These replication-competent adenoviruses can cause oncolysis of tumor cells as part of the virus life cycle, the new virus progeny released from the dead cells infects the neighboring cells and continues to replicate and spread until the tumor is eradicated. Furthermore, tumor antigens released from the dead cells can enhance antitumor immunity[3]. Until now, more than 10 kinds of replication-competent oncolytic viruses have been evaluated in different phases of clinical trials. This novel strategy of cancer treatment offers a promising treatment platform[4-6].

The key of replication-competent oncolytic adenovirus modification is to constrict viral replication to tumor cells. One approach is to regulate expression of viral genes essential for replication with tumor-specific promoters that are highly active in tumor cells, but inactive in normal cells. For example, human breast cancer has been targeted with the adenovirus Ad. DF3-E1 in which the E1A gene is under the control of the DF3/MUC1 gene promoter/enhancer[7]. Several other tumor-specific promoters are also being evaluated to restrict viral replication to their cognate tumors, such as prostate-specific antigen enhancer element for prostate carcinoma and α-fetoprotein for...
hepatocellular carcinoma\textsuperscript{8-10}. Although these results demonstrated that these reconstructed adenovirus had high selectivity, their major drawback was that they were only available for treatment of a narrow range of tumors, because only a limited number of tumors could express the targeted tumor markers. Telomerase is the broadest spectrum molecular marker of malignancies, which is highly activated in immortalized cell lines and most of the malignant tumors, but is inactive in normal somatic cells. Human telomerase reverse transcriptase (hTERT) is the major determinant of human telomerase activity\textsuperscript{11}. H\textsuperscript{TERT} promoter is highly active in tumor cells but inactive in normal cells, and utilizing hTERT promoter to drive antitumoral genes in cancer gene therapy can target to and selectively kill the cancer cells with positive telomerase activity\textsuperscript{12-14}.

In this study, we report a strategy for the development of a novel replication-competent oncolytic adenovirus. In this virus, the E1A gene was placed under the control of the human hTERT promoter to allow us to target a wide range of telomerase-positive tumors with little influence on the growth of normal cells.

**MATERIALS AND METHODS**

**Virus**

CNHK300 is a tumor-specific replication competent adenovirus variant that was reconstructed at our lab. hTERT gene promoter was inserted at the upstream of the E1A encoding region of the viral genome. Because different cell lines may show various levels of susceptibility to adenovirus infection and virus production, non-selectively replicating wild type 5 adenovirus (wtAd5) was compared with the tested conditionally replicating adenovirus CNHK300. CNHK300-GFP virus carried green fluorescent protein (GFP) as reporter gene which inserted the upstream of hTERT promoter of CNHK300. ONYX-015 is an E1B 55 kDa deleted adenovirus kindly presented by Professor Berk AJ (University of California, USA)\textsuperscript{15-17}. All adenoviruses were grown on the human embryonic kidney cell line HEK293. Virus titers were determined by plaque assay. Ratios of virus particle and plaque were between 20:1 and 100:1 normally. CNHK300 and ONYX-015 virus preparation were free of the wtAd5 and endotoxin contamination.

**Cells and cell cultures**

The human hepatocellular carcinoma cell lines HepG II (wild-type p53), Hep3B (mutated p53) and human normal fibroblast cell line BJ were purchased from the American Type Culture Collection (Manassas, VA). Transformed human embryonal kidney HEK293 cell line was obtained from Microbix Biosystem Inc. (Toronto, Ontario, Canada). HEK293, HepG II and Hep3B were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine. All the media were supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37°C in 5% CO\textsubscript{2}.

