Published in final edited form as:

J Mol Med (Berl). 2015 February ; 93(2): 211–223. doi:10.1007/s00109-014-1214-6.

Virotherapy Targeting Cyclin E Overexpression in Tumors with Adenovirus-enhanced Cancer Selective Promoter

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Abstract

Oncolytic virotherapy can selectively destroy cancer cells and is a potential approach in cancer treatment. A strategy to increase tumor-specific selectivity is to control the expression of a key regulatory viral gene with a tumor-specific promoter. We have previously found that cyclin E expression is augmented in cancer cells after adenovirus (Ad) infection. Thus, the cyclin E promoter that is further activated by Ad in cancer cells may have unique properties for enhancing oncolytic viral replication. We have shown that high levels of viral *E1a* gene expression are achieved in cancer cells infected with Ad-cycE, in which the endogenous Ad *E1a* promoter was replaced with the cyclin E promoter. Ad-cycE shows markedly selective oncolytic efficacy *in vitro* and destroys various types of cancer cells, including those resistant to ONYX-015/d11520. Furthermore, Ad-cycE shows a strong capacity to repress A549 xenograft tumor growth in nude mice and significantly prolongs survival. This study suggests the potential of Ad-cycE in cancer therapy and indicates the advantages of using promoters that can be upregulated by virus infection in cancer cells in development of oncolytic viruses.

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Authors' contributions

PHC, KMM and HSZ designed the study and drafted the manuscript. PHC, XFL, KMM, and HSZ participated in the revision of the manuscript. PHC, XD, MEE, and XMR carried out the experiments. PHC, XFL, KMM, and HSZ participated in the coordination of the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Keywords

oncolytic adenovirus; virotherapy; cyclin E; lung cancer; autophagy

Background

Oncolytic virotherapy with cancer-selective replicating viruses has emerged as a new hope for cancer treatment and has been considered an attractive drug platform [1, 2]. The therapeutic effects of oncolytic viruses are initiated from a small number of infected cancer cells and spread the infection to surrounding tumor cells. Gene-attenuated and replication-competent adenovirus (Ad), ONYX-015 (dl1520), has been applied in several preclinical and human clinical studies; H101, structurally similar to dl1520, has been commercially approved for cancer treatment in China [3, 4].

Although dl1520 has achieved some measure of success in solid tumor treatment, the native *E1a* promoter in dl1520 is constitutively active and thus leads to the safety concerns because virus may replicate in normal cells to cause toxicity [5-7]. In addition, some cancer cells are non-permissive or resistant to dl1520 oncolytic replication [8-10]. The major strategy to increase tumor specific replication of oncolytic Ads depends on transcriptionally regulating the essential *E1a* gene by tumor-specific promoters [11, 12]. The proteins encoded by the *E1a* gene are produced immediately after infection and modulate the cell cycle, recruit cellular proteins, and regulate expression of cellular and other viral genes to process viral replication [13]. By replacing the endogenous *E1a* promoter with a cellular promoter preferentially active in tumor rather than normal tissues, oncolytic Ads can carry out more selective replication in tumors. Some tumor specific promoter-based oncolytic Ads, such as OBP-301 (Telomelysin) driven by human telomerase reverse transcriptase (hTERT) promoter [14] and CV706 driven by prostate-specific antigen (PSA) promoter [15], have been evaluated in clinical trials. Nevertheless, most known tumor-specific promoters are only active in a narrow range of tumors with relative weak activity compared with the native viral *E1a* promoter [16-20]. Our published studies have shown that most cellular genes are strongly repressed in cells after virus infection [21]. The lack of consideration of the negative effects imposed by Ad infection on promoters used in oncolytic Ads may hinder the development of efficient oncolytic Ads.

Cyclin E is known to regulate cell cycle progression [22], DNA replication [23, 24], and centrosome duplication [25, 26]. Numerous types of cancers are highly associated with cyclin E overexpression [27]. Overexpression or dysregulation of cyclin E occurs in more than 90% of lung, liver, and gastrointestinal cancers, and also in more than 80% of glioma/blastoma, bone, and breast cancers [28]. Constitutive overexpression of cyclin E induces chromosome instability [29, 30], impairs normal cell cycle progression and triggers tumorigenesis in transgenic animal models [31-33]. Previously we have shown that cyclin E overexpression in human cancer cells is a molecular basis of selective replication of *E1b55K*-deleted Ads [8, 21]. Our study indicated that Ad *E1b55K* has a function in cyclin E induction and the replication of *E1b55K*-deleted oncolytic Ads is restricted in normal cells due to the failure of cyclin E induction. Ads particularly induce large form of cyclin E (EL). Cyclin E and cyclin EL are generated from alternative splicing. The translation of cyclin EL

