An E2F-responsive Replication-selective Adenovirus Targeted to the Defective Cell Cycle in Cancer Cells: Potent Antitumoral Efficacy but No Toxicity to Normal Cell

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ABSTRACT

To improve the transduction and distribution of adenovirus in a tumor mass, we generated an adenovirus to selectively replicate in tumors. We hypothesized that after infection the replicating adenovirus would spread throughout the tumor mass and cause direct oncolysis of tumor cells. E2F transcription factors are critical regulators of cell growth and are often overexpressed in cancer cells because of the frequent aberrations in the pRb/E2F/p16INK4a pathway. As a result, a majority of tumor cells exist in a high proliferative state. E2F-1 is a transcription factor that activates its own transcription and that of other genes involved in the G1 to S transition phase of the cell cycle. We constructed an adenovirus (AdE2F-1RC) so that E1A expression and viral replication were under the control of the human E2F-1 promoter element. AdE2F-1RC virus replicated as efficiently as the wild-type adenovirus and caused extensive cell killing in a panel of tumor cells in vitro. In contrast, nonproliferating normal epithelial, fibroblast, and endothelial cells, which express no E2F-1, were not able to support AdE2F-1RC replication. In animal studies, different dosing regimens of AdE2F-1RC administered to flank xenografts of ovarian and lung cancers led to a significant therapeutic advantage often surpassing that seen in animals treated with the wild-type adenovirus. This novel selectively replicating adenovirus offers a promising treatment platform for a variety of cancers of which the hallmark is uncontrolled cell growth.

INTRODUCTION

Replication-defective adenoviruses have been successfully exploited to deliver therapeutic genes for cancer gene therapy (1). Although adenovirus offers several advantages, progress in the clinic has been hampered by high viral immunogenicity, lack of cell specific infectivity, and poor viral distribution within the tumor-mass (2).

To confer specificity of infection and increase viral spread in the tumor mass, RSAds are now being actively developed as cancer therapeutic agents (3–6). These viruses have several advantages. First, viral replication amplifies the initial input dose allowing the use of a low dosing regimen. Second, lysis of the cell as part of the virus life cycle induces cell death. Third, the new virus progeny infects neighboring cells and continues to replicate and spread until the tumor is eradicated. Fourth, tumor antigens released from dead cells can enhance antitumor immunity. Fifth, these viruses can be additionally modified either to deliver cytotoxic transgenes (7, 8) or be enhanced for increased infectivity (9). Finally, replication is restricted to tumor cells thus avoiding toxicity to normal tissues.

Two commonly used approaches to restrict adenoviral replication to tumor cells are: (a) deleting entire (EIB) or partial regions (EIA) of essential viral genes that normally disarm host-defense mechanisms; and (b) to regulate expression of viral genes (EIA) essential for replication with heterologous promoters that are specifically active in tumor or tissues. For example in the well-characterized Onyx-015 (C1–1042) adenovirus (10), the EIB 55 kDa gene is deleted so that the virus replicates in p53-deficient tumor cells and not in normal cells, which contain wild-type p53. However, evidence from many studies indicates that the proposed basis for this selective replication is not solely determined by p53 deficiency (4, 11, 12). Nonetheless, its therapeutic potential often in conjunction with chemotherapy has been vigorously evaluated in >240 patients enrolled in Phase II/III clinical trials for a variety of carcinomas (13). Additional modifications of the Onyx-015 virus, including arming it with suicide genes (14, 15), deleting the EIB 19 kDa gene (16), and retargeting its receptor-mediated infection (17) have all provided increased therapeutic benefit. Another strategy used to prevent viral replication in normal cells is to delete the E1A-domain required to bind pRb and inhibit the S phase induction. However, viral replication will proceed in tumor cells, because the pRb/E2F/p16INK4a pathway is often defective. Such EIA mutant adenoviruses have proved to be highly effective in treating many mouse xenograft tumor models (18, 19), including metastatic disease models (20), and have exhibited low toxicities in normal cells (18, 20).

