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Conditional Replication of a Recombinant Adenovirus Studied Using Neomycin as a Selective Marker

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An E1B-defective adenovirus, named r2/Ad carrying the *neo* expression cassette, was constructed by homologous recombination. The construction, selection (using neomycin as a selective marker), and propagation of the recombinant virus was performed in human embryonic kidney 293 cells (HEK 293). An *in vitro* study demonstrated that this recombinant virus has the ability to replicate in and lyse some p53-deficient human tumor cells such as human glioma tumor cells (U251) and human bladder cells (EJ), but not in some cells with functional p53, such as human adenocarcinoma cells (A549) and human fibroblast cells (MRC-5). Also, based on the cytopathic effect (CPE), it was demonstrated, under identical conditions, that the U251 cells were more sensitive to r2/Ad replication than the EJ cells. In this paper, we report that r2/Ad could be very useful in studying the *in vitro* selective replication of E1B-defective adenovirus and has great potential in cancer gene therapy.

Keywords: Cancer gene therapy, E1B-defective adenovirus, Neomycin gene, Replicative adenoviral vector

Introduction

Human adenoviruses are attracting considerable attention because of their potential utility for gene transfer and gene therapy, for their development of live viral vectored vaccines, and for their protein expression in mammalian cells. Virologists have revealed many details about their molecular interactions with the cell and developed powerful technologies to genetically modify or regulate every viral protein (Pilder *et al.*, 1986; Goldsmith *et al.*, 1994; Heise *et al.*, 1997). In tandem, the limited success of nonreplicative adenoviral vectors in cancer gene therapy has brought the old concept of adenovirus oncolysis back into the spotlight (Curiel, 1999; Yoon *et al.*, 2000). Major efforts have been directed toward achieving conditional replication by the deletion of viral functions that are dispensable in tumor cells, or by the regulation of viral genes with tumor-specific promoters (Dmitriev *et al.*, 1998; Hemmi *et al.*, 1998; Staba *et al.*, 2000). Adenoviruses are DNA viruses that infect quiescent cells and induce them into the S-phase of the cell cycle so that viral DNA replication can proceed through mitosis. The region of human adenovirus that is largely responsible for this force entry into the S phase is the E1A protein, which binds pBR, p300, and other related proteins. The E1B gene on the virus genome encodes two major proteins, the 19K and 55K tumor antigens. Viral mutants that are defective for the expression of either E1B protein are deficient for transformation, thereby establishing a role for both E1B proteins in the transformation process. The E1B protein binds and inactivates the cellular tumor suppressor protein p53. This binding is essential for virus replication, possibly because E1A induces the p53-dependent apoptosis.

The p53 protein acts as a checkpoint (security guard) that is deployed when a cell's DNA is damaged to prevent the cell from becoming cancerous. It does this by either inhibiting cell growth until the damage is repaired or by causing the cell to commit suicide through a process called programmed cell death or apoptosis (Wiesmuller, 2001). One way p53 restrains damaged cells is by triggering the production of a protein called p21, which blocks cell division (Bunz *et al.*, 1998). The loss or inactivation of the p53 gene is the most common genetic defect in human malignancies, affecting more than 50% of all tumors (Attardi and Jacks, 1999). The p53 is thought to monitor the integrity of the cellular genome cycle arrest or apoptosis. Tumors that lack the functional p53 are

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Abbreviations: ori, origin of replication; pA, poly (A); wt-Ad5, wild-type adenovirus type 5; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; Pv, *Pvu*II; S, *Sal*I; X, *Xba*I; Xm, *Xma*I; CMV, cytomegalovirus; BGH, bovine growth hormone

unable to mount these responses; therefore, they respond poorly to radiation or chemotherapy.

The primary goal of this experiment was to construct a conditionally replicative adenovirus with a selection marker to eliminate wild-type adenovirus contamination in subsequent experiments, and to confirm or verify the view that E1B-defective adenovirus can selectively replicate in and lyse the p53-deficient human tumor cells, but not the cells with the functional p53.