is initiated at an ATG codon located in exon 2, while cyclin E is from the ATG codon in exon 3 [22]. This E1B55K function is not required in cancer cells as the cyclin E promoter is already highly activated or dysregulated in cancer cells. We have demonstrated that cyclin EL overexpression is induced by *E1b55K*-deleted oncolytic Ads and increases the cyclin E/CDK2 complex formation along with CDK2 phosphorylation at the T160 site [34]. Ad-induced cyclin EL activates CDK2 that further targets the transcriptional repressor pRb, turning on the downstream gene expression to adjust the cellular environment for productive oncolytic viral replication. We also reported that the cyclin E promoter is further augmented in cancer cells during viral reproduction processes [8, 35]. The unique properties of the cyclin E promoter in virus replication indicate its potential in oncolytic vector design.

We have developed *E1b*-deleted Ad-cycE in which the *E1a* gene is under the control of the human cyclin E promoter. As the cyclin E promoter is highly active in many types of cancer cells and would be further stimulated after Ad infection, Ad-cycE replication can be enhanced. Our results revealed that Ad-cycE selectively undergoes replication in various cancer cells, significantly repressed tumor growth and prolonged survival of nude mice bearing human lung cancer xenografts. The vector also shows selective and efficient antitumor effects in cancer cell lines reported to be non-permissive for dl1520 replication. This study not only demonstrated the potent antitumor efficacy of Ad-cycE targeting cyclin E overexpression in cancer cells, but also suggested a feasible promoter selection for the future design of oncolytic Ad vectors.

Results

Ad-cycE can destroy permissive and non-permissive cancer cells

Wild-type Ad type 5 (Adwt) and an *E1a*- and *E1b*-deleted non-replicating adenovirus expressing green fluorescent protein (AdGFP) were used as positive and negative controls, respectively. To detect the cytotoxicity of Ad-cycE, human A549 and H1299 lung cancer, MDA-MB-231 breast and Saos-2 osteosarcoma cancer cells were infected with Adwt, dl1520, Ad-cycE and AdGFP at the indicated MOI for 72-96 hr and stained with crystal violet (Fig. 1). As *E1b55K*-deleted dl1520 with the native *E1a* promoter can replicate in different types of cancer cells, we applied it as the oncolytic Ad control. Cell viability was then quantified and each group was compared to the mock-infected group. Adwt, as a positive control, induced strong oncolysis in all four cancer cell lines, whereas a non-replicative control AdGFP did not cause oncolysis. Similar cytotoxicity was observed with A549 and H1299 lung cancer cells infected with Ad-cycE or dl1520. However, Ad-cycE was more efficient than dl1520 in destroying Saos-2 osteosarcoma and MDA-MB-231 breast cancer cells (Fig. 1, boxed), which are non-permissive for efficient replication of dl1520 [8]. The quantified data of boxed areas indicated that with 2 MOI of virus infection, dl1520 decreased the cell viability of MDA-MB-231 to 85% while Ad-cycE decreased it to 59%; dl1520 decreased the cell viability of Saos2 to 97% while Ad-cycE decreased it to 69%. This experiment showed that Ad-cycE can efficiently kill both permissive and non-permissive cancer cells.

Ad-cycE selectively kills lung cancer cells and spares non-cancerous lung cells

Considering the level of cyclin E expression is dysregulated in cancer cells but is repressed in non-dividing normal cells [27], we examined whether E1A expression under control of cyclin E promoter in Ad-cycE can be selectively activated in lung cancer cells. A549 cell line is a human lung cancer cell line with dysregulated cyclin E that can be significantly induced after Ad infection [8]; WI-38 cell line has the properties of primary cells with a finite lifetime of 50 population doublings [36]. A549 cells were infected with AdGFP, Adwt, dl1520 or Ad-cycE at 5 MOI for 24 hr. Ad-cycE expressed high levels of E1A in A549 lung cancer cells but not in noncancerous WI-38, as dl1520 did (Fig. 2A). High levels of E1A expression from Adwt were detected in both WI-38 and A549 cells, and no E1A was expressed from non-replicative AdGFP, with deletion of the *E1a* and *E1b* regions. We noted that Ad-cycE produced E1A proteins with slightly higher molecular weights than those of dl1520 in WI-38 cells. The significance of this observation is not clear.