In another approach, viral replication has been restricted to tumor cells by regulating the transcription of the EIA gene with a heterologous promoter, which is highly active in given tumor and inactive in normal cells. For example, prostate cancer has been targeted with the adenovirus CN706 in which the EIA gene is under the control of the prostate-specific antigen enhancer element. CN706 has demonstrated a high safety index, selective replication, and a direct oncolytic activity that correlated with prostate-specific antigen level in prostate tumors (21). Moreover, CN706 selectivity has been additionally improved by regulating multiple adenoviral genes either with single or different tumor-specific promoters. For example, in CV787 virus (22), two different prostate-specific promoters control EIA and EIB genes, respectively. Alternatively, in CV890 adenovirus, a single α-fetoprotein promoter is used to control the EIA-IRE5-IEB cassette to target liver cancer (23).

Several other promoters including α-fetoprotein (24), osteocalcin (25), Muc-1 (26), 1,21-plasminogen activator inhibitor 1 (28), and carcinoembryonic antigen are being evaluated in other laboratories to restrict viral replication to their cognate tumors. Although all of these vectors are severely replication attenuated in normal cells and demonstrate high selectivity, their one drawback is that they are available for treatment of only a narrow range of tumors because only a limited number of tumors express the targeted tumor markers. However, targeting a rate-limiting gene of a key biochemical function
pathway that is defective in tumors can increase the range of application with a single RSAd.

E2F-1 is a transcription factor that binds to four E2F-1 binding sites, found in its own promoter, and it positively auto-regulates its own transcription during the G1 to S phase transition (29). In nonproliferating, normal cells, E2F-1 exists as a pRb/E2F-1 complex, and it is released when pRb is phosphorylated. The unbound form of E2F-1 transactivates several genes of the DNA synthesis pathway to thus exhibiting a deregulated G1–S phase. The Ad wild-type adenovirus, dl309. More importantly, Ad replicated in and killed a panel of tumor cells to the same extent as the own transcription during the G1 to S phase transition (29). In non-sites, found in its own promoter, and it positively auto-regulates its cation with a single RSAd.

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AdRSVtk and AdLaCZ viruses described elsewhere (41) are replication defective and contain RSV promoter-driven herpes simplex virus-thymidine kinase gene and cytomegalovirus-driven LacZ cassettes, respectively. All of these viruses were purchased from the Vector Core Facility (Wistar Institute, Philadelphia, PA).

**Immunoblot Analysis.** Nuclear protein extracts for E2F-1 analysis were prepared from log phase or confluent tumor cells and from growth-arrested normal cells (HMEC, SAEC, IMR90, NHLF, HDLF, HMEC, and WI38) essentially by the protocol described by Zumbansen and Stoffel (43).

To measure E1A protein levels, nonproliferating NHLF, HDLF, and WI38 cells were infected with different viruses at MOIs of 2000 to achieve at least 10% infectivity, predetermined with the replication-defective AdLaCZ virus. Later (48 h), cell extracts were prepared by scraping the cells into lysis buffer (2% SDS, 10 mM Tris-HCl (pH 6.8) and 0.05% Tween 20) and followed by heat inactivation at 60 °C for 10 min. The extracts were loaded on 10% SDS gel, separated on 10% SDS-polyacrylamide gel, and electrotransferred onto polyvinylidene difluoride (NEF Life Sciences, Boston, MA) membrane, and blocked in 5% milk in TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20) for 30 min. The membrane was incubated with either monoclonal anti-E2F-1 or polyclonal anti-E1A (clone KH95 and clone13S-5, respectively; Santa Cruz, Inc., Santa Cruz, CA), or monoclonal α-actin (American Pharmacia Biotech, Piscataway, NJ) primary antibodies for 1 h and repeatedly washed in TBST. After incubation for 30 min with appropriate secondary horseradish peroxidase-conjugated antibodies and extensive washing with TBST, immunocomplexes on the membrane were detected with the enzyme chemiluminescence reagent substrate according to the manufacturer’s instructions (NEF Life Sciences, Boston, MA).