Materials and Methods

Plasmids and bacterial strains The plasmids pcDNA3 and pSP72 are products of Invitrogen and Promega Companies, respectively. The plasmid pCMV55K was provided by Dr. Eileen White (Rutgers University, USA). The *Escherichia coli (E. coli)* TG1 or DH5α was used as the host for bacterial plasmid manipulations and was prepared by the calcium chloride ($CaCl_2$) precipitation method.

Culture media and reagents The Tryptone, Yeast-Extract for bacterial culture, and Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 medium, Minimum Essential Medium (MEM), and F12K medium for cell culture were purchased from GIBCO/BRL company and prepared according to the manufacturer's instructions. The Lipofectin reagent was obtained from GIBCO/BRL. The Geneticin 418 (G418) was obtained, prepared according to the manufacturer's instructions, and stored at −20°C.

Cell lines and viruses The following cell lines used in this study were obtained from the China Center for Type Culture Collection (CCTCC): the human embryonic kidney cell line (HEK 293) integrated with adenovirus early region 1 (growth medium DMEM), U251 (glioma tumor cells, $p53^-$, growth medium RPMI), EJ (bladder tumor cells, $p53^-$, growth medium RPMI), A549 (small lung cancer cells, $p53^+$, growth medium F12K), and MRC-5 (normal human fibroblast cells, containing functional p53, growth medium DMEM). The infectious wild-type adenovirus type 5 (wt-Ad5) was provided by Dr. Deng Xiao Zhao (Nanjing Military Medical Sciences, China). The human embryonic kidney cell line (HEK 293) was used throughout for co-transfection with DNAs of the recombinant transfer vector and the wild-type adenovirus, and for selection of recombinant adenoviruses under G418 pressure.

Co-transfection of HEK 293 cells Prior to co-transfection, the cells were washed three times with 2 ml fresh serum free DMEM without antibiotics. After incubation, 0.8 ml fresh serum DMEM free without antibiotics was added to the 0.2 ml lipid-DNA complexes and mixed gently. Next, 1 ml of the diluted lipid-DNA complexes was gently overlaid onto the monolayer cells. The cells were incubated at 37°C in a CO_2 incubator. After a 7 hr incubation, the co-transfection mixture was removed and replaced with 4 ml fresh DMEM that was supplemented with 10% horse serum (DMEM-10HS). The cells were again incubated for several days for co-transfection and homologous recombination between the flanking sequences of the left/right arms of the E1B region in the recombinant transfer vector and the intact E1B region in the wild-type adenovirus.

Plaque assay The HEK 293 cells with the recombinant transfer vector and infectious wild-type DNAs were co-transfected for 7 days until the cytopathic effect (CPE) could be seen. Then the co-transfection medium containing the mixed virus progenies was harvested and centrifuged at $5{,}000 \times g$. The mixed viral supernatant was used to infect the fresh HEK 293 cells at a multiplicity of infection (MOI) of 5. At 48 and 72 h postinfection, the old medium was removed and replaced with 2 ml fresh medium that was supplemented with 2% horse serum and low melting point gels. After 5-7 days of incubation, the cells were stained with crystal violet and the plaque assay for the isolation of the recombinant adenovirus was carried out.