We further examined whether Ad-cycE can successfully induce cyclin E overexpression in lung cancer cells. We have previously demonstrated that Ads specifically induce overexpression of the large form of cyclin E protein (cyclin EL) in the Ad-infected cells; and cyclin EL induction by Ads is a critical indicator of the viral replication process [8, 21, 34]. Cyclin E and cyclin EL are generated from alternative splicing with different ATG start codons in exons 2 and 3, respectively [22]. Failing to efficiently induce cyclin EL limits replication of *E1b55K*-deleted Ads in the normal cells [8, 34]. This *E1b55K* function is not required in cancer cells as the cyclin E promoter is already highly activated or dysregulated in cancer cells. Consistent with the results of E1A expression, Ad-cycE selectively induced cyclin EL overexpression in lung cancer rather than in normal lung cells (Fig. 2B).

Cytopathic effects (CPE), the classical characteristic of oncolytic virus replication, were recorded, showing morphological change as infected cells became rounded and detached from the cell monolayer (Fig. 2C). Adwt induced CPE in both A549 lung cancer cells and noncancerous WI-38 cells. Ad-cycE, as well as dl1520, induced significant CPE only on A549 lung cancer cells (Fig. 2C). By analyzing the cell viability, Adwt significantly decreased WI-38 cell viability to 30% ($P = 0.0000047$, compared with AdGFP), while Ad-cycE and dl1520 did not cause significant changes ($P = 0.2$ and 0.28 , respectively) (Fig. 2C). For A549 cancer cells, Ad-cycE decreased cell viability to 25%, while dl1520 decreased cell viability to 40%. It appears that Ad-cycE can destroy A549 lung cancer cells more efficiently than dl1520.

Next we investigated virus production in cancer and normal cells. Our results showed that in 72 hr Adwt yield increased 2250-fold, dl1520 833-fold and Ad-cycE 1333-fold in A549 cells; however only Adwt increased significantly in WI-38 cells (Fig. 2D). Taken these data together, Ad-cycE can selectively replicate in and destroy lung cancer cells, with significant sparing of noncancerous lung cells.

Ad-cycE favors autophagy in virus-induced oncolysis

Having observed efficient oncolysis caused by Ad-cycE, we next investigated potential mechanisms other than viral replication for this effect. Several groups have shown that E1A

expression triggers the accumulation of p53 protein and leads to p53-dependent apoptosis [37, 38] either by activating p53 transcription or preventing p53 from degradation [37-40]. E1A itself also can trigger apoptosis by p53-independent mechanisms [41, 42]. Hence initially we examined apoptosis activation after virus infection. The active form of caspase-3 and its substrate, the nuclear enzyme poly (ADP-ribose) polymerase (PARP) were detected by Western blot analysis. Caspase-3 has been implicated as an effector caspase which is activated by upstream caspase-8 or caspase-9 and serves as a convergence point to initiate different apoptotic signaling pathways [43, 44]. Therefore it is considered an important marker for cells entering into the apoptotic signaling pathway [44]. A specific antibody (#9661, Cell Signaling Technology, Danvers, MA) was used to detect the cleaved form of caspase-3 (17 kDa) from full length caspase-3 (35 kDa) [45]. As shown in Figure 3A, cleaved caspase-3 was detected in the groups infected with Adwt and dl1520 at different time points. Consistent with the pattern of cleaved caspase-3, the apoptosis-specific cleavage of its substrate PARP, a nuclear polymerase protein important for DNA repair in response to stress [46], was observed in both Adwt and dl1520-infected groups at 48 and 72-hr time points, showing smaller fragments of 85~90 kDa generated from the cleavage of the intact form of PARP with 115 kDa [47]. Surprisingly, cleaved caspase-3 and PARP in Ad-cycE-infected groups were relatively low or non-detectable even with the highest dose of virus infection (25 MOI) (Fig. 3A); suggesting Ad-cycE may not efficiently activate apoptotic pathways.

