[CANCER RESEARCH 63, 3073–3078, June 15, 2003]

Advances in Brief

Adenovirus with Insertion-mutated E1A Selectively Propagates in Liver Cancer Cells and Destroys Tumors *in Vivo*[1]

Tiejun Zhao,[2] Xiao-Mei Rao, Xiaoming Xie, Ling Li, Timothy C. Thompson, Kelly M. McMasters, and Heshan Sam Zhou[3]

Center for Cell and Gene Therapy [T. Z., L. L.], Departments of Immunology [X. X.] and Urology [T. C. T], Baylor College of Medicine, Houston, Texas 77030, and Brown Cancer Center [X-M. R., K. M. M., H. S. Z.], Departments of Medicine [X-M. R., H. S. Z.] and Surgery [K. M.], University of Louisville, Kentucky 40202

Abstract

The adenovirus E1A proteins are involved in the transcriptional activation of viral and cellular genes needed for controlling cell cycle and virus replication. Undifferentiated embryonic carcinoma cells have the ability to produce an E1A-like activity that can induce the expression of E1A-targeted adenoviral and cellular genes in the absence of the E1A products. Differentiated embryonic carcinoma cells lose the ability to produce the E1A-like activity. In this study, we investigated the E1A-like activity in cancer cells with an adenovirus having a mutated *E1a* gene. The mutation is generated by the insertion of a large DNA fragment in the *E1a* gene and interrupts the COOH-terminal region of both the E1A 12S and 13S proteins. The *E1a*-mutated virus can efficiently replicate in HepG2 and Hep3B liver cancer cells and produce high titers of virus. Replication of the *E1a*-mutated virus inhibits tumor formation and destroys tumors *in vivo*. The results obtained in this study imply that cancer cells may produce an E1A-like activity to support the selective replication of mutated virus in cancer cells. In addition, we found that although the E1a-mutated virus could not replicate in Huh1.cl2 liver cells, the viral DNA could amplify in the cells. This result suggests that replication of adenoviral DNA is necessary, but not sufficient, for generating infectious viral progeny and destroying tumor cells.

Introduction

The human adenovirus genome is composed of double-stranded, linear DNA ~36 kb in size. The viral genes are divided into early (E) and late (L) genes relative to the onset of viral DNA replication. The E1 region contains two sets of genes, *E1a* and *E1b,* regulating cell proliferation and apoptosis; the *E2* genes encode three proteins directly involved in viral DNA replication. Adenoviral vectors have been developed for the delivery of therapeutic genes into tumor cells to enhance the ability of host immune systems against tumors, render cancer cells sensitive to selective drugs, or replace the lost function of cancer suppressor genes. The inability of replication-deficient adenoviruses to sufficiently transduce the bulk of tumor cells, a direct result of tumor size and impenetrability, is a major obstacle to successful cancer gene therapy. Tumor-selective replication of adenovirus is a promising solution to the current problems in cancer gene therapy.

ONYX-015 (originally called dl1520; Ref. 1) contains a deletion in *E1b* gene coding for the E1B55K protein, which binds the tumor suppressor protein p53 (2, 3). It was hypothesized that a virus without E1B55K cannot interact with p53 and therefore was unable to replicate in p53 WT[4] cells but could replicate in tumor cells with already dysfunctional p53 (4, 5). The mechanism for ONYX-015 replication in cancer cells needs to be further studied because multiple reports have suggested that ONYX-015 could replicate in both p53 WT and mutated cells (for a review, see Ref. 6). In Phase I–III clinical trials, the ONYX-015 virus was generally well tolerated at high doses, but single-agent efficacy with the virus alone has been limited (7).

Adenovirus *E1a* is the first transcription unit to be expressed on adenoviral infection of cells. The E1A proteins are involved in diverse cellular functions, including induction of the host cell to enter the S phase of the cell cycle, protection of the infected cell from various antiviral defenses, and transcriptional activation of viral genes needed for viral DNA replication (8). There are two major *E1a* products, the 12S and 13S proteins. The E1A 13S protein contains three conserved regions (CR1, CR2, and CR3), whereas the E1A 12S protein contains two of the conserved regions (CR1 and CR2). Both E1A 12S and 13S proteins bind to the pRb protein through CR1 and CR2 to release E2F from the E2F-pRB complex (9, 10). The E2F is then free to activate the viral E2 promoter and some cellular promoters for virus propagation. Fueyo *et al.* (11) hypothesized that adenovirus with deleted E1A CR2 would be able to replicate in cancer cells with disrupted pRb function, while sparing the normal cells with functional pRb. They constructed an adenovirus (AdΔ24) with deletion of a 24-bp region in the CR2 and tested it with mutated and WT pRb cells. They reported that AdΔ24 replicated in and lysed pRb-mutated cancer cells with great efficiency.

In this study, we investigated the possibility of an adenoviral E1A-like activity in cancer cells that may enhance virus-selective replication. Shenk *et al.* (12) have demonstrated that *E1a*-deleted adenovirus could replicate in HeLa cells and release viral progeny when cells were infected at high MOI. Undifferentiated embryonic carcinoma F9 cells, as well as human cancer cells, have long been recognized to allow a high level of uninduced expression of the heat shock (*hsp70*) gene (*i.e.,* HeLa cells) and harbor the E1A-like activity (13). Glotzer *et al.* (14) recently showed that a heat-shock response is a specific virus function for ensuring proper synthesis of viral proteins and virus replication. Therefore, *E1a*-mutated viruses may replicate in cancer cells that produce an E1A-like activity. To test the E1A-like activity and selective replication of *E1a*-mutated adenovirus in cancer cells, we have studied viral DNA replication, virion release, and tumor repression with an adenovirus in which a large DNA fragment has been inserted into the *E1a* gene. We found that the insertion-mediated virus can replicate in liver cancer cells (HepG2 and Hep3B) as efficiently as a WT adenovirus can. We also found that the mutant failed to produce viral progeny in Huh1.cl2 liver cancer cells and in other normal cells, but the viral DNA could be amplified in Huh1.cl2

Received 1/29/03; accepted 5/1/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by NIH Grant P50-CA58204 and a Research Funding from the J. Graham Brown Cancer Center.