**In Vitro Cell Viability Assay.** Tumor cells were plated at 60–70% confluence in six-well plates and 24 h later, infected with different adenoviral vectors at various MOIs. Normal, HUVEC, and W138 cells were plated in 96-well plates and cultured for another 2 and 4 days, respectively, before being infected. Tumor cells were trypsinized and plated at a density of 3000 cells/100 µl/well in 96-well plates. After 6 (HUVEC) and 9 (W138) days, cell viability was measured by the MTS assay using the Nonradioactive Cell Proliferation Cytotoxicity Assay kit (Promega, Madison, WI) based on the dehydrogenase enzyme activity of metabolically active cells. The percentage of cell survival was calculated using the formula: % cell survival = A10000–A10000 / A10000 × 100%.

**In Vitro Cytopathic Assay.** Ren, HeLa, A549, and SKOV3 cells were plated at a density of 2–3 × 10^5 in six-well plates and 24 h later infected with dl309 or AdE2F-1RC at various MOIs. Normal cells (W138, IMR90, NHDF, NHLF, SAEC, and HMEC) were contact inhibited by growing them for 3–4 days in 10% serum, and infected with dl309 and AdE2F-1RC at MOIs of 1000 (HMEC) and 2000 (W138, IMR90, NHDF, NHLF, and SAEC). The assay was terminated once dl309-infected cells showed significant CPE (~90–95%). All of the cells were fixed in acetonemethanol (1:1 v/v) for 10 min at room temperature and stained with 1% crystal violet (Sigma, St. Louis, MO) for 1–2 min, the plates dried, and photographed.

**Adenoviral Replication Assay.** Contact-inhibited normal cells and log phase tumor cells (plated at 60–70% confluence) were infected with dl309 or AdE2F-1RC at various MOIs. After infection (48 h), medium and cells were scrapped into 1-ml medium, subjected to three freeze-thaw cycles, and centrifuged to collect the supernatant. Serial dilutions of the supernatant were assayed for live virus particles by standard plaque assays on 293 cells. For each cell line, the efficiency of replication of the AdE2F-1RC virus was expressed as VRI, which was calculated using the formula: VRI = number of AdE2F-1RC plaques per 48 h/ml of tumor cells × 100%.

**Treatment of Established Tumors with AdE2F-1RC Virus.** Female CB17-SCID (Taconic Laboratory, Germantown, NY) mice, aged 6–8 weeks, were acclimatized in a pathogen-free animal facility of the Wistar Institute for at least 1 week before each experiment. All of the animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals (NIH) and approved by the Animal Use Committees of the Wistar Institute and the University of Pennsylvania. Four million A549 or 7 × 10^6 SKOV3 cells in 200 µl of serum-free medium were injected s.c. into flanks of 21 SCID mice. Tumor sizes were measured regularly, and the volume was calculated using the formula for an ellipsoid, i.e., tumor volume (mm^3) = π/6 × W × L^2, where L = length of the tumor and W = width of the tumor. When the tumors had reached approximately 50–100 mm^3, mice (n = 7) were randomly divided into 3 treatment groups: (a) medium only; (b) dl309; and (c) AdE2F-1RC. Viruses (10^9 pfu) in 150 µl of serum-free medium were administered intratumorally for a total of three times every other day and the tumor size monitored. Animals were sacrificed ~3 weeks after viral injection and the tumors weighed.

In a second experiment, 3.5 million A549 cells were injected in the flanks of SCID mice and allowed to reach a size of 30–50 mm^3. Flank tumors in four groups of mice (n = 7) were injected directly with a single dose of 10^9 pfu of dl309, AdE2F-1RC, AdRSVtk, or medium only. Tumor volumes were monitored regularly for 60 days. This experiment was repeated, but the tumors were allowed to grow to an average size of 900 mm^3 before they were injected with a single dose of 10^9 pfu of different viral vectors. Tumor size was monitored periodically for the following 50–55 days.

