Development of a packaging cell line for propagation of replication-deficient adenovirus vector

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Abstract

**A human embryonic kidney cell line 293 is widely
used for adenovirus production and propagation.
With this cell line, however, replication-competent
virus (RCV) is frequently generated, especially dur-
ing large-scale production and successive propaga-
tion because 293 cells contain not only E1 gene but
also non-E1 adenovirus gene. Homologous recom-
bination between non-E1 region of 293 genomic
DNA and its homologous region in the recombinant
adenoviral vector generate RCV. To overcome this
problem, we developed a new packaging cell line,
Hela-E1, which contains minimum E1 region and
from which non-E1 adenoviral region that is homol-
ogous with recombinant adenovirus vector was
excluded. No RCV was detected during adenovirus
propagation in Hela-E1 compared to in 293. In addi-
tion, adenovirus-p53 produced in HeLa-E1 was able
to overexpress p53 protein when introduced into an
ovarian cancer cell line, SKOV3. These results may
have a significant impact on the development of
packaging cell lines for replication-deficient aden-
ovirus production.**

Keywords: adenovirus; replication competent virus;
packaging cell line; HeLa-E1 cell

Introduction

A recombinant adenovirus vector has been extensively
utilized as a delivery vehicle for gene transfer,
vaccination, and gene therapy (Zhang, 1999). The
widely used recombinant adenovirus vector was derived
from subgroup C and is highly infectious and capable of
delivering therapeutic genes to many different cell types.
Among subgroup C, the serotypes 2 and 5 have been
used for vector development because they are not
associated with any severe pathology in humans
(Horwitz, 1990). In some cases, however, the gene-
ration of RCV (replication-competent virus) has been a
major obstacle in production and successive pro-
pagation of adenovirus vectors because it causes cell
toxicity by itself and leads to immunological response
(Smith *et al.*, 1993; Yang *et al.*, 1994).

The general method to obtain recombinant adeno-
viruses involves homologous recombination in pac-
kaging cells capable of complementing defective
adenoviruses (Berkner and Sharp, 1983). Homolo-
gous recombination results in generation of a replica-
tion-defective adenovirus that can not replicate in host
cells unless the E1 gene critical for adenovirus
replication is supplied. However, RCV production was
often detected during a large-scale adenovirus pro-
pagation or successive production in universal pac-
kaging cell line, 293 (Lochmuller *et al.*, 1994). This is
due to a fact that 293 cells contain and express the
left 11% of the Adenovirus type5 (Ad5) genome and
this region contains a part of adenovirus E1 region
(1.6-9.7 m.u.) as well as non-E1 region (0-1.6 m.u.,
9.7-11 m.u.). RCV is presumably generated by the
homologous recombination between non-E1 region of
293 genomic DNA and homologous region of a
recombinant adenovirus vector (Figure 1).

In the present study, we reported a strategy for
making a new packaging cell line, HeLa-E1, which does
not contain non-E1 region but a minimum E1 gene (1.6-
9.7 m.u.) of adenovirus type 5 (Ad5). We also demon-
strated that no RCV was generated during adenovirus
production in HeLa-E1 as compared to in 293. More-
over, Adenovirus-p53 (Ad-p53) produced in HeLa-E1
induced an overexpression of p53 protein when trans-
fected into an ovarian cancer cell line, SKOV3. These
results indicate that HeLa-E1 can act as a packaging
cell line that excludes RCV generation and the
strategy used here can be useful for making other new
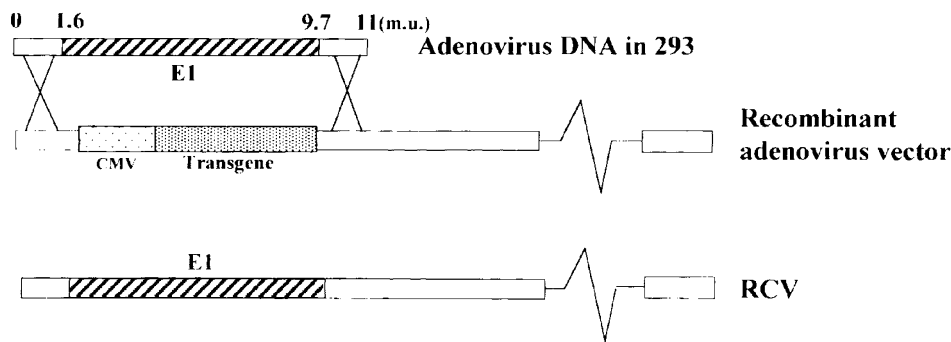packaging cell lines for recombinant adenovirus
production.