[2] Present address: Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montreal, Quebec H4P 2R2, Canada.

[3] To whom requests for reprints should be addressed, at University of Louisville Health Sciences Center, Brown Cancer Center, Baxter Building I, RM: 204D, 570 South Preston Street, Louisville, KY 40202. Phone: (502) 852-5745; Fax: (502) 852-2356; E-mail: hsamzhou@yahoo.com.

[4] The abbreviations used are: WT, wild type; MOI, multiplicity of infection; PFU, plaque forming unit(s); IFU, infectious unit(s); Ad5, adenovirus type 5; CPE, cytopathic effect.

cells. This result implies that amplification of adenoviral DNA is necessary, but not sufficient, for generating infectious viral progenies and destroying tumor cells.

Materials and Methods

Cells, Plasmids, and Viral Vectors. 293 cells contain chromosome-integrated adenovirus sequences extending from the left end to nucleotide 4344 (15). E2T is an E1 and E2a double-complementing cell line derived from the 293 cell line (16). Hep3B, HepG2, and Huh1.cl2 (17) are liver cancer cells derived from human hepatocellular carcinoma. Both Hep3B and HepG2 were obtained from the American Type Culture Collection (Rockville MD), and Huh1.cl2 was a gift from Dr. T. Tamaoki (University of Calgary, Calgary, Alberta, Canada). Hep3B is p53 negative, and HepG2 is a p53 WT cell line (18). The p53 status for Huh1.cl2 has not been determined.

The pBHGE3 plasmid (19) contains adenovirus genomic DNA in plasmid form. Plasmid pΔE1spA1 is an adenovirus shuttle plasmid with an E1 deletion from 342 to 3523 bp (19). The above adenovirus plasmids were kindly provided by Dr. F. Graham. Cotransfection of pBHGE3 and pΔE1spA1 plasmids into E2T cells resulted in an adenovirus with deletion of the entire E1 region designated AdΔE1 (20). Adhz3 (AdFG140) was rescued from the pFG140 plasmid (21) in 293 cells after transfection. The virus contains a 2.2-kb fragment inserted in the *Xba*I site, at 1339 bp counted from the left end of the adenovirus genomic DNA (GenBank accession no. M73260). The 2.2-kb fragment is the backbone of pFG140 plasmid containing replication origin and ampicillin marker for the plasmid replication and selection in bacteria. The 2.2-kb fragment inserted in Adhz3 disturbs the sequence coding for E1A 12S and 13S proteins (8). Adhz20 was rescued from pBZ66 plasmid in E2T cells after transfection (16). Adhz20 contains a deletion of the entire E2a open reading frame but is otherwise identical to Adhz3. The WT human Ad5 was obtained from American Type Culture Collection. The four viruses Ad5, Adhz3, Adhz20, and AdΔE1 are depicted in Fig. 1.

Virus Purification, Titer, and Structure Determinations. The viral-infected cells were collected and underwent three rounds of freezing and thawing to release viral particles. After serial dilution, virus titer was assayed by measurement of IFU in 293 or E2T cells cultured in 96-well plates (22) or PFU. Plaque purification and assay were performed in six-well plates using E2T cells as described previously. Plaques appeared ~5–7 days for WT adenovirus and the E1-deleted Adhz3 and AdΔE1 and 7–10 days for the E1 and E2a double-deleted Adhz20. Purified viruses were amplified in 293 or E2T cells for large preparation. Two days after infection, >90% cells showed CPEs. Viruses were purified from these cells by two rounds of CsCl centrifugation (23) and passed through desalting columns (Econo-Pac, 10 DG; Bio-Rad, Hercules, CA). One pair of PCR primers, Ad401F (5′-CTCAGGTGTTTTCCGCGTTC) covering 401–420-bp sequences of the adenovirus genomic DNA and Ad1370R (5′-CAGCTATCCGTACTACTATT) covering 1370–1351 bp, was used to confirm the E1 structure in the vectors.

Assessments of CPEs. Various cells were seeded onto six-well plates at 3×10^5 cells/well and cultured overnight. Subsequently, the cultures were infected with the indicated viruses for 4 h, after which the medium and unattached virus particles were removed. The cells were fed with fresh medium and observed daily for CPE.

Viral DNA Replication Assay. The cells were divided into 10-cm dishes at 2×10^6/dish. The next day, the cells were infected with virus at an MOI of 1. The cells were collected at different times after infection. Subsequently, the DNAs were isolated from the cells for Southern blot analysis. DNA samples (5 μg each), digested with *Kpn*I, were added to 0.8% agarose gel for electrophoresis and transblotted to a Hybond-N$^+$ membrane (YA3609; Amersham Pharmacia Biotech, Arlington Heights, IL). To prepare the probe for the Southern blot, 100 ng of pBHG10 (19) DNA were also digested with *Kpn*I, denatured for 5 min in boiling water, and then cooled on ice for 5 min. The reaction buffer, labeling reagent, and cross-linker were added to the DNA following the protocol of the AlkPhos DIRECT kit (RPN 3690; Amersham Pharmacia Biotech). The reaction was carried out at 37°C for 30 min. Prehybridization was performed for 15 min at 55°C, with 0.125 ml of hybridization buffer/cm^2. The probe was added to the hybridization buffer at 10 ng/ml, and hybridization was performed overnight at 55°C with gentle agitation. For detection, the blot was drained after hybridization, and CDP-Star detection reagent was added onto the blot membrane at 30 μl/cm^2 and kept at room temperature for 5 min. Then, the membrane was exposed to film for ~1 min before development.