**Statistical Analysis.** The results of the MTS assay and tumor weights are presented as mean ± SE (error bars). Comparisons were made using ANOVA with appropriate pot-hoc testing (Fisher’s PLSD).

**RESULTS**

**Construction of the AdE2F-1RC Virus.** We developed an RSAd in which the E1A gene was placed under the control of the human E2F-1 promoter (Fig. 1). Adenoviral DNA was extracted, and recombination was confirmed by restriction enzyme digestion and by PCR amplification of the region between the E2F-1 promoter and the E1A gene using the 7-S and 8-AS primers shown in Fig. 1. Absence of wild-type virus was confirmed by performing PCR for the E3 gene.

**Rationale for Assessing the Selective Replication of the AdE2F-1RC Virus.** We reasoned that comparison of AdE2F-1RC replication between tumor cells overexpressing E2F-1 protein and cells expressing reduced levels of E2F-1 protein would provide a reasonable assessment of the selectivity of this transcriptionally regulated adenovirus. Because, in the clinical setting, the adenovirus would normally be administered to tumor cells in the milieu of normal, nonproliferating cells (i.e., endothelial cells, epithelial cells, and fibroblasts), we reasoned that these cells would be the best controls to verify this. To do this, we measured the E2F-1 protein levels in nuclear extracts prepared from tumor and normal cells by immunoblot analysis. E2F-1 protein levels varied widely in lung, mesothelioma, cervical, and ovarian cancer models is extremely limited. Thus, studying the effects of high doses of the AdE2F-1RC virus on normal human cells provides the best approach for safety studies.

**Tumor Cells Express High Levels of E2F-1 Protein.** One consequence of a deregulated pRb/E2F1/p16INK4a pathway seen in many tumor cells is the dissociation of E2F-1 protein from pRb/E2F complex with an increased level of “free” E2F-1 protein (32, 33). To verify this, we measured the E2F-1 protein levels in nuclear extracts prepared from tumor and normal cells by immunoblot analysis. E2F-1 protein levels varied widely in lung, mesothelioma, cervical, and ovarian cancer cells as shown in Fig. 2A. However, in all of the tumor cells, a much higher level of E2F-1 was expressed compared with normal cells (Fig. 2B). In addition, in two tumor cell lines (REN and A549), E2F-1 protein levels in the log phase of cell growth (Fig. 2A, Lanes 1 and 4, respectively) did not change significantly when these cells were grown to confluence for 3 days before analysis (Fig. 2A, Lanes 2 and 5, respectively).

E2F-1 protein was not detected in 4 of the 6 (Fig. 2B, Lanes 1–3 and 4) nonproliferating normal epithelial and fibroblast cells. A low level was detected in two fibroblast cell lines (Fig. 2B, Lanes 4 and 5). A positive control (HeLa) is shown in Fig. 2B, Lane 7, and actin loading control is shown in the bottom panel of Fig. 2B. It is well known that E2F-1 activity is very tightly regulated during the cell cycle progression from G1 to S phase in normal cells (44). This data shows that whereas E2F-1 protein was generally undetectable in a majority of growth-arrested normal cells, E2F-1 protein levels in tumor cells were unaffected by the growth state of the cell.
**E2F-1 Promoter Is Activated in Tumor Cells.** To assess the transcriptional activity of the E2F-1 promoter, the human E2F-1 promoter was subcloned upstream of the luciferase gene in the pGL2-Basic plasmid. Using the luciferase reporter assay, the E2F-1 promoter activity was compared with the ubiquitous SV40 promoter in a panel of tumor cells and normalized with the cotransfected, pSV promoter. It is not clear if the E2F-1 promoter or is because of the leakiness of the E2F-1 promoter. We would require a construct with no EIA promoter sequences.