Selection of recombinant virus To select the recombinant virus from the wild-type virus, the mixed viral progenies that were harvested from the transfection medium were subjected to a limited dilution. The monolayers-culture of the HEK 293 cells that were grown to 50% confluency in a 24 well plate were infected with the mixed viral progenies at a multiplicity of infection (MOI) of 0.5 to 0.1. They were allowed to stand for 2 hr in a CO_2 incubator for the virus attachment. The cells were washed twice with fresh DMEM serum free (DMEM-SF) to remove the uninfected particles. Then 1 ml of a fresh medium that was supplemented with 5% horse serum (DMEM-5HS, maintain medium) and 2.5 mg/ml G418 was added to the cells and incubated at 37°C in a CO_2 incubator. After 5-7 days of infection, the infection medium was collected and centrifuged at $5{,}000 \times g$ for 5 min. Next, 1/1000th of the mixed viral supernatant was used to infect the fresh monolayer culture of cells. Subsequent passages were performed using 1/1000th of the viral supernatant that was harvested from the previous infection. For each passage, about 2.5 mg/ml of G418 was added to the medium. The infected cells that showed the cytopathological effect (CPE) were harvested. After eight passages, a culture of the recombinant virus carrying the *neo* gene was obtained. The viral stock was diluted serially (10^{-1} to 10^{-8}) in a fresh medium containing 5% horse serum and 2.5 mg/ml G418, and then used to infect fresh HEK 293 cells in a 96 well microliter plate at 37°C in a CO_2 incubator. After 7 days, the medium from the infected well with the highest dilution was taken and regarded as pure recombinant adenovirus.

Restriction digestion and southern blotting The HEK 293 cells were infected separately with the purified recombinant virus and wild-type viruses. After 7 days of infection, the infection medium was collected and centrifuged at $5{,}000 \times g$ for 5 min. The supernatant containing the virus particles was subjected to centrifugation at $38{,}000 \times g$ for 1 h. DNAs of the purified recombinant and wild-type virus were extracted separately from the virions, then digested overnight with *Hin*dIII at 37°C. The digested fragments were separated on 0.5% agarose gels and bidirectionally blotted onto nitrocellulose (NC) filters overnight by standard procedures. A radiolabeled probe (using *neo* gene) was prepared with a random priming DNA kit, according to the manufacturer's instructions (Boerhinger Mannheim Co., Indianapolis, USA). Southern hybridization was carried out by the standard method.

Expression of target gene and SDS-PAGE analysis In order to confirm that the *neo* gene was correctly integrated into the E1B region of the wild-type adenovirus and that it can express, the 100 μl supernatant containing the purified recombinant virus that was harvested from the infected cells was used to infect a fresh monolayer culture of the HEK 293 cells in a 3 ml DMEM of a 6 well plate at 37°C. For the control experiment, 100 μl of the wild-type virus was used to infect the fresh HEK 293 cells in a separate well. After 48, 72, and 96 h infections, the cells were harvested separately and centrifuged at 5,000 × *g* for 5 min. The cell extracts were boiled in a water bath for 5 min and analyzed in a 12% polyacrylamide gel for identification of the expressed target proteins (neo protein from the recombinant virus or E1B55K protein from the wild-type virus).

Dot hybridization To identify the transcription of the *neo* gene in the recombinant virus, the monoculture HEK 293 cells were infected with 200 μl of the recombinant virus, and the total cellular RNA at different times of postinfection (48, 72, and 96 h, respectively) were extracted and subjected to dot hybridization, according to standard procedures. The probe of the *Pst*I 364 bp fragment of the *neo* gene was labeled with the α-^{32}P-dATP by the random primer method (Boerhinger Mannheim).

Microscopy study of recombinant virus The recombinant adenovirus lacking the intact E1B region was tested in p53-deficient human tumor cells (U251, human glioma cells; EJ, human bladder cells), normal human fibroblast lung cells (MRC-5), and p53$^+$ human tumor cells (A549, small lung cancer cells). This was done in order to study its selective replication and lysis in p53$^-$ human tumor cells, but not the cells with functional p53 or p53$^+$ tumor cells. Each cell type was grown to 80% confluency in an appropriate growth medium that was supplemented with 10% calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in a 24-well tissue culture dishes at 37°C in a CO_2 incubator. For each cell type, two wells that were labeled M and C were used. The cells in the M wells were infected with the purified recombinant virus at a multiplicity of infection (MOI) of 1; the cells in the C wells were left uninfected as a control. Both cells were incubated at 37°C in a CO_2 incubator and observed under both low and high power microscopes every day for 5-7 days for selective oncolytic effects and efficiencies.