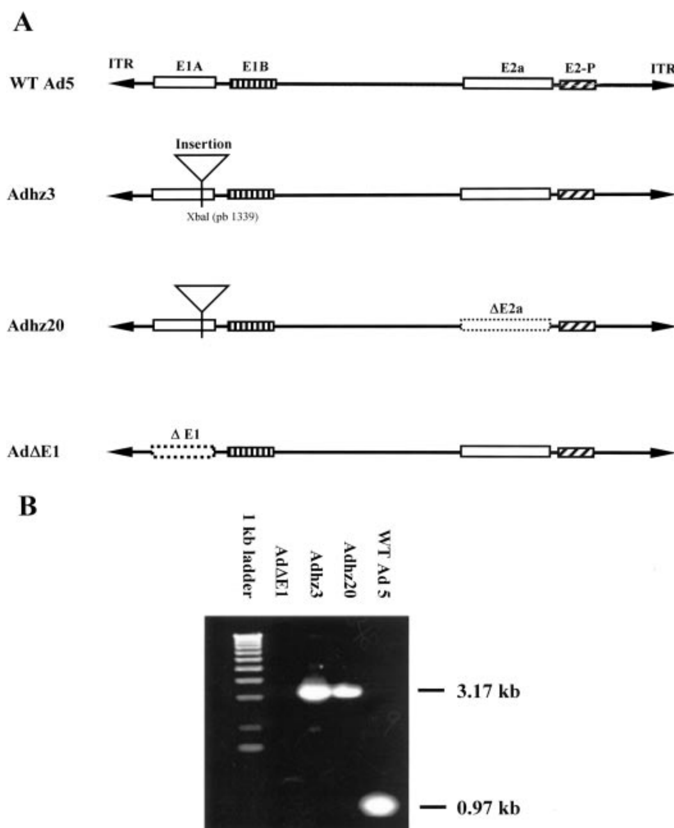


Fig. 1. Structure of viruses. *A,* depiction of viruses. The 103-bp ITRs are located on the left and right termini of the Ad genome. *Open bars, E1a* gene; *hatched bars, E1b* gene. △, insertion in the *E1a* gene. *Dashed open bar,* deletion of E2a open reading frame. *B,* PCR analysis of insertion and deletion in the *E1a* gene of these viruses. Two PCR primers (Ad-401F and Ad-1370R) that flank the insertion site were used for analysis of this region. AdΔE1 with deletion of E1a and E1b could not be amplified by PCR. Insertion of a 2.2-kb segment in Adhz3 and Adhz20 produced a PCR fragment of 3.17 kb. PCR reaction with the WT Ad5 produced a 0.97-kb fragment.

Animals. The animal studies were carried out at the animal facility of Baylor College of Medicine in accordance with institutional guidelines. Male athymic nude mice, 3 weeks old, were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals had unlimited access to food and water and were housed at a maximum of 5 mice/cage in a barrier care room. The mice were used when they were 6 weeks old.

Mouse Tumor Model Studies. The antitumoral efficacy of Adhz3 and Adhz20 was tested *in vivo*. Hep3B cells were harvested from tissue culture flasks with trypsin treatment. The viability of the cells at this point was determined by trypan blue exclusion and was typically >95%. The cells were then washed with serum-free medium and suspended at 1×10^7/ml in serum-free medium. Cells (0.1-ml samples at 1×10^6) were injected under the skin of nude mice (6 weeks old). Tumor volume was determined by measuring tumor size with calipers. When tumor size reached ~40 mm^3 (~21 days), 1×10^9 PFU of Adhz3 or Adhz20 was injected directly into tumors ($n = 10$ for each group). After 3 days, a second injection with the same amount of virus was delivered. The animals were sacrificed when the primary tumor reached 10% of body weight or at 3 months when the study was ended.

Results

Structure of Viruses. Adhz3 was rescued from plasmid pFG140 that contains an insertion of a 2.2-kb segment in the E1A region at 1339 bp of the Ad5 genome. Insertion in this region interrupts translation of the COOH terminus for both E1A 12S and 13S proteins (8, 21). Adhz20 was rescued from plasmid pHZ66 with a deletion of the E2a open reading frame (Fig. 1*A*). Except for the E2a deletion,

Adhz20 is identical to Adhz3. As *E2a* gene expression is essential for viral replication, Adhz20 was amplified in E2a-complementing E2T cells. The WT Ad5 and a viral vector AdΔE1 with the entire E1 region deleted were also used in this study as controls (Fig. 1*A*).

Because of DNA sequence overlap in the E1 region between Ad vector DNA and the complementing cellular DNA, propagation of the E1-mutated vectors in 293 cells may introduce E1 WT sequences into the vectors through homologous recombination. Two PCR primers (Ad-401F and Ad-1370R) that flank the insertion site of Adhz3 and Adhz20 were designed for analysis of this region. All of these Ad vectors contained the appropriate E1 region as shown by PCR analysis. As expected, the E1-deleted AdΔE1 without DNA sequences complementary to the primers did not generate any PCR fragments. PCR reaction with the WT Ad5 produced a 0.97-kb fragment. Insertion of a 2.2-kb segment at 1339 bp in the E1a region of Adhz3 and Adhz20 increased the size of the PCR fragment to 3.17 kb (Fig. 1*B*).