**Detection of hTERT mRNA in various cell lines**

RT-PCR amplification method was used to detect the hTERT mRNA. Total RNA was extracted from cell lines by the Trizol Reagent (Invitrogen Corp., San Diego, CA). First strand cDNAs synthesis and PCR reaction were performed using the SuperScript one-step RT-PCR with Platinum Taq Kit (Invitrogen Corp.) according to the manufacturer’s protocol. The reaction mixture was heated at 50°C for 30 min, and then 30 cycles of PCR were performed, including denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s, and then 72°C for 2 min. For PCR reaction, sense and antisense oligonucleotide primers (5’-CGGAAGAGGTGTCTGGAGCAA-3’; 5’-GGATGAAGCGGAGTCTTGGA-3’) were designed. The PCR products (150 bp in length) were electrophoresed on 10% agarose gel.

**Detection of E1A expression by Western blotting**

HepG II, Hep3B and BJ cells were plated at a density of 5 × 10\textsuperscript{4} in 6-well plates (Falcon) and 24 h later infected with CNHK300, ONYX-015 or wtAd5 at a MOI corresponding to IC\textsubscript{50}. After a 2 h incubation at 37°C in 5% CO\textsubscript{2}, culture medium were removed, the cells were then washed twice with serum-free medium and incubated at 37°C in 5% CO\textsubscript{2}. Forty-eight hours later, culture medium were removed, total cell lysates were prepared using 150 µL/well of Mammalian Protein Extraction Reagent (M-PER, PIERCE, Rockford, IL). Twenty µL of aliquots were subjected to detection on 10% SDS-PAGE and E1A protein was detected with M73 antibody against adenovirus E1A (Santa Cruz Biotechnology, Santa Cruz, CA). The signals were visualized with LumiGLO chemiluminescence reagent and peroxide (Cell Signaling Technology, Bevery, MA).

**Proliferation of CNHK300-GFP and GFP expression**

Hep3B and BJ cells were plated at a density of 2 × 10\textsuperscript{5} in 6-well plates (Falcon). When the cells reached 80% confluence 2-4 d later, the cells were infected with CNHK300-GFP at a MOI of 0.01, 1, 10 plaque forming unit (pfu)/cell, respectively. The cells were observed and photographed at 3, 7 and 10 d after infection.

**In vitro viral replication assay in different cell types**

HepG II, Hep3B and BJ cells were plated at a density of 5 × 10\textsuperscript{4} in 6-well plates (Falcon) and 24 h later infected with CNHK300, ONYX-015 or wtAd5 at MOI of 5 pfu/cell. After a 2 h incubation at 37°C in 5% CO\textsubscript{2}, culture medium were removed, the cells were then washed twice with serum-free medium and incubated at 37°C in 5% CO\textsubscript{2} for varying periods of time (0 h, 12 h, 24 h, 48 h, 96 h), the cultures at each time point were harvested and lysed by three cycles of freeze and thaw. The supernatant of each time point was tested for virus production on 293 cells for 10 d with plaque assay. The relative ability of the viruses to amplify in these cells after infection was calculated by comparing the ratio of viral yields to initial virus input.

**In vitro cell viability assay**

MTT assay was performed to determine cell viability at
various viral MOIs. HepGII, Hep3B and BJ cells were plated at a density of $1 \times 10^4$ in 96-well plates (Falcon) and 24 h later, the cells were infected with CNHK300 at increasing MOIs. After 7 d of incubation, cell viability was measured by the MTT assay using the non-radioactive cell proliferation kit (Roche Molecular Biochemicals) according to the kit protocol and the spectrophotometrical absorbance of the samples using a microtiter plate (ELISA) reader at 570 nm (the reference wavelength was 650 nm). The percentage of cell survival was calculated using the formula: Cell survival (%) = (A value of infected cells/A value of uninfected control cell) × 100%. Eight replicate samples were taken at each MOIs, and each experiment was performed at least three times.

RESULTS

Detection of hTERT mRNA in various cell lines
RT-PCR was performed to detect the hTERT mRNA expression in HepGII, Hep3B cells and normal cells. As shown in Figure 1, telomerase hTERT mRNA expression was demonstrated in both cancer cells tested. In contrast, BJ fibroblast cells did not express hTERT mRNA.