Figure 1. Scheme of RCV production mechanism by homologous recombination. Replication competent adenovirus is produced by homologous recombination between adenovirus gene of 293 and its homologous region of a recombinant adenovirus vector.

Materials and Methods

Cell lines

The cervical cancer cell line HeLa and the ovarian cancer cell line SKOV3 were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum. The human embryonic kidney cell line, 293 was maintained in Eagle's Minimum Essential Medium (EMEM, Biowhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum. All cell lines were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA).

Adenovirus-βgal (Ad-βgal) and Ad-p53

The adenovirus containing wild-type p53 cDNA (Ad-p53) was constructed as described previously (Hwang *et al.*, 1998). Briefly, the shuttle vector pδA–CMVp53 and an adenovirus vector pJM17 were used to cotransfect a packaging cell line, 293, by the calcium phosphate method. A replication deficient adenovirus clone was isolated and propagated in 293 cells. Ad-RSV-βgal, a β-galactosidase-expressing adenovirus was kindly provided by Wei Wei Zhang (GenStar Therapeutics Corporation, San Diego, California, USA).

Cloning of E1 and construction of HeLa-E1 cell line

E1 gene of adenovirus was cloned from 293 genomic DNA by polymerase chain reaction (PCR) using a pair of E1 primers, 5'-CCGACACCGGGACTGAAAATGAG-3' (542-564 bp) and 5'-CCACACATTTCAGTACCTCA-ATC-3' (3526-3504 bp). E1 PCR product was directly cloned into pCR3.1-Uni vector (Invitrogen, Groningen, Nether-lands) for eukaryotic gene expression. To construct a stable cell line, HeLa-E1, approximately 2 x 10^5 cells of HeLa were seeded in 60 mm dish before 24 h of transfection. Cells were transfected with FuGene6 reagent (Roche, Mannheim, Germany). After 48 h of transfection, transfected cells were screened with 400 μg/ml of geneticin (GIBCO-BRL, Gaithersburg,

MD, USA). Finally one clone was selected and propagated for further experiment.

Western blot analysis

Cells were lysed in RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP40, 0.1% SDS, and 0.5% sodium-deoxycholate]. Equivalent amounts of whole cell extracts (10 μg for each; 5 μg for 293) were resolved on 12% polyacrylamide gel and transferred to Hybond-ECL nitrocellulose filter membrane (Amersham, Buckinghamshire, UK). The membranes were blocked in 25 mM Tris (pH 8.0) containing 125 mM NaCl, 0.1% Tween 20, and 5% skim milk. Protein bands were probed with either anti-E1A antibody (PharMingen, San Diego, CA. USA) or anti-p53 antibody (Novocastra, Newcastle, U.K.) followed by the labeling with horseradish per-oxidase-conjugated anti-mouse antibody (Amersham, Buckinghamshire, U.K). Bands were visualized using an ECL kit (Amersham, Buckinghamshire, U.K.) according to the manufacturer's instructions.

Assay for replication competent virus (RCV)

The generation of replication competent virus (RCV) in adenovirus produced either in HeLa-E1 or in 293 was detected by the presence of E1A gene in the DNA isolated from virus using PCR method. The sequences of E1A primers and non-E1 primers were reported previously (Hwang *et al.*, 1998). To check β-galactosidase gene, PCR was done using a pair of β-gal primers, 5'-CTGTATG-AACGGTCTGGTCT-3' (1803-1822 bp) and 5'-ATCTGTGAAAGAAAGCCTGA-3' (2303-2284 bp) generating 500 bp DNA segments containing β-galactosidase gene. Viral DNA was isolated by proteinase K (2 mg/ml) treatment in the presence of 0.5% SDS followed by phenol extraction and ethanol precipitation.

Results

Establishment of HeLa cells which express E1

HeLa-E1 was made by the introduction of E1 gene into cervical cancer cell line, HeLa. E1 gene of adenovirus type 5 was cloned by conventional PCR technique from 293 genomic DNA using primers described in Materials and Methods. The PCR product was directly cloned into pCR3.1-Uni vector for mammalian expression. After transfection into HeLa, cells were selected with geneticin (400 μg/ml). Finally one clone was isolated and propagated in 100 mm dish for further experiment. The expression of E1A gene in HeLa-E1 cells was confirmed by western blot analysis (Figure 2). HeLa-E1 cells appeared to induce an overexpression of E1A compared to the non-transfected HeLa cells as a control, which showed no E1A expression. However, the transient expression of E1A in HeLa induces a weak E1A expression.