Replication of the E1a-mutated Virus in Liver Cancer Cells. We first tested replication of the E1a-mutated adenovirus in various cancer cell lines. Two normal human cell lines, liver line WRL-68 and skin line Hs27, also were included as controls. Adhz3 caused CPEs in multiple cancer cells, including HeLa, Hep3B, HepG2, DU145, and A549, at 2–3 days after infection at an MOI of 10. However, the E1a-mutated virus could not cause CPE in the normal cells (WRL-68 and Hs27) and other cancer cells (Huh1.cl2, MRC-5, LNCaP, and PC-3) at an MOI of 10 (data not shown). The strongest CPE effects were observed in two liver cancer cell lines, Hep3B and HepG2, even at an MOI of 0.5.

In another experiment, all three cell lines were infected with Adhz3 at an MOI of 1. Complete CPE was observed in the Hep3B and HepG2 cultures at day 3 after infection with Adhz3, but no CPE was seen in the Huh1.cl2 cells (Fig. 2*A*). Whether replication of the E1a-mutated virus still depends on viral replication proteins or Hep3B and HepG2 cells have developed a specific mechanism to support E1a-mutated virus DNA replication was unclear. To study this question, Adhz20 (with deleted E2a coding for the single-stranded DNA-binding protein) was used to infect the three liver cancer cell lines. DNA-binding protein is one of the three viral proteins essential for viral DNA replication. Adhz20 did not cause any CPE in all three cell lines (Fig. 2*A*). The CPE caused by Adhz3, but not Adhz20, in Hep3B and HepG2 suggested that replication of the E1a-mutated virus still depends on the viral replication mechanism.

To compare the titers of viruses propagated in Hep3B cells, we used WT Ad5, Adhz3, Adhz20, and AdΔE1 to infect Hep3B cells at an MOI of 1. After 3 days postinfection, the cells infected with WT Ad5 and Adhz3 showed complete CPE and flowed in the medium as we expected, whereas the cells infected with Adhz20 and AdΔE1 did not. The titers of Adhz3 generated in Hep3B cells were similar to that of the WT Ad5 (Fig. 2*B*). Hep3B cells increased the titer of the E1a-mutated vector Adhz3 about four to five orders of magnitude during the infection. However, the titers of Adhz20 and AdΔE1 were even lower than the titer of virus added in infection (10^6 IFU, indicated with a dashed line). The above studies further confirmed that Adhz3 with insertion in E1a is capable of selectively killing liver cancer cells, whereas the controls of the E2a-deleted virus Adhz20 and E1-deleted virus AdΔE1 cannot replicate in the liver cancer cells.

Viral DNA Replication Is Essential, but not Sufficient, for Viral Progeny Production. The E1a-mutated Adhz3 could propagate well and cause significant CPE in Hep3B cells but not in Huh1.cl2 cells (Fig. 2*A*). This may be caused by poor adsorption and entry of the vector into the Huh1.cl2 cells or a lack of viral genome DNA replication in this cell line. To compare vector entry and DNA replication, Hep3B and Huh1.cl2 cells were infected independently with Adhz3 or Adhz20 at an MOI of 1. After 4 h of infection, the unabsorbed virus particles in the media were removed, and fresh media were added. The infected cells were collected at days 0 (4 h), 1, 2, and 4 after infection. The total DNAs were isolated from collected cells, digested with the restriction enzyme *Kpn*I, and applied on agarose gel at 5 μg of DNA/well for Southern blot analysis.

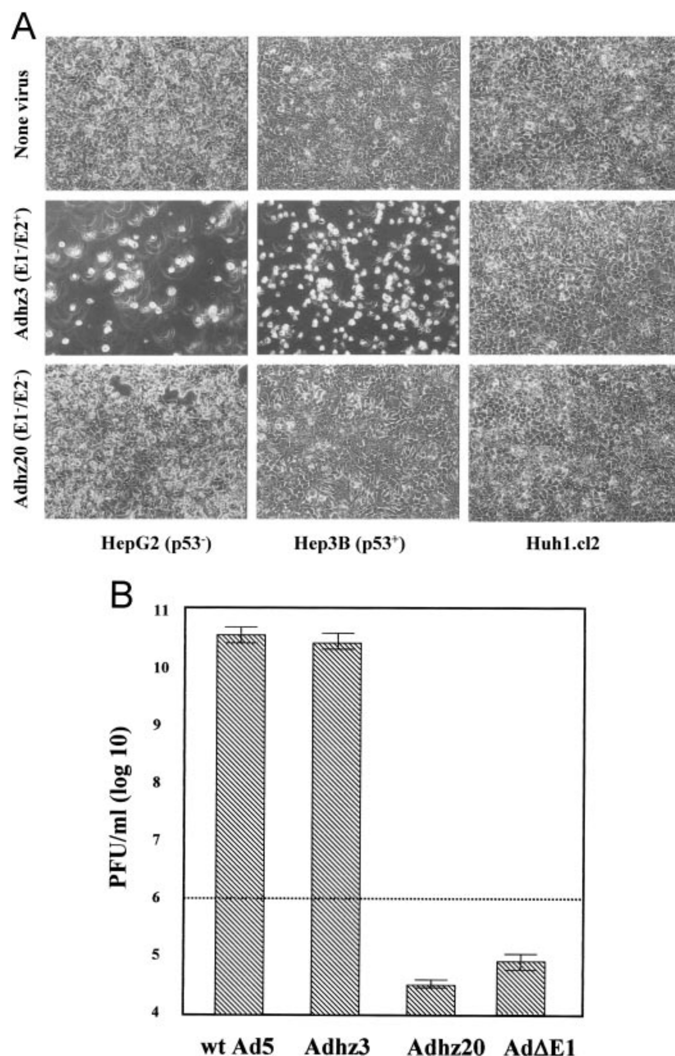
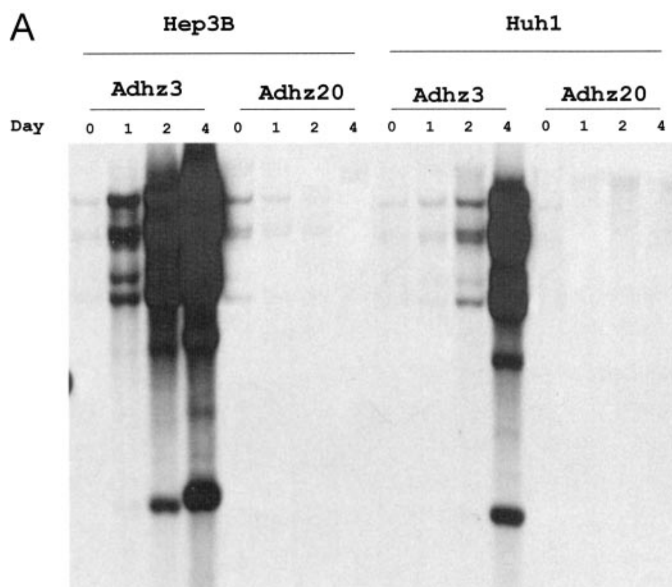