Considering the lack of significant change in apoptosis markers associated with Ad-cycE, we then investigated the protein level changes of the autophagy marker microtubule-associated protein 1 light chain 3 (LC3). Autophagy is a process involving the lysosomal degradation and recycling of cytoplasmic organelles and cellular proteins [48]. In our previous study we have shown that autophagy may generate decomposed cellular molecules as nutrients to support adenovirus replication [49]. Western blot was used here to determine the conversion of LC3-I to LC3-II, which is one of the representative characteristics of autophagy activation. During the autophagy process, LC3-I is cleaved by cysteine protease Atg4 to generate lipidated form LC3-II that localizes to autophagosome membranes [50, 51]. Therefore the LC3-II/LC3-I ratio can be used to estimate the abundance of autophagosomes [52-54]. Figure 3A showed two forms of LC3, the upper band corresponding to LC3-I (18 kDa) and lower band corresponding to LC3-II (16 kDa) [52]. Compared with the mock-infected group, LC3-I cleavage was detected in all virus-infected groups and became more significant with the increase of virus doses over the time (Fig. 3A). Densitometric analysis of these bands provided the quantitation data to support this phenomenon. Compared to mock-infected group, LC3-II/LC3-I conversion increased from 24 hr to 72 hr and in general depended on doses of viruses (Fig. 3A, bottom). Adwt initiated significant fold increases in LC3-II/LC3-I conversion ratios at 24 hr, while the changes induced by dl1520 and Ad-cycE were not obvious at the early time point. However, at 72 hr after infection, Ad-cycE increased the LC3-II/LC3-I ratio 12 fold, similar to Adwt; while dl1520 only caused less than 2-fold change of the LC3-II/LC3-I ratio (Fig. 3A, bottom).

We further examined the level of autophagy adaptor sequestosome 1 (SQSTM1/p62). SQSTM1/p62 is a ubiquitin binding protein that binds directly to LC3 to facilitate the degradation of ubiquitinated proteins by autophagosome [55, 56]. SQSTM1/p62 itself is

degraded by the autophagic process, which makes the decrease of SQSTM1/p62 as an important marker for autophagy flux [55-58]. Consistent with the pattern of LC3-II/LC3-I conversion, Ad-cycE, as well as Adwt, significantly induced SQSTM1/p62 degradation compared with dl1520 (Fig. 3B). Mock and AdGFP treatments, as negative controls, did not affect the levels of the autophagy markers. Taken together, these results suggest that Ad-cycE may favor autophagy rather than apoptosis in its oncolysis process.

Ad-cycE suppresses tumor growth *in vivo*

The therapeutic effects of Ad-cycE were further examined in human A549 lung tumors in an athymic nude mice model. Once palpable tumors were established, mice were randomized and injected intratumorally with 5×10^8 IFU of Ad-cycE or control AdGFP in 50 μ L PBS every 2 days for a total of 4 treatments. The tumor sizes were monitored over time and the time of death of mice was recorded; once the tumor volume exceeded 1000 mm³, the mouse was euthanized. The initial reduction of tumor volumes in Ad-cycE treated group was observed at day 9 after the first treatment. Mice treated with Ad-cycE exhibited significant suppression of tumor growth with 95% reduction in the mean tumor volume as compared with mice treated with the control AdGFP vector at day 51 after the first treatment ($P = 0.0008$, Fig. 4A). Representative photographs of mice on the day of sacrifice revealed that Ad-cycE treatment suppressed tumor growth compared to AdGFP treatment (Fig. 4B). Moreover, the survival of animals was also monitored for 120 days and analyzed by Kaplan-Meier method. Only one of six mice in the control group survived to that time point, whereas all mice treated with Ad-cycE survived (Fig. 4C). Thus the Ad-cycE treatment significantly improved the survival time, compared with that of AdGFP treatment ($P = 0.002$). In the Ad-cycE-treated group with a total of seven mice, two of them were tumor free (not visible), four had repressed or stable tumors during the 120 days of follow-up, and only one mouse had uncontrolled tumor growth (Fig 4D).

In an additional experiment, tumors from the mice that were injected with AdGFP or Ad-cycE were harvested at day 7 after the last treatment and analyzed with immunohistochemistry to detect the expression of Ad hexon protein. Hexon protein is the major coat protein of Ad capsids and represents the classic marker of virus particles produced in cancer cells [59]. The stain of hexon proteins localized within the cytoplasm as the brown dots (Fig. 5, panel c and d). The results showed a higher level of hexon expression in the tumor sections generated from A549 tumor treated with Ad-cycE but not with AdGFP, indicating Ad-cycE replication within tumor tissues. These results demonstrate that Ad-cycE replicates in tumor tissues and strongly inhibits tumor growth, resulting in prolonged mice survival and potential antitumor efficacy.