**Transcriptionally Targeted AdE2F-1RC Has a Potent Antiproliferative Effect in Tumor Cells but not in Normal Cells.** The extent of the antiproliferative effect of AdE2F-1RC was determined by comparing the growth rates of AdE2F-1RC and dl309 infected cells. Normal endothelial (HUVEC) and fibroblast (W138) cells were infected with AdRSVtk, dl309, or AdE2F-1RC, at MOIs of 10 and 2000, respectively. Cell viability of endothelial cells and fibroblasts was measured 6 and 9 days, respectively, by the MTS assay. This is shown in Fig. 3. The dl309 virus exhibited a strong antiproliferative effect in both cell lines with 60–80% of the cells dead at the end of the experiment. In contrast, AdE2F-1RC had minimum effect on the growth of cells and appeared to behave in the same manner as the replication defective adenovirus, AdRSVtk.

To test the anticancer effects of AdE2F-1RC virus, different cancer cells in log phase of growth were infected with AdRSVtk, dl309, or AdE2F-1RC at various MOIs, and cell viability was measured within 9 days. Because it is difficult to grow tumor cells until they are contact inhibited, as they continue to grow in other planes often forming...
colonies, we tested our viruses on subconfluent tumor cell monolayers. Tumor cells infected with the dl309 showed a marked decrease in cell viability by day 6, and >45–90% of cells were dead by day 9 (Fig. 3). Cell death was a result of viral replicative oncolysis because the replication-defective adenovirus, AdRSVtk, showed minimum effect on tumor cell viability. The cell killing effects of AdE2F-1RC and dl309 viruses on SKOV3, A549, and HeLa cells were comparable. Lowest cell-killing activity was seen in SKOV3 cells with both viruses, suggesting that at the MOIs used the virus production and spread may be a limiting factor. In H661 cells, the antiproliferative effect of AdE2F-1RC was less than that for the dl309, but overall cell viabilities were reduced to 15–38% of uninfected control.

Replication of AdE2F-1RC Is Abrogated in Nonproliferating Normal Cells but not in Tumor Cells. To assess the replicative ability of AdE2F-1RC, we compared the replication of AdE2F-1RC with that of dl309 in different types of normal and tumor cells. Contact-inhibited normal cells (WI38, IMR90, NHLF, NHDF, HUVEC, HMEC, and SAEC) were infected with AdE2F-1RC and dl309 at MOIs of 10 for HUVEC, 1000 for HMEC, and 2000 for the remainder of the cell lines. Cell lysates were titrated on 293 cells 48 h after infection, and the VRI (calculated as a ratio AdE2F-1RC pfu/48 h:d309 pfu/48 h) calculated is shown in Fig. 4.

Although high MOIs were required, we were able to infect all of the normal cells with dl309. This was evident from the robust replication of dl309 in all of the normal cell lines except in SAEC, which displayed a markedly lower dl309 titer (<2 × 10^10 pfu/ml) than seen with all of the other cell lines (range of 2–9 × 10^10 pfu/ml). In contrast, AdE2F-1RC replication was severely attenuated in WI38, IMR90, NHDF, and NHLF cells with low to undetectable levels in the remainder of the cell lines. For example, in WI38, the virus replicated 80–100-fold less than dl309 (Fig. 4, Lane 1). The somewhat limited extent of viral replication seen in normal cells may be a result of a portion of cells that remain permissive to viral replication, because they may not be growth arrested and may support low E2F-1 activity. We also detected low levels of E1A production in nonproliferating normal fibroblast cells (Fig. 2C), although most of these cells do not express E2F-1 protein (Fig. 2B).

On the other hand, AdE2F-1RC virus replicated as well as the dl309 in tumor cells (SKOV3, REN, HeLa, and A549) infected at 60–70% confluence with different viral vectors and processed for plaque assay on 293 cells, 48 h later (Fig. 4). In A549, AdE2F-1RC replication was reduced 2.5–3.0-fold compared with the dl309 (Fig. 4, Lane 1)-infected cells.