Results

Construction and verification of recombinant transfer vector The flow chart for the construction of the recombinant transfer vectors and recombinant viruses is shown schematically in Fig. 1.

An agarose gel electrophoresis of the recombinant plasmid by restriction digestion confirmed that the *neo* expression

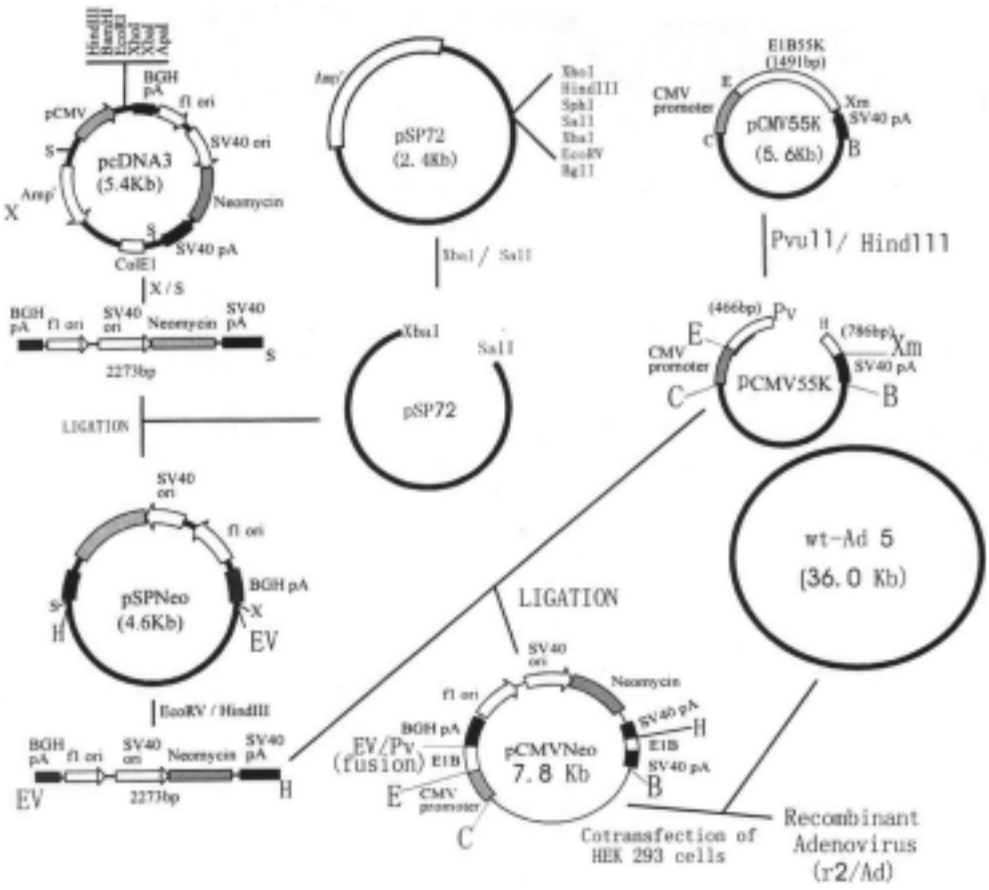

Fig. 1. Flow chart for construction of the recombinant transfer vector pCMVNeo and recombinant adenovirus r2/Ad.