Replication deficient adenovirus production in HeLa-E1

To demonstrate replication-deficient adenovirus production in HeLa-E1, we prepared adenovirus-β-galactosidase (Ad-βgal), which is able to express β-galactosidase gene. To check RCV contamination of Ad-βgal stock, viral DNA was isolated and PCR was done using non-E1 primers, βgal primers and E1A primers (Figure
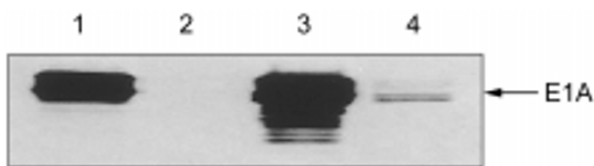
3). E1A fragment was not detected, instead DNA bands for non-E1 and β–galactosidase gene was generated as 861 bp and 500 bp fragments, respectively. These results indicate that the clone selected does not contain any RCV. Replication deficient Ad-βgal was infected into HeLa-E1 or 293 cells with 100 m.o.i (multiplicity of infection). After 48 h of infection, culture media and pellet were collected and separated by centrifugation. Adenovirus DNA was extracted from the supernatant, and then PCR was done to detect RCV production with primers described in Materials and Methods. No RCV production was detected in Ad-βgal produced in HeLa-E1, whereas Ad-βgal produced in 293 cells contained RCV (Figure 4A). To confirm the generation of RCV during successive adenoviral production, the rest of supernatant was used for second infection. After 48 h of second infection, adenovirus DNA was isolated from the supernatant. When PCR was done with same primers as described above, again there was no RCV production in Ad-βgal produced in HeLa-E1 in contrast to in 293 (Figure 4B). These results clearly demonstrate that the production of adenovirus vector using HeLa-E1 cells excludes the generation of RCV.

Figure 2. E1A expression in HeLa-E1 cell line. (Lane 1); 293 cell line, (Lane 2); HeLa cell line, (Lane 3); HeLa-E1 cell line, (Lane 4); HeLa cell line which is transiently transfected with E1. Each cell was collected and lysed in RIPA buffer. Equivalent amounts of whole cell extracts (10 μg for each; 5 μg for 293) were applied to western blot analysis for E1A detection.
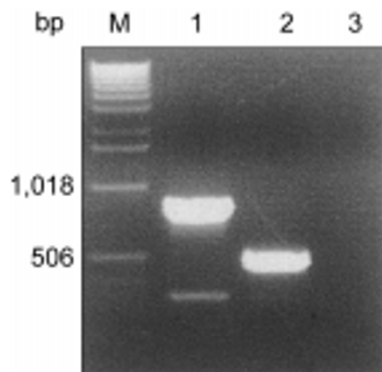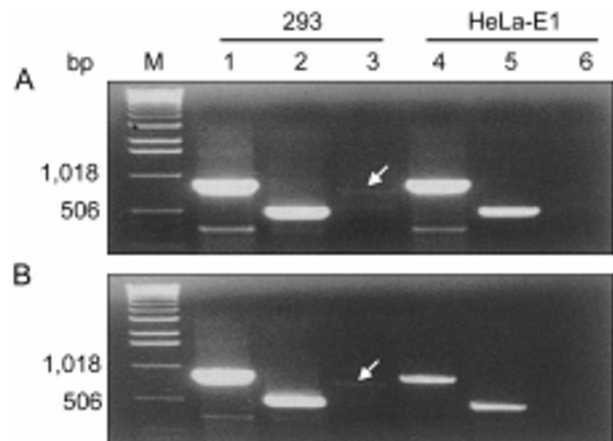

Figure 3. Identification of replication deficient adenovirus by PCR. PCR was done using the DNA extracted from the Ad-βgal with non-E1 adenovirus primers (lane 1), βgal primers (lane 2) and E1A primers (lane 3). A 861 bp of non-E1 region and 500 bp of βgal region were amplified whereas a 752 bp of E1A was not produced.