Discussion

One of the significant features of cyclin E is its differential expression in cancer and normal cells. While dramatic overexpression or dysregulation is detected in numerous cancers, cyclin E expression is strongly repressed in non-dividing normal cells [27]. We have previously shown that the cyclin E promoter (-363 to +87) is more active in cancer cells and the promoter activity is further augmented in cancer cells after Ad infection [8, 35]. The aim

of this study was to evaluate Ad-cycE, a novel *E1b*-deleted oncolytic Ad with its *E1a* gene controlled by the human cyclin E promoter. To date, most known designs of tumor-specific oncolytic Ad vectors do not consider the effect of virus infection on the selected promoters. This is the first oncolytic Ad using the human cyclin E promoter combined with the strategy of deletion of the entire *E1b* gene. As the cyclin E promoter is already activated in cancer cells and its activity can be further augmented by Ad itself, the oncolysis of Ad-cycE can be dually enhanced in cancer cells. Our data showed that Ad-cycE selectively replicated in multiple cancer cells, but not in normal cells. Ad-cycE also inhibited tumor growth in nude mice and prolonged survival time.

Most tumor-specific oncolytic Ads are developed based on promoters preferentially active in a specific type of cancer [11]. Although regulating essential viral gene expression by a tumor-specific promoter is feasible, this approach can be hindered by the promoter activity [16-20]. We have previously shown that many cellular promoters are repressed in cells after Ad infection [21]. Therefore, the efficacy of cancer promoter-driven Ads is limited; only Ad H101 with the native *E1a* promoter has been approved as a commercial agent for cancer virotherapy. We previously constructed an Ad vector with the prostate cancer-specific ARR2PB promoter driving *E1a* gene and observed poor E1A expression and virus replication compared with Adwt and Ad-cycE (Supplemental data, Fig. S1).

The newly developed Ad-cycE elicited better oncolytic efficacy in different types of cancer cells, including cancer cells that are not permissive for dl1502 or H101 replication (Fig. 1). We have previously reported that the activity of the cyclin E promoter is enhanced during viral reproduction process [8, 35] and Ad-induced cyclin E activates the pRb/E2F pathway that in turn enhance cyclin E expression via a feedback regulation loop [34]. This study and our previous publications suggest that oncolytic Ad-cycE driven by the cyclin E promoter has a broader range of application since it can be activated in cancer cells with dysregulated cyclin E.

In this study, we also identified the difference in the mechanisms of oncolysis between Ad-cycE and dl1520. Oncolytic Ads can mediate oncolysis by several mechanisms. The major mechanism relies on viral replication that subsequently causes cell lysis and release of progeny virions from the infected cells [60]. It is believed that several viral proteins also directly cause cytotoxicity to tumor cells, resulting in apoptosis as the second major mechanism in oncolysis [61, 62]. We observed that Adwt and dl1520 significantly activated the cleavage of caspase-3 and PARP, which is an important apoptosis event (Fig. 3A). We also noticed that this apoptosis event is a dynamic process and highly correlated to viral oncolysis. The oncolysis and apoptosis marker both occurred with higher MOI of virus infection while with lower MOI of virus infection it showed delayed oncolysis together with later activation of apoptosis marker compared with higher MOI-infected group. To our surprise, although Ad-cycE elicited efficient oncolysis, we did not detect dramatic cleavage of caspase-3 and PARP, as that was observed with both Adwt and dl1520 (Fig. 3A). It is well-known that E1A can trigger apoptosis by either p53-dependent [37, 38] or -independent [41, 42] mechanisms. The mutation studies suggested that the binding of E1A to p300 or pRb is crucial for E1A-induced apoptosis [41, 63]. In this study, we noted Ad-cycE preferentially generates larger forms of E1A transcripts distinct from that of Adwt and

dl1520 (Fig 2A). Considering the important features of E1A to the apoptosis, we tend to speculate that E1A transcripts produced by Ad-cycE may bear different properties from that produced by Adwt or dl1520, both with the native *E1a* promoter. Though Ad-cycE does not induce apoptosis as efficiently as dl1520, we observed that it favors autophagy more than dl1520 (Fig. 3). We have previously reported that complete apoptosis aborts viral reproduction and that the production of abundant viral progeny only occurs in cells that do not undergo apoptosis [64]. The lack of significant apoptosis in Ad-cycE-infected cells may allow viruses to carry out better replication and permit cells to undergo autophagy, which may generate decomposed cellular molecules as nutrients to support virus replication [49]. Autophagy involves lysosomal degradation, recycling of cellular components and type II programmed cell death [48, 65]. The increased release of virus particles from dead cells benefits viral spread in tumors [66]. Thus we reasoned that autophagy may play multiple crucial roles in Ad-cycE-induced oncolysis. The clear mechanism still required more detailed studies.