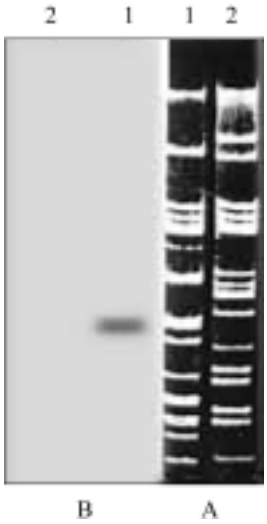

Fig. 2. Southern blotting of recombinant virus r2/Ad and wild-type virus DNAs. (A) Ethidium bromide-stained agarose gels. (B) Directional Southern blot of the gel (Radioautograph). 1. Recombinant r2/Ad DNA/*Hin*dIII. 2. Wild-type virus DNA/*Hin*dIII. The *neo* gene in r2/Ad was hybridized with the probe due to the presence of *neo* gene, but the wild-type virus DNA contains no *neo* gene and so there was no hybridization signal observed.

cassette was correctly inserted into the E1B region of pCMV55K at the *Pvu*II-*Hin*dIII sites. Digestion of the plasmid DNA with *Kpn*I+*Hin*dIII resulted in the release of 2273bp of the *neo* expression cassette on a 0.8% agarose gel. The recombinant transfer plasmid obtained via ligation was designated as pCMVNeo.

DNA analysis of recombinant virus r2/Ad Figure 2 indicates that there is a hybridization signal to the recombinant r2/Ad DNA with the *neo* probe, but no hybridization signal to the wild-type virus DNA. Therefore, the Southern hybridization result as a confirmatory test demonstrated that the *neo* gene was correctly integrated into the virus genome, resulting in a mutant adenovirus lacking its intact functional E1B region.

Transcription and expression of *neo* gene The dot blot hybridization result indicated that the *neo* gene in r2/Ad was transcribed at different times of postinfection (i.e. at 48, 72, and 96 h postinfection, respectively), but in the HEK 293 cells there was no hybridization signal, due to the absence of the *neo* gene (see Fig. 3). Therefore, the amount of mRNA that is produced in HEK 293 cells infected by r2/Ad increases with time. As shown in Fig. 3, the mRNA is higher at 96 h postinfection and less at 24 h postinfection.

Figure 4 shows that the NEO protein with a relative molecular weight of 28.5 kDa is expressed in HEK 293 cells infected with r2/Ad under the control of the SV40 promoter, which is present in the expression cassette. There was no

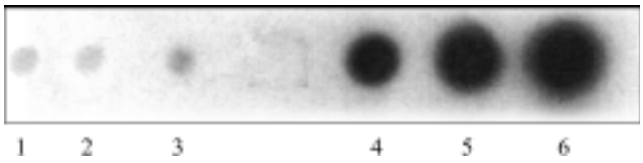

Fig. 3. RNA dot hybridization. Total RNA from cells infected with recombinant or wild-type virus was isolated and directly transferred onto nitrocellulose (NC) membrane and hybridized with α-^{32}P-labeled 364bp pCMVNeo *Pst*I 364bp fragment. Lanes 1, 2, and 3 represent control experiment of HEK 293 cells without infection by r2/Ad or wild-type virus. Lanes 4, 5, and 6 represent HEK 293 cells infected by r2/Ad at 48, 72, and 96 h postinfection, showing *neo* transcription products.

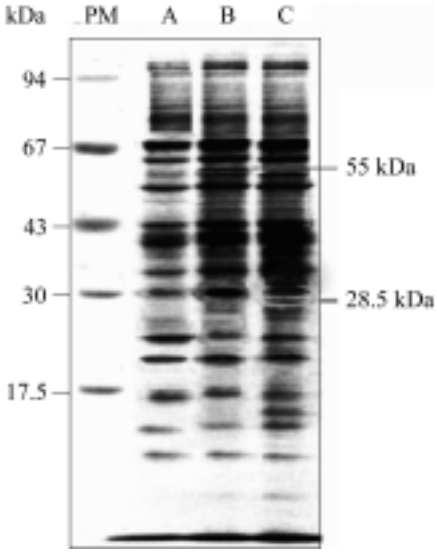

Fig. 4. SDS-PAGE analysis of the expressed target proteins in HEK 293 Cells. PM = protein marker. (A) Normal cells HEK 293 cells without infection by r2/Ad or wt-Ad5 as control (B) HEK 293 cells infected by wt-Ad5 showing a 55 kDa protein of the expressed E1B (C) HEK 293 cells infected by r2/Ad showing a 28.5 kDa protein of the expressed NEO without the 55 kDa of E1B, demonstrating that the *neo* gene was correctly integrated into the wild-type virus genome resulting in the deletion of E1B region.