Transcriptionally Targeted, AdE2F-1RC Causes Extensive CPE in Tumor Cells but not in Normal Cells in Vitro. To test the selectivity and safety feature of AdE2F-1RC we determined its CPE in several normal cells. We tested the virus on epithelial and fibroblast cells that were contact inhibited, and in a nonproliferative state. Normal cells grown to contact inhibition were infected in parallel with either AdE2F-1RC or dl309, or remained uninfected. Cells were examined regularly for CPE, and when the dl309-infected cells showed almost complete cell death the experiment was halted, and cells were fixed and stained. All of the normal cells were susceptible to dl309 infection and succumbed to its lytic effect within 8–12 days after infection (Fig. 5A). In contrast, the morphology of the AdE2F-1RC-infected cells appeared to resemble that of uninfected cells. In fact, remarkable integrity of the monolayer was seen in some cells for up to 12–15 days. The SAEC cell line did not appear as healthy as the uninfected cells, although it did not have any cells that were floating as seen in the dl309-infected wells. Overall, no significant CPE was observed in any of the AdE2F-1RC-infected normal cells (Fig. 5A).

To monitor the cytolysis activity of AdE2F-1RC, different tumor cells (HeLa, A549, and REN) were infected in parallel with either AdE2F-1RC or dl309, or remained uninfected. Cells were monitored for CPE. Complete CPE was observed in all of the tumor cells (Fig. 5B). In REN cells, a dose-dependent CPE was observed (Fig. 5B). Moreover, REN cells appeared to be more sensitive, as equivalent killing was observed in A549 and REN cells (by day 8) even though REN cells were infected at a 50-fold lower MOI. In addition, the AdE2F-1RC virus was just as effective in killing tumor cells as the dl309.

Replication-selective Virus AdE2F-1RC Suppresses Growth of Tumors in Vivo. To evaluate the antitumoral effect of the AdE2F-1RC, subcutaneous tumors were established in flanks of SCID mice using lung (A549) and ovarian cancer (SKVO3) cells. Fourteen (A549) and 17 (SKOV3) days later, macroscopic tumors with a volume of approximately 50–100 mm^3 were injected intratumorally with medium only, dl309, or AdE2F-1RC every other day for a total of three injections. Tumor size was measured with calipers every 3–4 days for 34 days. Tumor growths in dl309- and AdE2F-1RC-treated groups were compared with medium only treated groups. As shown in Fig. 6, by 31–34 days, there were significant reductions in the tumor size in dl309 and AdE2F-1RC versus medium alone-treated groups for both A549 and SKOV3 tumor models. At the end of the experiment, in dl309- and AdE2F-1RC-treated groups, tumor sizes were significantly reduced by 50% (P < 0.05 for both treatments) and 75–85% (P < 0.01 for both treatments) of control values in A549 (Fig. 6A) and SKOV3 (Fig. 6B) tumor models, respectively. Because in vivo efficacy in dl309- and AdE2F-1RC-treated groups were very similar, comparisons between these groups were not made. Although not significant, AdE2F-1RC treatment provided a slightly improved therapeutic benefit compared with dl309 in the SKOV3 tumor model (Fig. 6B).

To verify the tumor size measured by calipers, tumors were excised on day 31 (A549) and 34 (SKOV3), and weighed. Tumor weights in the dl309- and AdE2F-1RC-treated groups were significantly smaller than in the medium alone group for both tumor models (Fig. 6, A and B, right panels). In the dl309-treated group, A549 and SKOV3 tumor weights were reduced significantly by 60% and 85%, respectively (both comparisons, P < 0.001). Similarly, significant reductions in
the tumor weights of A549 (55%) and SKOV3 (90%) were obtained in AdE2F-1RC-treated groups (both comparisons, \( P < 0.001 \)).

To confirm intratumoral viral replication, we harvested tumors at an early and at a late time point from both tumor models. Tumor sections were subjected to immunohistochemistry for the detection of adenoviral hexon protein using the antibody described previously (42). Tumors excised from the dl309- or AdE2F-1RC-treated groups demonstrated a strong presence of hexon protein in the tumor mass. Interestingly, tumors harvested 12 days after viral injection also showed marked hexon staining suggesting viral replication persisting for a prolonged period after injection, in vivo of tumors (data not shown).