We extended our findings from *in vitro* studies to evaluate antitumor effects of Ad-cycE in a human lung cancer xenograft mouse model. Seven days after the last treatment we could detect the increased level of hexon protein expression in the tumor sections treated with Ad-cycE compared to those treated with control virus AdGFP. Signals of hexon proteins correlating with the production of virus particles were detected in the cytoplasm of tumor cells. During the long-term follow up (120 days), we noted that once virus treatment completely eliminated tumor masses, mice could maintain tumor-free status. Two mice had no visible tumors at the end of the study. We observed that four mice had repressed or stable tumors during the period of follow-up 120 days. Our results showed that Ad-cycE elicited potent antitumor efficacy, resulted in tumor regression and prolonged survival time in mice bearing tumors.

Conclusions

In the present study we have shown that Ad-cycE exhibits potent antitumor efficacy *in vitro* and *in vivo*. Our study is the first to demonstrate the application of a promoter that can be augmented by Ad itself in cancer cells. As cyclin E is highly overexpressed in a variety of cancers and its promoter activity is further increased during virus infection, Ad-cycE has the potential to destroy various types of cancers. In a recently separate study, we observed that Ad-cycE can also selectively replicate in murine ED-1 cancer cells and repress ED-1 tumor growth (Cheng *et al*, study will be reported separately). ED-1 cell line was derived from lung cancers of cyclin E transgenic mice [67, 68]. Thus, the therapeutic effects of Ad-cycE can also be evaluated with cancer model in immunocompetent animals that may better mirror the human pathogenesis of the viral infection. Further studies will characterize the clinical importance of Ad-cycE in future cancer treatments.

Methods

Cell lines and culture conditions

Human embryonic kidney HEK 293 (ATCC no. CRL-1573), lung fibroblast WI-38 (ATCC no. CCL-75), lung cancer A549 (ATCC no. CCL-185) and H1299 (ATCC no. CRL-5803),

prostate cancer DU-145 (ATCC no. HTB-81) and LNCaP (ATCC no. CRL-1740), osteosarcoma Saos2 (ATCC no. HTB-85), and breast cancer MDA-MB-231 (ATCC no. HTB-26) cell lines were purchased from the American Type Culture Collection (Rockville, MD). HEK 293, A549 and MDA-MB-231 cells were cultured in DMEM. Saos 2 cells were cultured in McCoy's 5A. LNCaP and H1299 cells were cultured in RPMI-1640 medium. WI-38 cells were cultured in minimal essential medium (MEM) Alpha GlutaMAX with 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate. All media were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml). Cells were cultured in a 5% CO₂ incubator at 37°C. All cell culture reagents were obtained from Gibco BRL (Bethesda, MD) and Corning Cellgro (Manassas, VA).

Adenoviral vectors

Wild-type adenovirus type 5 (Adwt, ATCC no. VR-5) was used as a replication-competent control. AdCMV/GFP, an Ad vector with *E1* deletion carrying a green fluorescent protein (GFP) constructed in our laboratory [49], was used as a replication-defective control. Ad dl1520 is an *E1b55K* mutant which contains an 827-bp deletion and a point mutation to generate a premature stop codon in the *E1B55K* coding region [69]. Adhz55 is an *E1b*-deleted oncolytic vector carrying prostate-specific ARR2PB promoter [70] driving an intact *E1A* expression cassette constructed in our laboratory. Ad-cycE is a novel *E1b*-deleted oncolytic vector carrying a human cyclin E promoter driving an intact *E1A* expression cassette. To prepare Ad-cycE vector, 671 bp of human cyclin E promoter (GenBank ID: X95406) [71, 72] were released by digesting pGL-2-cycE promoter 10-4 plasmid (Addgene, Cambridge, MA) with *Sma*I and the fragment was ligated into the shuttle plasmid pMEa301 which contains promoterless *E1a* region and the deletion of *E1b* to generate pBZ400 plasmid. The pBZ400 plasmid contains cyclin E promoter, together with the entire *E1a* open reading frame (ORF) and *E1b* deletion. pBHGE3 plasmid [73] containing Ad genomic DNA and pBZ400 plasmid were cotransfected into 293 cells. Ad-cycE was rescued from cells after transfection and contains a human cyclin E promoter to control *E1a* open reading frame with the deletion of *E1b* region. All of the vectors created and used in this study are based on the backbone of wild-type Ad type 5.