Fig. 2. *A,* comparison of CPEs of Adhz3 and Adhz20. Adhz20 could not replicate in all tested cells. Adhz3 caused CPE in Hep3B and HepG2 cells and resulted in the cells floating. Adhz3 could not cause CPE in Huh1.cl2 cells. All microscopy is originally at a magnification of ×40 at 3-day postinfection. *B,* virus produced in Hep3B cells. The Hep3B liver cancer cells were infected with WT Ad5, Adhz3, Adhz20, or AdΔE1 at an MOI of 1. The cultures were collected after 3 days of infection and went through three cycles of freezing and thawing to release viruses from the cells. Lysates were serially diluted to determine the titers in an E1- and E2a-complementing cell line, E2T. The titers of Adhz3 generated in Hep3B cells were similar to those of the WT Ad5. The titers of Adhz20 and AdΔE1 were lower than the levels of virus added in infection. *Dashed line,* the level of viruses added in the cultures.

The Southern blot results showed that viral particle entries in Hep3B and Huh1.cl2 cells were equal, as demonstrated by the viral DNA density at day 0 (Fig. 3*A*). The results also showed that Adhz3 DNA replicated very well, and the viral DNA significantly increased after 4 days in Hep3B cells. As Adhz3 could not cause CPE in Huh1.cl2 cells, we expected that the Adhz3 might not replicate in Huh1.cl2 cells. However, we were surprised to find that the Adhz3 viral DNA could also replicate in Huh1.cl2 cells, resulting in an ~1000-fold increase of DNA in 4 days, although at a much lower rate compared with that of the same virus in Hep3B cells (Fig. 3*A*). The Adhz20 with the deleted *E2a* gene could not replicate in both cell lines.



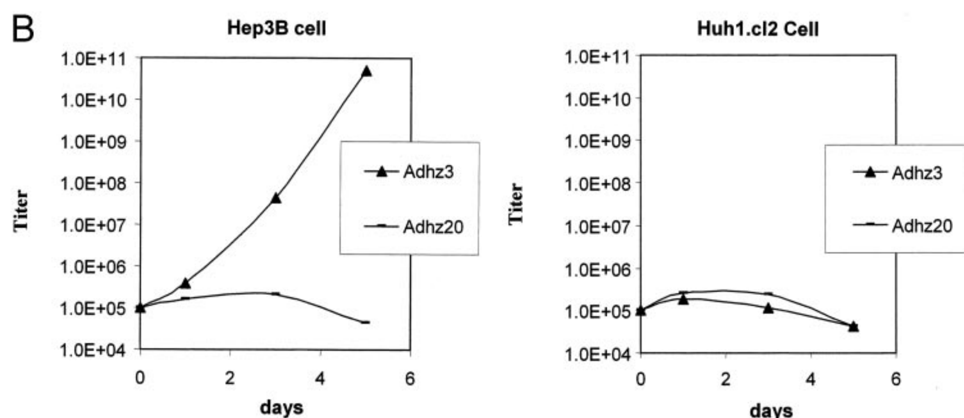


Fig. 3. *A,* Southern blot analysis of the viral DNA amplified in Hep3B and Huh1.cl2 cells. The probe was pBHG10 plasmid DNA, which contained most of adenovirus 5 genome. The DNAs were isolated from cells at days 0, 1, 2, and 4 after infection and fragmented with the restriction enzyme *Kpn*I. Viruses could infect both Hep3B and Huh1.cl2 cells as indicated by the weak bands at day 0. Adhz20 with deletion of the *E2a* gene could not replicate in Hep3B and Huh1.cl2 cells. Replication of E1a-mutated Adhz3 was effective in Hep3B cells. *B,* growth of viruses in Hep3B and Huh1.cl2 cells. Growth of Adhz3 and Adhz20 in Hep3B cells is shown on the *left,* and growth of the viruses in Huh1.cl2 is shown on the *right*. Virus was collected at the times indicated, and titer (IFU) assays were performed in 96-well plates.