**Effect of a Single Dose Treatment of AdE2F-1RC on Tumor Growth.** To determine the in vivo efficacy of a low dose of AdE2F-1RC treatment on tumors, at different stages of growth, the experiment was repeated with A549 cells. A single dose (instead of \( 3 \times 10^3 \) pfu of viral vectors given previously) of vectors or medium alone was administrated intratumorally into very small tumors (30–35 mm³). In another similar experiment the tumors were allowed to grow to a larger size (\( \sim 900 \) mm³) before being treated with virus. Changes in tumor size of the groups with microscopic tumor treatment and groups with larger tumor treatment are shown in Fig. 7, A and B, respectively. Microscopic tumors treated with \( 10^3 \) pfu of AdE2F-1RC and dl309 were significantly smaller from the medium alone-treated groups by
Fig. 6. Established flank xenograft tumors in SCID mice treated with multiple doses AdE2F-1RC. A, subcutaneous tumors (50–100 mm³) growing in the flanks of SCID mice injected with 4 × 10⁶ A549 cells were infected with 10⁶ pfu of AdE2F-1RC or dl309 intramuscularly, three times every other day. B, the experiment was repeated with s.c. tumors grown with ovarian cancer cells, SKOV3. Average relative tumor volume sizes measured externally with calipers are shown in A (A549) and B (SKOV3). Right panels show tumors weights at the end of experiment (virus-treated groups versus control, ***, P < 0.001; tumor weight is mean; n = 10; bars, ±SE).

DISCUSSION

Our study is the first report of an RSAd, in which the “promoter-based regulation of E1A,” approach is used to target the deregulated G₁ to S phase in tumor cells. We demonstrated that AdE2F-1RC replicated selectively in tumor cells and not in normal cells expressing high and low levels of E2F-1 protein, respectively. Additionally, in two mouse xenograft models, AdE2F-1RC exhibited significant in vivo therapeutic benefit often equivalent to wild-type adenovirus treatment. These studies validate several design features of AdE2F-1RC.

The wild-type adenovirus dl309 replicated in all of the normal cells tested. We reasoned that normal resting cells would be a good model for AdE2F-1RC toxicity tests, because these cells do not express E2F-1 (44, 45) and are found in the tumor environment. In contrast to dl309, the replication and CPE of AdE2F-1RC was significantly attenuated in normal cells suggesting that the E2F-1 promoter was not optimally activated. One reason is that the presence of pRb/E2F-1 complex in nonproliferating normal cells renders E2F-1 inactive. Our results agree with studies carried out by Fueyo et al. (18) who showed that an E1A mutant virus, AdΔ24 (unable to bind pRb), did not replicate in growth-arrested CCD32-Luc lung fibroblasts. Nonetheless, we were concerned that E1A expression was not completely abolished in AdE2F-1RC-infected normal fibroblasts (Fig. 2C). This may represent infection of a small population of proliferating cells, which had escaped contact inhibition. However, E1A immunostaining in normal AdE2F-1RC-infected NHLF resting cells was not informative (data not shown). Although we formally did not test viral replication in proliferating normal cells, Heise et al. (20) have demonstrated that their dl922–947 E1A-mutant virus (unable to bind pRb) induced S phase, and replicated in proliferating epithelial and endothelial cells. Howe et al. (19) also showed that another E1A-mutant virus dl1107 (unable to bind pRb) replicated in proliferating MRC9 fibroblasts. Mechanistically, this may reflect the activity of increased levels of free E2F-1 in S phase cells. The extent to which either a leaky E2F-1 promoter, endogenously activated E1A promoter, or interference from neighboring adenoviral transcriptional elements contributed toward E1A induction in AdE2F-1RC-infected resting normal cells is unclear.