Cytotoxicity assay

Cells were seeded into 24-well plates at a density of 2.5×10^4 (cells/well) and cultured under the indicated conditions. Cytotoxicity was assessed with crystal violet staining [74]. Cells were fixed with 3.7% paraformaldehyde and stained with 1% crystal violet followed by washing with water to remove excess dye. The dye was solubilized with 2% SDS and the absorbance of the solubilized stain was measured at 590 nm using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT). The OD values were quantitated into the cell viability % by the formula, cell viability % = (OD value of experimental group / OD value of control group) $\times$ 100%. The mock-control group was calculated as 100% of cell viability in the assay [75].

Western blot analysis

Infected cells were harvested at indicated time points and lysed with CDK2 lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40, 0.1% Brij 35, 5 mM sodium glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol). The Western blot analyses were performed as described previously [76]. Briefly, 80 µg of cell lysates were electrophoresed through 10-12% SDS-polyacrylamide gels and transferred onto an Immobilon-P Membrane (Millipore, Billerica, MA). The primary antibodies used in this study were rabbit anti-cyclin E (M-20) (Santa Cruz Biotechnology, Santa Cruz, CA), LC3 and actin (Sigma, St. Louis, MO), SQSTM1/p62, cleaved caspase-3 (Asp175) and poly (ADP-ribose) polymerase PARP (46D11) (Cell Signaling Technology, Danvers, MA) and mouse anti-adenovirus type 5 E1A (BD Pharmingen, San Jose, CA). Actin was used as an internal control. The membranes were then incubated with anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG peroxidase-linked species-specific whole antibody (GE Healthcare, Piscataway, NJ). Chemiluminescent detection was performed with ECL reagents according to the supplier's recommendations (GE Healthcare).

Viral titration

Total infected cells and culture supernatants were collected at the indicated time points and lysed to release virus particles with three cycles of freezing and thawing. The viral titers were determined by the infective unit method as described previously [77, 78]. Briefly, HEK 293 cells were seeded in 96-well plates at a density of 10³ (cells/well) and then infected with 10-fold serially diluted viruses. CPE was recorded and scored after incubation for 7 days.

Lung cancer xenograft study

Female athymic nude mice (NCR-nu/nu) were obtained from National Cancer Institute (Bethesda, MD). 5 × 10⁶ A549 human lung cancer cells were subcutaneously injected into the flanks of mice (aged 6 weeks). Once palpable tumors were established and reached approximately 20 mm³, the mice were randomized into 2 groups to receive 5 × 10⁸ IFU of AdGFP (n = 6) or Ad-cycE (n = 7) in 50 µL PBS every 2 days for total 4 treatments. The tumors were measured every 3 days until the tumor volume was greater than 1000 mm³. The tumor volume was determined by externally measuring in 2 dimensions with a caliper and calculated based on the following equation: $V = (L \times W^2) / 2$, where L is length and W is width of the tumor. Animal experiments were performed according to the institutional guidelines approved by the University of Louisville Institutional Animal Care and Use Committee.

Immunohistochemistry

Tumors were harvested one week after the fourth treatment, embedded in optimal cutting temperature compound (O.C.T.) (Sakura Finetek, Torrance, CA), and stored at -20°C. The tumor sections and immunohistochemical staining were performed as previously reported [79]. Briefly, slides were air-dried, fixed in cold acetone at 4°C for 20 min. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 mins and washed with PBS three times, followed by incubation with SuperBlock (37515, Pierce Biotechnology, Rockford, IL) at room temperature for 30 min. All antibodies were applied in SuperBlock.

Sections were incubated with goat-anti-adenovirus polyclonal antibody (AB1056, Millipore, Billerica, MA), diluted 1:800, for 1 hr at room temperature. The signals were amplified by a biotinylated anti-goat IgG diluted 1:200 in conjunction with VECTASTAIN avidin-biotin complex method kit (Vector Laboratories, Burlingame, CA). Visualization was achieved using 3,3-diaminobenzidine tetrahydrochloride (ImmPACT DAB peroxidase substrate; Vector Laboratories). Slides were counterstained with hematoxylin and photographed at X200 and X400 magnification and in an Olympus BX53 microscope (Olympus, Center Valley, PA).