Fig. 3*A* shows that Adhz3 DNA replication in Huh1.cl2 cells is delayed and decreased in comparison with Hep3B cells. We further tested infectious virus production in Hep3 and Huh1.cl2 cells at different time points. The Hep3B and Huh1.cl2 cells infected with either Adhz3 or Adhz20 viruses were collected for measuring viral progeny production after infection. Serial dilutions were titered on E2T cells. The results showed that Adhz3 could propagate in Hep3B and increase its titer to 10^{10} IFU/ml. However, Adhz3 could not produce infectious virus particles in Huh1.cl2 cells even at a later time point (Fig. 3*B*). The cells also did not show CPE at 5–7 d after infection. In addition to finding that the Adhz3 viral DNA could replicate in the Huh1.cl2 cells with decreased efficiency, we also found that the viral late protein (fiber) produced by Adhz3 was significantly lower in Huh1.cl2 than in Hep3B cells (data not shown).

E1a-mutated Virus Inhibits Tumor Growth *in Vivo*. The Hep3B cells were harvested and injected under the skin of nude mice. When tumor size reached ~40 mm^3, the tumor was injected with Adhz3 or Adhz20 (two injections speared 3 days, 1×10^9 PFU in each injection). Afterward, tumor size was checked once a week. The tumors injected with Adhz3 grew much slower (Fig. 4*A*). However, the tumors injected with Adhz20 grew very rapidly. At days 50–60, the mice treated with Adhz20 either died or had to be sacrificed because of large tumor size. In contrast, all of the mice treated with the Adhz3 virus survived >70 days, and 7 of 10 mice survived >100 days, the time the experiment ended (Fig. 4*B*).

In another experiment, we let the tumors grow to larger sizes (~250 mm^3). In this case, intratumoral injection of Adhz3 did not efficiently inhibit tumor growth, and all mice died before 100 days (data not shown). As Adhz3 viral particles produced in the infected Hep3B cells likely further infected neighboring tumor cells *in vivo,* why was Adhz3 unable to efficiently inhibit larger tumors? One explanation could be the poor distribution and spreading of virus in the larger tumor mass. To test this, Hep3B cells were first infected either with Adhz3 or Adhz20 for 4 h. Then the virus-infected Hep3B cells were washed with PBS to remove virus particles in the culture medium and mixed with uninfected Hep3B cells. The infected and uninfected Hep3B cells were mixed at a ratio of 1:10 in the mixture. Then 1×10^6 of the total well-mixed cells were injected under the skin of nude mice. By doing so, the viral-infected Hep3B cells were in close contact with the uninfected cells. Viruses replicating in and released from the infected cells could have easily infected the other tumor cells. The mixture containing Adhz3-infected cells was injected into the right side of the mice, and the mixture containing Adhz20-infected cells was injected into left side. After 50 days, all of the tumors grew on the left sides that were injected with the cells infected by the nonreplicated Adhz20 virus. However, not a single tumor formed on the right side injected with Hep3B cells with partial infection of Adhz3 (Fig. 4*C*). The results demonstrated that Adhz3 can inhibit the tumor formation and spread from infected cells to noninfected cells *in vivo* only when these cells are closely contacted.