In one respect, AdE2F-1RC and the adenovirus CV890 (23) have similar construction designs in that both have an intact E1A-IRES-E1B cassette. When hepatoma and normal HBL100 fibroblasts were infected with CV890, E1A and E1B genes were expressed only in hepatoma cells. In our study, E1B 55kDa and E1B 19kDa proteins were expressed in dl309-infected quiescent normal NHLF fibroblasts and in A549 cells. In comparison, when cells were infected with AdE2F-1RC, both E1B proteins were expressed in A549 cells, and a low level of only E1B 55kDa was expressed in NHLF (data not shown). This may reflect the differential activation of the E1B promoter by E1A protein (46). With a view to improving selectivity we are currently examining the effect of removing the E1A-TATA box from AdE2F-1RC.

We found that AdE2F-1RC was as effective as dl309 in killing different tumor cells (Fig. 5B) with various levels of E2F-1 (Fig. 2A). Also, the high selective replication index, seen in tumor cells, correlated reasonably well with deregulated E2F-1 levels in tumor cells. These results suggest that AdE2F-1RC potency in tumor cells corre-
The AdRSVtk/control-treated experiment was terminated by day 110, as p53/E2F-1 RC cells. The potency effects of AdpRb, an colon cancer-specific promoter to drive the promoter by the E4-6/7 protein (47). Thus, the dual regulation of E1A quiescent cells. This is because free E2F-1 is recruited to the E2 DNA synthesis) genes, these promoters, because of the free E1A sites are present in the promoters of E2 (Ref. 46; essential for carcinomas after repeated intratumoral injections. They showed selective expression and low toxicity in normal tissues. After AdE2F-1β-gal injection into rat glioma tumors, β-galactosidase expression was observed only in tumor cells with minimal expression in normal brain tissues. Other RSAds developed to target the G1-S transition phase (18–20) have all used the “partial E1A gene deletion” approach. All of these vectors have demonstrated high therapeutic benefits in preclinical studies, although the effects, including immunological, of E1A mutant proteins in normal cells and any deleterious loss of radio or chemo-sensitization have not been studied. In this regard our vector expresses wild-type E1A, which has many other beneficial antitumoral activities as shown by its use in clinical trials for breast cancer (53). To reduce E1A-mutant protein toxicity in normal tissues, modifications that have been made include transcriptional control with the E2F-1 promoter (ONYX-838; Ref. 54) and retargeting infectivity by genetically altering the AdΔΔ24 virus fiber-knob (55). A different modification describes the transcriptional control of a fusion protein, E1A-pRb, which is unable to destabilize the pRb/E2F-1 complex in normal cells with a multiplicity p53 response element (56).

AdE2F-1RC and other RSAds targeting the G1-S phase pathways may prove to be more effective in treating a broad range of malignancies than vectors with tumor-specific promoters, which have a narrow range of application as they rely on overexpression of a specific tumor or tissue protein (21–28). However appealing the notion of an agent that is applicable to a broad range of tumors, caution has to be exercised in assuming that the proposed mechanism of selective replication will hold true in the many different genetic backgrounds in tumors. For example, ONYX-015, which was engineered to target a broad range of tumors with p53 defect has in practice proven to be somewhat controversial in treating every tumor with p53 defect (4, 5, 11, 12). Nonetheless, ONYX-015 has proven to be beneficial, especially when it is genetically modified to improve viral spread (57–59), viral release (16, 50), and cell killing (14, 15), or is combined with chemotherapeutic agents (4, 5, 13).

The strategy of using RSAds as oncolytic agents has many advantages for cancer therapy. As shown in this study, even if only a small portion of cells is infected (single dose injection), viruses replicate and kill the cells and replicating viruses spread to adjacent cells. Our
approach of targeting E2F-1, a critical cell cycle pathway protein, with a tumor-specific promoter controlling the EIA gene allows the targeting of a wide variety of cancers, of which the hallmark is a deregulated cell cycle.

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An E2F-responsive Replication-selective Adenovirus Targeted to the Defective Cell Cycle in Cancer Cells: Potent Antitumoral Efficacy but No Toxicity to Normal Cell

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