Detection of E1A expression by Western blotting
The Western blotting revealed that CNHK300 could express E1A in the HepGII and Hep3B cells but not in the BJ human normal fibroblast cells. As controls, BJ cells were also infected with wtAd5, and the results indicated that wtAd5 could effectively express E1A in BJ cells. These findings demonstrated that CNHK300 selectively expressed E1A in telomerase positive hepatocellular cancer cells, whereas wtAd5 did not show any selectivity in E1A expression (Figure 2).

Proliferative selectivity of CNHK300-GFP
As shown in Figure 3, CNHK300-GFP could infect both BJ and Hep3B cells and expressed GFP within 3 d. But after 7 d of infection, CNHK300-GFP virus could proliferate effectively and express GFP protein. When the CNHK300-GFP was released from the infected cancer cells, it could infect the adjacent cells and enter into new life cycles. Therefore, the cancer cells showed obvious proliferation and cytopathologic effect (CPE) at the 7th day. Because many cancer cells died from virus infection after 10 d of infection, the GFP protein was degraded and extincted gradually. Whereas in normal cell lines, CNHK300-GFP proliferation was attenuated, and BJ cells did not show CPE after days of infection (Figure 3).

Replication of CNHK300 attenuated in normal cells but not in tumor cells
To determine whether the CNHK300 viruses we reconstructed replicate preferentially in tumor cells and replicatively attenuated in normal cells, we performed virus yield assay in HepGII and Hep3B tumor cell lines and BJ normal cell lines, and because different cell lines may show different susceptibility to adenovirus infection and replication ability to correct the difference in infectivity and virus production between various tumor cell lines and normal cell lines, wtAd5 served as a control. As shown in Figure 4, CNHK300 replicated by 40 625 and 65 326 folds on tumor cell HepGII and Hep3B, respectively, similar to those of wtAd5. However, the CNHK300 replication (180-folds) was attenuated significantly as compared with wtAd5 (4000-folds) in BJ normal cell lines.

Specificity of cytotoxicity of CNHK300 by MTT assay
MTT assay was performed to characterize the specificity...
of CNHK300 on tumor cells with no or less toxicity than normal cells. As shown in Figure 5, CNHK300 could cause significant cytolysis in HepGII and Hep3B tumor cell lines with a MOI of 0.5 pfu/cell and 0.0002 pfu/cell, respectively. However, cells infected with CNHK300 showed an over 50% cell viability rate at the same time points with MOI of 100 pfu/cell, suggesting that more than 200,000,000 folds of CNHK300 were needed to kill half of normal fibroblast cells compared with HepGII and Hep3B tumor cells.

DISCUSSION

Many independent studies indicated that telomerase was active in over 85% of malignant tumors, but not in normal tissues except hematopoietic stem cells and germ cells in the ovary and testis. Telomerase activation has been regarded as the critical characteristic of malignant tumors. hTERT is the major determinant of human telomerase activity, and hTERT promoter has been successfully used to drive antitumoral genes in cancer gene therapy to target to and selectively kill the cancer cells with positive telomerase activity. In our study, we constructed a replicative adenovirus CNHK300, in which, the E1A gene was placed under the control of the human hTERT promoter to allow us to target a wide range of telomerase-positive tumors. A key requirement for clinical application of tumor-specific oncolytic virus is their attenuation in non-target tissues. So, in vitro viral replication assay and in vitro cell viability MTT assay were used to test the replicative specificity and cytototoxicity of CNHK300 in cancer cell lines and attenuated cytolysis in normal cells. Our experiments achieved similar results with some foreign studies. As shown in our results, CNHK300 showed strong differences in replication and cytotoxicity in different hepatocellular cell lines. Though we tested higher titters of CNHK300 in HepGII cells than in Hep3B cells after 48 h of virus exposure, CNHK300 did not show stronger cytotoxicity on HepGII cells. There might be some other cell-killing mechanisms besides oncolytic effect due to virus replication.

Hematopoietic stem cells and germ cells in the ovary and testis are all telomerase-positive cells. Therefore, CNHK300 may replicate in above normal cells if it infects these cells. Fortunately, CNHK300 can not get into these cells because they lack adenoavirus-specific CAR receptor on their cell surface.