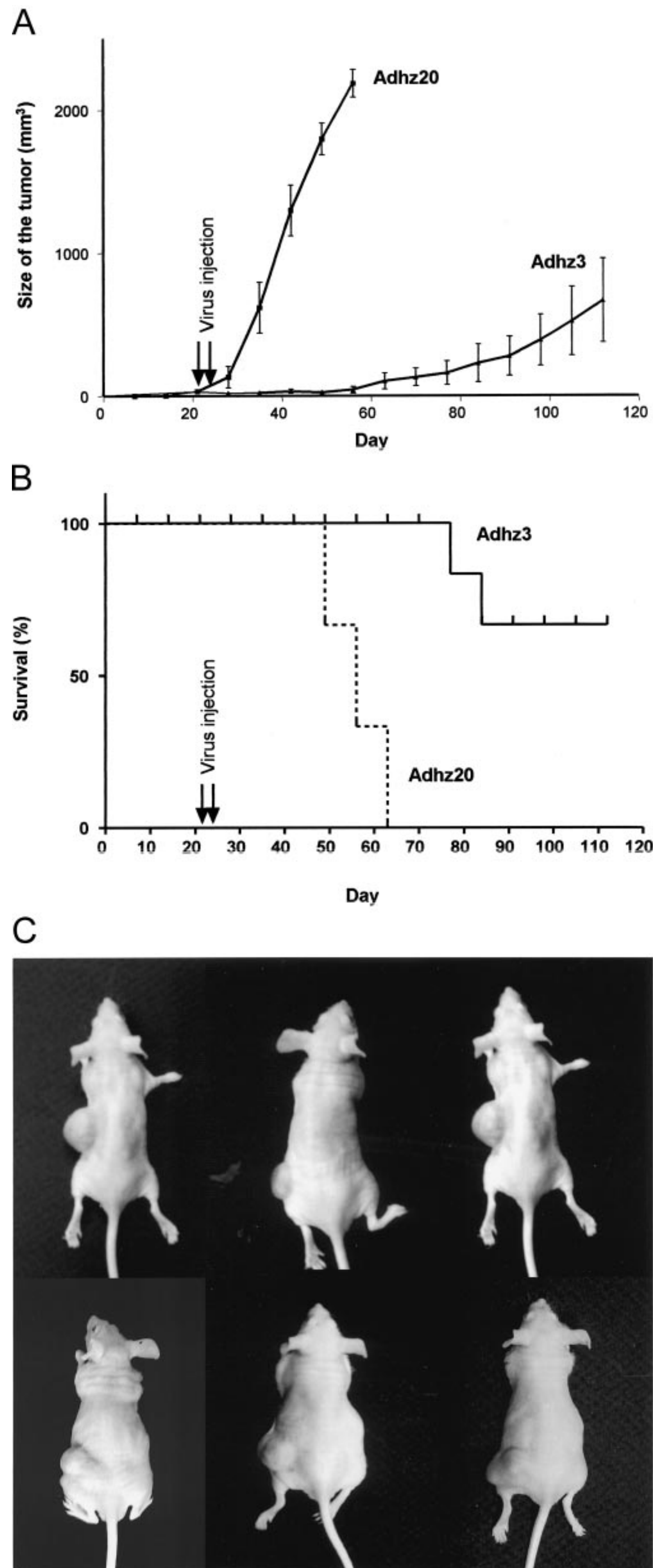


Fig. 4. In *A,* the Hep3B cells were injected under the skin of nude mice. After 3 weeks, the tumors were injected with either Adhz3 or Adhz20 virus twice. The tumor size was followed. In *B,* the mice treated with the control virus Adhz20 either died or were sacrificed because of large tumor size after 60 days. Seven of 10 mice treated with the E1a-mutated Adhz3 virus survived >100 days. *C,* inhibition of tumor formation by viral infection. The Hep3B cells were infected with either the E1a-muated Adhz3 or control Adhz20 with deletion of E2a. The infected cells then were mixed with uninfected Hep3B cells at a ratio of 1:10 (infected:uninfected). The mixtures were injected under the skin of nude mice. The mixture containing Adhz3-infected cells was injected into the right side of the mice, and no tumors formed on this side. The mixture containing Adhz20-infected cells was injected into the left side, and all of the injections resulted in tumor formations.

Discussion

The study of mutated adenovirus replication in cancer cells is of great interest because the mechanisms involved may reflect fundamental properties of tumor cells that we do not fully understand, and such study also may lead to the development of more efficient and specific adenovirus vectors for cancer gene therapy. Adenoviruses with mutated *E1a* (11) or *E1b* (4) are able to replicate in cancer cells. In this study, we analyzed the replication of a virus with insertion-mutated *E1a* in liver cancer cells. In Adhz3, a large insertion of a 2.2-kb segment at 1339 bp of adenovirus genome interrupts expression of E1A 12S and 13S (8, 21). Therefore, the activities of the E1A proteins would have been at least partially inactivated. Unlike WT adenovirus, Adhz3 did not efficiently replicate in Huh1.cl2 and other cells but was still able to replicate in the aggressively growing liver cancer cells, Hep3B and HepG2 (Fig. 2). Adhz3 replication in liver cancer cells significantly inhibited tumor formation and growth *in vivo* (Fig. 4). This is the first report to show that adenovirus with an insertion-mutated *E1a* selectively replicated within and killed cancer cells.

This selective replication of Adhz3 in Hep3B and HepG2 cells implies that these liver cancer cells may produce a cellular factor with an E1A-like activity that can partially replace the functions of proteins coded by the *E1a* gene (12). Imperiale *et al.* (13) observed that *E1a* gene products induced the expression of the viral *E2a* gene and the cellular heat shock (*hsp70*) gene and that this E1A-like transcriptional activity is harbored in F9 cells in the absence of E1A. Studies on an E1A-like activity led to the discovery of the E2F/pRb pathway in cell cycle regulation. E1A proteins bind to pRb to free E2F from the E2F/pRb complex. The released E2F protein interacts at the E2F sites of the viral E2a promoter via the E2F/DP complexes and increase transcription of the viral gene essential in viral DNA replication (10, 24). However, the identification, isolation, and characterization of this E1A-like activity remain to be carried out.

Our study suggests that liver cancer cells may harbor an E1A-like activity that allows replication of the E1a-mutated Adhz3 in these cells. However, Adhz3 could not cause CPE and generate infectious virions in Huh1.cl2 and other cells. One explanation is that Huh1.cl2 cells may lack the adenovirus receptor CAR on its surface; therefore, virus adsorption and entry into the cell are prohibited. The Southern blot results demonstrated that the viruses entered the Huh1.cl2 as well as Hep3B. We also found that the *E1a*-mutated virus could readily replicate its DNA within the cells (as demonstrated when the viral DNA increased ~1000-fold at 4 days after infection; Fig. 3). But Adhz3 could not cause CPEs and produce infectious viral progeny in Huh1.cl2 cells. Our study suggested that Huh1.cl2 cells might not provide late proteins to package the replicated viral DNA into infectious particles. This study demonstrated that viral DNA replication in cancer cells is essential, but not sufficient, for the generation of infectious virus particles and induction of oncolysis.

The major advantage of cancer-selective replication viruses is that infection of a small portion of tumor cells could result in a spreading of the virus to the rest of the cancer cells, eventually killing all of them. In this study, the E1a-mutated virus could efficiently infect, amplify, and spread in liver cancer cell monolayers. *In vivo* study showed that when tumor size was small, ~40 mm^3, direct intratumoral injection of the *E1a*-mutated virus could strongly inhibit tumor growth. However, when tumor mass was >250 mm^3, the virus could not efficiently inhibit tumor growth. The spread of virus appears limited within a large solid tumor mass. When Hep3B cells were first infected with the *E1a*-mutated virus and then mixed with uninfected cells, no solid tumor was formed from the mixed cell injections. This suggested that the virus did replicate in tumor cells and could release

virions to infect the uninfected cells *in vivo*. It is possible that the overgrown tumor may have developed the ability to block infection by the virus. Other studies have shown that E1b19K-deleted adenovirus Ad337 was unable to eradicate established flank tumors, although the virus could efficiently replicate in A549 lung cancer cells *in vitro* (25). Our and other studies suggested that barriers within the established tumor, such as the connective tissue and tumor matrix, may limit the spread of virus.

Although *E1a*- or *E1b*-mutated viruses have an advantage of replicating in different cancer cells and could be developed for cancer therapy, the mechanisms by which these mutated viruses replicate in cancer cells remain unclear. This study implies that liver and other cancer cells may provide cellular factors that complement the function of the attenuated E1a to allow viral replication. Characterizing the E1A-like factors in cancer cells will help us further understand cancer biology and enhance the development of viral vectors to selectively kill off cancer cells.