Figure 4. Test for the presence of replication competent virus in Ad-βgal produced in HeLa-E1 or 293. PCR was done using the viral DNA of Ad-βgal produced in 293 or HeLa-E1 after first infection (panal A) and second infection (panal B) with non-E1 primers (lane 1, 4), βgal primers (lane 2, 5) and E1A primers (lane 3, 6). E1A fragments produced in 293 are indicated by arrows.
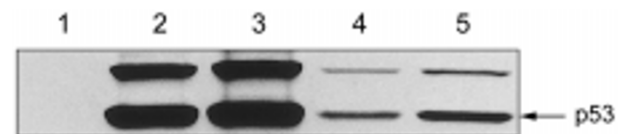

Figure 5. p53 expression by Ad-p53 infection in SKOV3 cell line. SKOV3 was treated with PBS (lane1) or supernatant of Ad-p53 produced in 293 (Lane 2, 3) or HeLa-E1 (lane 4, 5). 1.5ml supernatant(lane 2, 4) or 3 ml supernatant (Lane 3, 5) of Ad-p53 was used for each infection. Each sample was collected and lysed in RIPA buffer. Each of 10 ug protein was applied for western blot analysis for p53 detection.

Identification of adenovirus-p53 produced in HeLa-E1

To demonstrate the expression of p53 gene, an ovarian cancer cell line, SKOV3 was infected with adenovirus-p53 produced in either HeLa-E1 or 293 cells. After 48 h of infection, total cells were lysed for whole cell extracts and then western blot analysis was done with anti-p53 antibody. As shown in Figure 5, a high level expression of p53 protein was induced in SKOV3 cells transfected with Ad-p53 that was generated in HeLa-E1 or 293. However, there was no detectable expression of p53 protein in SKOV3 treated with PBS as a negative control. Taken together, these results indicate that HeLa-E1 can be used for packaging cell line for the generation of replication-deficient recombinant adenovirus. It is noteworthy that HeLa-E1 shows lower productivity than 293 as a packaging cell line. However, this problem can be overcome by selecting stable cell lines which contain many E1 gene copies in their genome. The most critical point is that this strategy can be used for making packaging cell lines that exclude RCV generation during recombinant adenovirus propagation.

Discussion

The most widely used adenovirus packaging cell line is 293, which was derived from human embryonic kidney (HEK) cells by transformation with Ad5 fragments. 293 cells contain the left 11% of Ad5 genome. RCV is produced by homologous recombination between Ad5 fragment in 293 cells and recombinant adenovirus vector during multiple passages. To solve this RCV problem, we proposed a new strategy to make a packaging cell line for RCV-free adenovirus propagation. We selected a cervical cancer cell line HeLa to develop a new packaging cell line for RCV-free recombinant adenovirus production because HeLa shows high susceptibility to adenovirus relative to other stable cell lines. HeLa-E1 was made by the introduction of minimum E1 (1.6-9.7 m.u.) into HeLa. When HeLa-E1 cells were employed as packaging cells, there was no detectable RCV generation during recombinant adenovirus production, as determined by the PCR analysis. On the contrary, an easily detectable level of RCV appeared to be generated during the first propagation in 293 cells. Although several previous reports showed that RCV was produced at the late passage during large-scale or successive production of recombinant adenovirus in 293 cells (Lochmuller *et al.*, 1994), in our experiment, RCV generation was detected from the early passage. This is due to the possibility that RCV can be produced in any passage duirng the propagation in 293 cells. In addition, we confirmed that a recombinant adenovirus possessing p53 gene (Ad-p53) produced in HeLa-E1 cloud be

successfully transfected into a human ovarian cancer cell line SKOV3, and induce an overexpression of p53 protein, suggesting that a new packaging cell line HeLa-E1 is applicable as a packaging cell line for the production of RCV-free recombinant adenovirus. However, since the produc-tivity of recombinant adenovirus in HeLa-E1 is lower than in 293 cells, it needs to be further studied to increase the productivity. It is highly likely that a possible approach to overcome this problem may be simply to select a cell line that contains many E1 gene copies in their genome like 293 cell line.

In conclusion, in this report, we proposed a strategy for preparing a packaging cell line which can exclude RCV generation and thus its contamination during large-scale as well as multiple-passage production of recombinant adenovirus. This strategy could be applicable to normal cells because the expression of E1 gene by itself is able to transform normal cells to immortalized cells as 293 cell lines. However, we need to handle many obstacles to apply this strategy to the gene therapy for human. Particularly, at first we should make a packaging cell line derived from normal human cells, because we cannot use any products obtained from cancer cells for the human gene therapy. In addition, we should consider the virus productivity, life cycle, and longevity of cells when we prepare a packaging cell line.

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