Adenovirus is widely used in generating replication-selective viruses. In recent years, two major strategies have come into force. In the first one, viral genes that become dispensable in tumor cells, such as the genes responsible for activating the cell cycle through p53 or Rb binding, have been completely or partially deleted. E1b 55 kDa deleted adenovirus Onyx-015 was proposed to replicate selectively in p53-deficient cells. Yet, several studies indicated that Onyx-015 was independent of the p53 status. In the second strategy, transcription of viral genes has been controlled by replacing the native viral promoters with tumor-specific promoters. In contrast, great progress has been made in the study of tumor-specific promoter regulated replication-selective adenovirus.

In this study, the authors tested the antitumor activity of the hTERT promoter regulated replication-selective adenovirus, Paul Hallenbeck (Genetic Therapy-Novartis, Gaithersburg, MD) and Daniel R. Henderson (Calydon, Sunnyvale, CA) have initiated the efforts in this direction using the α-fetoprotein (AFP) and prostate specific antigen (PSA) promoters to drive the adenovirus E1A gene. It has been proved that modified adenovirus using this strategy has some advantages compared with Onyx-015.

COMMENTS

Background

Viruses can infect, replicate in and kill human cells through diverse mechanisms. Clinicians have treated hundreds of cancer patients with a wide variety of wild-type viruses over the last century, but the approach was abandoned due to toxicity. However, with the advance of recombinant DNA technology, it became possible to genetically engineer viruses to enhance their safety and antitumoral potency. Conditionally replicating adenoviruses (CRAds) represent a promising new platform for the treatment of malignant tumors. Recently, the promoter of human telomerase reverse transcriptase (hTERT) was used to restrict adenoviral replication to telomerase-positive cancer cells through controlling the adenoviral E1A gene. Compared with other promoters, the hTERT promoter is highly active in more than 85% of different human cancers, but inactive in most normal somatic cells, therefore, this mechanism can be applied to a wide range of cancers. In this study, the authors tested the antitumor activity of the hTERT promoter regulated tumor-selective RCAd CNHK300, and confirmed its highly efficient antitumor activity in vitro in HCC cell lines.
Research frontiers
In recent years, two major strategies have come into force. In the first one, viral genes that become dispensable in tumor cells, such as the genes responsible for activating the cell cycle through p53 or Rb binding, have been completely or partially deleted. In the second strategy, transcription of viral genes has been controlled by replacing the native viral promoters with tumor-specific promoters. McCormick’s group at Onyx proposed that an E1b 55 kDa deleted adenovirus Onyx-151 would replicate selectively in p53-deficient cells. Recently, the progress made in the study of tumor-specific promoter regulated replication-selective adenovirus has proved that modified adenovirus using this strategy has some advantages compared with Onyx-151.

Innovations and breakthroughs
Tumor specificity is a major challenge in viral therapy. For adenovirus, one strategy is the development of promoter-driven replicative adenovirus to achieve selective-replication adenovirus. The promoters of AFP, MUC-1 and PSA have been studied to control the adenovirus E1A expression targeting hepatocellular, breast and prostate carcinomas, respectively. In CNHK300, the E1A gene was placed under the control of the human hTERT promoter to allow us to target a wide range of telomerase-positive tumors with little influence on the growth of normal cells.

Applications
The results demonstrated CNHK300 could replicate in liver cancer cells efficiently as well as wtA5 and kill hepatocellular carcinoma, but had severely attenuated proliferation and cytolysis in normal cells. It may have a strong potential for targeting therapy of a wide range of cancers including hepatocellular carcinoma.

Peer review
This is a paper worthy of publishing. The design of the study in all aspects is methodologically sound. hTERT promoter-regulated replicative adenovirus CNHK300 can specifically kill a wide range of cancer cells including HCC. This new cancer therapy strategy may be proven to be very promising in clinical practice.

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