expression of the 55 kDa of the E1B region with the r2/Ad infection. On the other hand, the wild-type virus expressed only the 55 kDa of E1B, but no NEO protein. Therefore, the transcription and expression of the *neo* gene without the E1B expression in the HEK 293 cells infected by r2/Ad demonstrated that the *neo* expression cassette was integrated into the virus genome, resulting in the deletion of the E1B region. The resultant virus is a mutant without the functional E1B region.

Selective replication of r2/Ad in some p53**-deficient human tumor cells** At 24 h postinfection, the oncolytic effect was

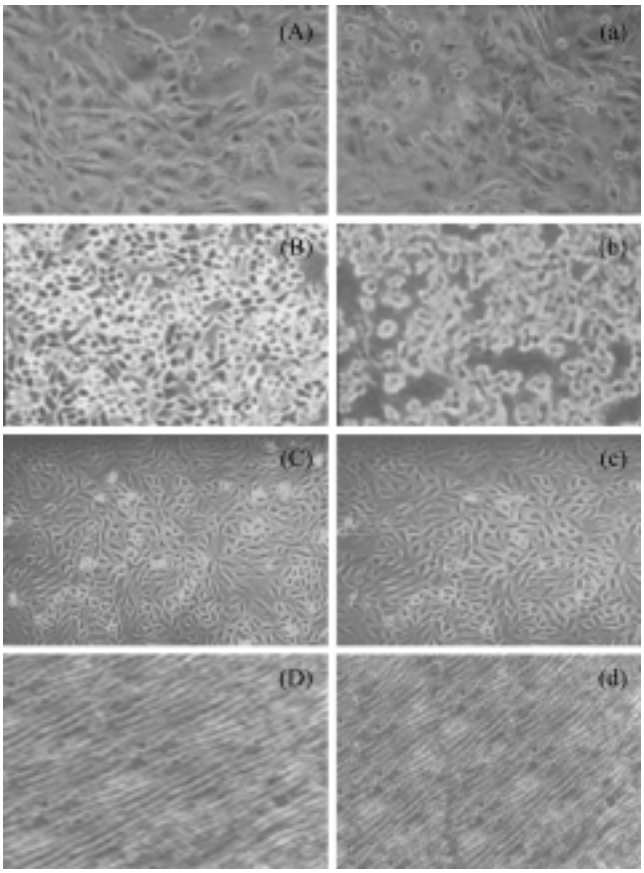

Fig. 5. Microscopy study of *in vitro* selective replication of recombinant adenovirus r2/Ad. (A) U251 cells without infection as control; (a) U251cells infected by r2/Ad at 72 h postinfection; (B) EJ cells without infection as control; (b) EJ cells infected by r2/Ad at 72 h postinfection; (C) A549 cells without infection as control; (c) A549 cells infected by r2/Ad at 72 h postinfection; (D) MRC-5 cells without infection as control; (d) MRC-5 cells infected by r2/Ad at 72 h postinfection.

apparent in U251 and EJ cells. But, in the normal cells (MRC-5) or $p53^+$ tumor cells (A549) there was no infection. At 72 hr or 96 hr postinfection, most of the U251 and EJ cells became infected with CPE. When the postinfection time was increased to 7 days, then almost all of the U251 and EJ cells died as a result of the r2/Ad infection. But, in the normal cells (MRC-5) or $p53^+$ tumor cell (A549) there was no clear evidence of CPE to demonstrate the r2/Ad infection (see Fig. 5).