Statistical analyses

Quantitation results were reported as means $\pm$ standard deviation (S.D.). Statistical difference of the experiment was assessed with a Student's *t*-test. Survival data were plotted based on Kaplan-Meier method, followed by the analysis of log-rank test with SPSS statistical software (version 19.0; SPSS, Inc., Chicago, IL) [80]. Statistical significance of difference was set at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIH Grant R01 CA129975 (HSZ). P. H. Cheng is partially supported by the K. C. Huang Scholarship from the University of Louisville. We thank the other members in our laboratory for their help in the experiment: Jorge G. Gutierrez, Stephen L. Wechman, Hongying Hao, and Deyi Xiao.

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Figure 1. Cytotoxicity of Ad-cycE on multiple cancer cell types

A549, H1299, Saos2, and MDA-MB-231 cells were seeded at a density of $2-5 \times 10^4$ and infected with Adwt, dl1520, Ad-cycE or AdGFP at 0, 0.5, 1, 2, 4, and 8 MOI for 72-96 hr. Cytotoxicity were determined with crystal violet staining and photographed. Data were quantitated and presented as cell viability % values relative to the mock-control group $\pm$ S.D. of duplicate samples.

Figure 2. Characterization of cancer selectivity of Ad-cycE on WI-38 and A549 cells

(A) Cells were infected with AdGFP, Adwt, dl1520 or Ad-cycE at 5 MOI and collected at 24 hr p.i. for detecting E1A protein and actin; (B) samples were collected at 72 hr p.i. for detecting cyclin E and actin. Actin was used as a loading control. (C) Cells were infected with AdGFP, Adwt, dl1520 or Ad-cycE at 8 (for WI-38) or 5 MOI (for A549). CPE was observed at 72 hr p.i. and photographed with an inverted microscope Olympus CKX41. The cell viability % was determined and the values represent the means $\pm$ S.D. of triplicate samples compared with the AdGFP-infected group. (D) Virus yields were determined at 0 and 72 hr p.i. with the infection unit method. The virus production ratios represent virus yields at 72 hr p.i. relative to virus yields at 0 hr p.i. The values represent the means $\pm$ S.D. of triplicate samples. * $P < 0.05$ compared with Adwt-infected group, Student's *t*-test.

Figure 3. Effects of human Ads on apoptosis and autophagy in A549 lung cancer cells

(A) Cells were mock-infected or infected with Adwt, dl1520 or Ad-cycE at 1, 5, 25 MOI and collected at 24, 48, and 72 hr p.i. The cell lysates were immunoblotted for cleaved caspase 3, PARP, LC3 and actin. Actin was used as a loading control. The scanned band intensity was quantitated and LC3-II/LC3-I conversion ratios were calculated. The table of values represents fold increase of LC3-II/LC3-I conversion ratios compared with mock-infected group. (B) Cells were mock-infected or infected with AdGFP, Adwt, dl1520 or Ad-cycE at 5 MOI and collected at 72 hr p.i. The cell lysates were immunoblotted for SQSTM1/p62, LC3 and actin. Actin was used as a loading control.

Figure 4. Antitumor effects of Ad-cycE on subcutaneous A549 human lung cancer in nude mice
Mice carrying established A549 tumors were treated with control virus AdGFP (n=6) or Ad-cycE (n=7) on days 0, 2, 4, and 6. The total viral dose was 2×10^9 IFU / mouse. (A) The vertical arrow represents each treatment. Tumor volume (V) was plotted against time and was determined by the equation $V = (L \times W^2) / 2$, in which L represents the length, and W represents the width of the tumor. The values represent the means produce $\pm$ S.D. * $P < 0.05$ compared with AdGFP control group, Student's *t*-test. (B) Representative mice from each group were photographed at the time of sacrifice. (C) Kaplan-Meier survival curves were plotted for both treatments (AdGFP n = 6, Ad-cycE n = 7, * $P < 0.05$, log-rank test). (D) Follow-up of tumor volume curve for individual mouse in Ad-cycE-treated group.

Figure 5. Immunohistochemical analysis to detect the expression of Ad hexon protein

Tumors from the mice that were injected with AdGFP or Ad-cycE were harvested 7 days after the last treatment. Anti-adenoviral hexon antibody was used to detect the production of adenoviral particles in tissue sections. The representative photographs were taken at original magnification X200 or X400.