Acknowledgments

We thank Dr. Malcolm Brenner and Thomas Gegeny for critical reading and editing this manuscript, respectively.

References

1. Barker, D. D., and Berk, A. J. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. Virology, *156:* 107–121, 1987.
2. Steegenga, W. T., Riteco, N., Jochemsen, A. G., Fallaux, F. J., and Bos, J. L. The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. Oncogene, *16:* 349–357, 1998.
3. Querido, E., Blanchette, P., Yan, Q., Kamura, T., Morrison, M., Boivin, D., Kaelin, W. G., Conaway, R. C., Conaway, J. W., and Branton, P. E. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev., *15:* 3104–3117, 2001.
4. Bischoff, J. R., Kirn, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J. A., Sampson-Johannes, A., Fattaey, A., and McCormick, F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. Science, *274:* 373–376, 1996.
5. Ries, S. J., Brandts, C. H., Chung, A. S., Biederer, C. H., Hann, B. C., Lipner, E. M., McCormick, F., and Korn, W. M. Loss of p14ARF in tumor cells facilitates replication of the adenovirus mutant dl1520 (ONYX-015). Nat. Med., *6:* 1128–1133, 2000.
6. Dix, B. R., Edwards, S. J., and Braithwaite, A. W. Does the antitumor adenovirus ONYX-015/dl1520 selectively target cells defective in the p53 pathway? J. Virol., *75:* 5443–5447, 2001.
7. Kirn, D. Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? Gene Ther., *8:* 89–98, 2001.
8. Shenk, T. Adenoviridae: the viruses and their replication. *In:* D. M. Knipe and P. M. Howley (eds.), Fields Virology, Ed. 4, Vol. 2, pp. 2265–2300. Philadelphia: Lippincott Williams & Wilkins, 2001.
9. Bagchi, S., Raychaudhuri, P., and Nevins, J. R. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. Cell, *62:* 659–669, 1990.
10. Bandara, L. R., and La Thangue, N. B. Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature, *351:* 494–497, 1991.
11. Fueyo, J., Gomez-Manzano, C., Alemany, R., Lee, P. S., McDonnell, T. J., Mitlianga, P., Shi, Y. X., Levin, V. A., Yung, W. K., and Kyritsis, A. P. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect in vivo. Oncogene, *19:* 2–12, 2000.
12. Shenk, T., Jones, N., Colby, W., and Fowlkes, D. Functional analysis of adenovirus-5 host-range deletion mutants defective for transformation of rat embryo cells. Cold Spring Harb. Symp. Quant. Biol., *44:* 367–375, 1980.
13. Imperiale, M. J., Kao, H. T., Feldman, L. T., Nevins, J. R., and Strickland, S. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. Mol. Cell. Biol., *4:* 867–874, 1984.
14. Glotzer, J. B., Saltik, M., Chiocca, S., Michou, A. I., Moseley, P., and Cotten, M. Activation of heat-shock response by an adenovirus is essential for virus replication. Nature, *407:* 207–211, 2000.
15. Louis, N., Evelegh, C., and Graham, F. Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. Virology, *233:* 423–429, 1997.
16. Zhou, H., and Beaudet, A. A new vector system with inducible cell line E2T for production of safer and higher titer adenoviral vectors. Virology, *275:* 348–357, 2000.
17. Ido, A., Nakata, K., Kato, Y., Nakao, K., Murata, K., Fujita, M., Ishii, N., Tamaoki, T., Shiku, H., and Nagataki, S. Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human α-fetoprotein gene promoter. Cancer Res., *55:* 3105–3109, 1995.
18. Farshid, M., and Tabor, E. Expression of oncogenes and tumor suppressor genes in human hepatocellular carcinoma and hepatoblastoma cell lines. J. Med. Virol., *38:* 235–239, 1992.
19. Bett, A. J., Haddara, W., Prevec, L., and Graham, F. L. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc. Natl. Acad. Sci. USA, *91:* 8802–8806, 1994.
20. Zhou, H. S., Zhao, T., Rao, X. M., and Beaudet, A. L. Production of helper-dependent adenovirus vector relies on helper virus structure and complementing. J. Gene Med., *4:* 498–509, 2002.
21. Graham, F. L. Covalently closed circles of human adenovirus DNA are infectious. EMBO J., *3:* 2917–2922, 1984.
22. Sandig, V., Youil, R., Bett, A. J., Franlin, L. L., Oshima, M., Maione, D., Wang, F., Metzker, M. L., Savino, R., and Caskey, C. T. Optimization of the helper-dependent adenovirus system for production and potency in vivo. Proc. Natl. Acad. Sci. USA, *97:* 1002–1007, 2000.
23. Graham, F. L. Manipulation of adenovirus vectors. *In:* E. J. Murray (ed.), Methods in Molecular Biology, Vol. 7, pp. 109–128. Clifton, NJ: The Humana Press, Inc., 1991.
24. Murray, E. J., Stott, D., and Rigby, P. W. Sequences and factors required for the F9 embryonal carcinoma stem cell E1a-like activity. Mol. Cell. Biol., *11:* 5534–5540, 1991.
25. Harrison, D., Sauthoff, H., Heitner, S., Jagirdar, J., Rom, W. N., and Hay, J. G. Wild-type adenovirus decreases tumor xenograft growth, but despite viral persistence complete tumor responses are rarely achieved–deletion of the viral E1b-19-kD gene increases the viral oncolytic effect. Hum. Gene Ther., *12:* 1323–1332, 2001.