Comparison of *neo* transcripts in U251 and EJ cells infected by r2/Ad In order to confirm by CPE assay that the r2/Ad replication efficiency is higher in U251 cells than in EJ cells, the total cellular RNA was isolated from the U251 and EJ cells that were infected by r2/Ad at MOI of 1, and hybridized using the pCMVNeo *Pst*I 364bp fragment as a probe. Dot hybridization results demonstrated that the transcription of neo in U251 is higher than that in the EJ cells (Fig. 6). This, therefore, suggests that the U251 cells are more

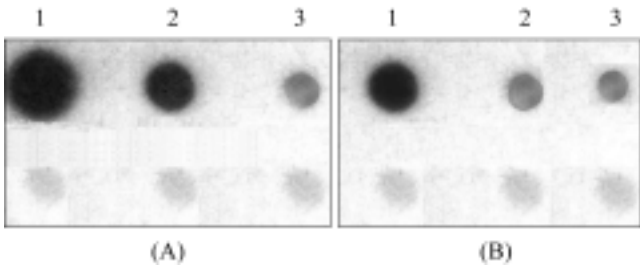

Fig. 6. Comparative analysis of *neo* transcripts in U251 and EJ cells infected by r2/Ad. (A) and (B) are neo transcripts in U251 and EJ cells, respectively. Top row indicates neo transcription products and the bottom row indicates the control experiment. Lanes 1, 2, and 3 represent hours of neo transcription at 96, 72, and 48, respectively.

sensitive to r2/Ad replication than the EJ cells. This result has some significance as it demonstrates that the mutant adenovirus r2/Ad lacking the functional E1B region does not only selectively replicate in and lyse the $p53^-$ tumor cells, but its replication efficiency is not the same in the different p53-deficient tumor cells. This means that some $p53^-$ tumor cells are more sensitive than others to the mutant adenovirus that lacks the E1B region. The reason for this difference is not understood well, but it is possibly due to unknown cellular factors in some $p53^-$ tumor cells that may likely slow down or speed up the mutant adenovirus r2/Ad replication efficiency.

Discussion

The recombinant transfer vector, named pCMVNeo, was constructed after subsequent sub-cloning of a 2273bp *neo* expression cassette containing an SV40 promoter, a neomycin gene, and an SV40 poly (A) signal. The *neo* expression cassette was inserted in the E1B region of the adenovirus genome at *Pvu*II- *Hin*dIII sites after deletion of a 320 bp *Pvu*II- *Hin*dIII fragment, which resulted in the production of right and left flanking sequences of the E1B region in the pCMVNeo, respectively. The deleted 320 bp fragment is somewhat short, but the flanking sequences are long enough for homologous recombination and to maintain the recombinant virus stability in the infection medium. In general, the longer the flanking sequences are on both arms, then the greater the frequency of homologous recombination becomes.

The recombinant virus r2/Ad was constructed by homologous recombination between the flanking sequences of the E1B in pCMVNeo and the intact E1B region of the wild-type virus by co-transfection of the HEK 293 cells with the transfer vector pCMVNeo and wild-type virus DNAs. The recombinant virus r2/Ad displayed no visible difference with the wild-type virus; therefore, it cannot be selected by traditional methods. By serially passaging the mixed viral progenies from the co-transfection medium at a very low

multiplicity of infection (MOI) under G418 pressure, then the recombinant virus r2/Ad containing the *neo* gene was selectively amplified to a high proportion. Although the adenovirus can package and be stable with inserts of up to 1.8-2.0 kb (Ghosh-Choudhury, *et al.*, 1987), in this work, 2.2 kb of the *neo* expression box was integrated into the wild-type adenovirus genome with deletion of the E1B region. The fact that r2/Ad can propagate after many passages under G418 selection demonstrates its stability in the infected cells or culture medium. Results demonstrated that the *neo* gene was integrated into the wild-type adenovirus genome at the E1B region, and that the recombinant adenovirus that lacks the E1B region was successfully constructed and purified to a greater homogeneity. Also, these results showed that the adenovirus could package with an insert of up to 2.0-2.2 Kb.

The r2/Ad that lacks the intact functional E1B was tested for its ability to selectively replicate in and lyse only the $p53^-$ tumor cells, but not the cells with the functional p53. The wild-type adenovirus was also tested in U251 or EJ cells, MRC-5 cells, and A549 cells. They produced about 100 times infectious virus in all cell types. The replication efficiency of r2/Ad and the wild-type virus was tested in U251 cells. In contrast, both r2/Ad and the wild-type virus grew efficiently in the U251 cells with detectable CPE at 24 h postinfection. But, when 2.5 mg/ml G418 was added to the cultured (infection) medium, then the cells that were infected by r2/Ad continued to grow or survive with high CPE even 96 h postinfection due to the expression of the *neo* gene which conferred resistance to the G418 pressure. But, the ones that were infected with the wild-type virus died at 48 h postinfection, due to the lack of the *neo* gene. The ability of r2/Ad to replicate in $p53^-$ tumor cells (U251 cells) in the presence of G418 proved that r2/Ad (or the *neo* gene) is cytotoxic to cells, and that the infection of U251 or EJ cells was not due to the wild-type adenovirus contamination. The recombinant adenovirus dl1520 that lacks the E1B region for cancer gene therapy (Bischoff, *et al.*, 1996) has no selection marker to avoid wild-type adenovirus contamination during subsequent experiments. It is important, therefore, to construct a mutant adenovirus with a selection marker to eliminate wild-type adenovirus contamination. In this work, we introduced a *neo* gene into the wild-type adenovirus genome with the deletion of the E1B region for selection and propagation of r2/Ad and to avoid wild-type virus contamination in the subsequent experiments.

A comparative study of the r2/Ad replication efficiency in the U251 and EJ cells ($p53^-$ tumor cells), based on a CPE assay, also indicated that the U251 cells are more sensitive to r2/Ad replication than the EJ cells at the same MOI. Slight increases in MOI of 2 or 3 of r2/Ad in the EJ cells, and keeping or reducing the MOI in U251 at 1 or 0.5, also showed that U251 is more sensitive to r2/Ad than the EJ cells (data not shown). To further confirm this result, the total cellular RNA from the U251 and EJ cells that were infected with r2/Ad at MOI of 1 and 2, respectively, was isolated separately. A dot blot analysis confirmed that the level of neo that is transcribed in the U251 cells at MOI of 1 was higher than that in the EJ cells at MOI of 2. Most cancer gene therapy researchers that use mutant adenovirus only focused on the *in vitro* study of selective replication of the mutant adenovirus in $p53^-$ tumor cells, but they did not compare the replication efficiency of the mutant adenovirus in these p53-deficient tumor cell lines. In this experiment, the selective replication, as well as the comparative study on the replication efficiency of the mutant adenovirus r2/Ad that lacks the functional E1B, was tested in different p53-deficient tumor cells. Replication efficiency was performed to determine which $p53^-$ tumor cell line is more sensitive to r2/Ad.

Until recently, the prevailing view was that adenovirus had developed strategies to negate p53-dependent effects in order to circumvent cellular mechanisms for the inhibition of viral replication. Recent clinical studies also do not support the view that the E1B-defective adenovirus replicates in normal cells with functional p53. They also confirm that the E1B-defective adenovirus has no measurable toxicity in animals or humans, and in some clinical studies can be shown to replicate in tumors that lack functional p53. Research from several laboratories, however, found that a variety of tumor cell lines with the wild-type p53 allow efficient replication of the E1B-defective adenovirus (Goodrum and Ornelles, 1998; Rothmann *et al.*, 1998; Turnell *et al.*, 1999). It was speculated that genetic lesions affecting molecular factors within the p53 pathway (other than mutations of p53 itself) can render cells permissive for E1B-defective adenovirus (Ries and Korn, 2002).

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