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, RSAd's 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 (E1B) or partial regions (E1A) of essential viral genes that normally disarm host-defense mechanisms; and (b) to regulate expression of viral genes (E1A) 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 E1B 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 I/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 E1B 19 kDa gene (16), and re-targeting 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 E1A 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 E1A 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 E1A 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 E1A and E1B genes, respectively. Alternatively, in CV890 adenovirus, a single α-fetoprotein promoter is used to control the E1A-IRES-E1B cassette to target liver cancer (23).

Several other promoters including α-fetoprotein (24), osteocalcin (25), Muc-1 (26), t-plasmin (27), secretory leukocyte protease inhibitor (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
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 coordinate cell cycle transit through the S phase (30, 31). In contrast, it is released when proliferating, normal cells, E2F-1 exists as a pRb/E2F-1 complex, and thus exhibiting a deregulated G1-S phase. The Ad element to allow us to target a variety of tumors expressing E2F gene is placed under the control of the human E2F-1 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-E2F-1 generally contributes to tumorigenesis, although experimental evidence indicates that forced overexpression of E2F-1 induces apoptosis in tumor and normal cells (39).

Taking advantage of this, a replication-defective adenovirus containing the E2F-1 promoter-driven herpes simplex virus-thymidine kinase gene has been used to target E2F-1-expressing cancer cells (40). Although tumor cell-specific thymidine kinase expression was achieved and treatment with gancyclovir administration reduced tumor growth in a glioma mouse model, poor viral transduction still posed a problem. We have extended this observation to establish direct oncolysis using a RSAd to target E2F-1-expressing tumors without additional transgene delivery.

Here, we report a strategy for the development and preclinical testing of a novel RSAd named AdE2F-1RC. In this virus the E1A gene is placed under the control of the human E2F-1 promoter element to allow us to target a variety of tumors expressing E2F-1, thus exhibiting a deregulated G1-S phase. The AdE2F-1RC virus replicated in and killed a panel of tumor cells to the same extent as the wild-type adenovirus, dl309. More importantly, AdE2F-1RC showed extremely low levels of replication and CPE in nonproliferating normal endothelial, epithelial, and fibroblast cells. In addition, AdE2F-1RC was as potent as the wild-type adenovirus in inhibiting ovarian and lung tumor growth in mouse xenograft models. AdE2F-1RC offers a new treatment platform applicable for any cancer cell of which the hallmark is a deregulated control of cell growth.

MATERIALS AND METHODS

Cell Lines. The human cell lines A549 (lung adenocarcinoma), 293 (transformed human embryonal kidney), SKOV3 (ovarian adenocarcinoma), HeLa (epithelial cervical carcinoma), WI38 (diploid lung fibroblasts), 1MR90 (lung fibroblasts), and H661 (lung adenocarcinoma) were purchased from the American Type Culture Collection (Manassas, VA). The normal cells, NHDF, NHLF, SAEC, HMEC, and HUVEC were purchased from Clonetics (Bio-Whittaker, Walkersville, MD) and grown in culture medium recommended by the manufacturer. The malignant human mesothelioma cell line, REN, has been described elsewhere (41). HeLa, 293, IMR90, H661, and WI38 cells were maintained in DMEM plus 10% FBS. A549, SKOV3, and REN were maintained in RPMI 1640 with 10% FBS. Medium for all of the American Type Culture Collection cell lines was supplemented with 2 mm L-glutamine, and 1% penicillin (100 units/ml) and streptomycin (100 μg/ml). HUVEC cells were maintained in M199 medium containing 15% FBS, epidermal growth factor (20 ng/ml; Collaborative Biomedical Products, Bedford, MA), and heparin sulfate (50 μg/ml).

Cloning of the E2F-1 Promoter. A 269-bp fragment of the human E2F-1 promoter (GenBank accession no. S74230) was amplified by PCR from human genomic DNA using the forward (5'-GTT-TAA-TTA-CTA-CGA-GGC-TAC-CAT-CCG-GAC-AAA-GCC-TGC-G-3') and reverse (5'-ACT-TTA-TTA-AAA-GCT-TAG-ATC-TGC-AGG-GCT-CAA-TGC-TCG-GC-3') primers (29). PacI and XhoI (forward primer), and PacI, HindIII, and BglII sites (reverse primer) were incorporated into the primers to facilitate additional cloning of the E2F-1 promoter. The amplification conditions were an initial step of 94°C for 5 min followed by 35 cycles, with each cycle of 94°C for 30 s and 70°C for 0.5 min, and a final extension step at 72°C for 10 min. The amplified product was subcloned into the pCRII-TOPO plasmid as per the manufacturer’s instructions (Invitrogen, Carlsbad, CA). A positive clone pCR1IE2F-1 was confirmed to contain the E2F-1 promoter by restriction enzyme digestion and DNA nucleotide sequencing. The E2F-1 promoter was digested with XhoI and HindIII from pCR1IE2F-1, and cloned upstream of the luciferase gene into similarly cut pGL2-Basic plasmid (Promega, Madison, WI) to generate pGL2E2F-1.

Analysis of the E2F-1 Promoter Activity. The activity of the E2F-1 promoter was measured by transiently cotransfecting 0.2 μg of pSVβ-gal plasmid (for transfection-control) with 1.0 μg of either pGL2E2F-1 or pGL2 promoter (positive control), or pGL2-Basic (negative control) into a panel of tumor cells using lipofectin (Life Technologies, Inc, Rockville, MD). Cell extracts were assayed 48 h later for luciferase and β-galactosidase activities using the Luciferase Assay System and β-Galactosidase Assay System, respectively, according to the manufacturer’s instructions (Promega, Madison, WI). Luciferase values were normalized for β-galactosidase activity.

Construction of the AdE2F-1RC Vector. The pXC1 plasmid has adenovirus 5 sequences from 22–5790 bp containing the E1 gene (Microbix Biosystems Inc., Toronto, Ontario, Canada). A unique PacI site was introduced at nucleotide position 522, essentially as described by Rodriguez et al. (21) to generate the plasmid pXC1522. A PacI site at nucleotide position 522 was confirmed by restriction digestion with PacI and by DNA nucleotide sequencing. The E2F-1 promoter was cut out of the pCR1IE2F-1 plasmid with PacI and ligated to pXC1522 plasmid (also cut with PacI) to obtain pXC1522E2F-1. The orientation of the E2F-1 promoter was confirmed by DNA nucleotide sequencing.

To construct the AdE2F-1RC virus, homologous recombination was performed between pXC1522E2F-1 plasmid and the right hand side of ClaII digested H5.010CMVFGP adenovirus DNA in 293 cells by standard techniques, including the green/white selection process (42). Individual white plaques, visible in 7–10 days, were grown in 293 cells until they exhibited CPE. Low molecular weight DNA isolated from these cells was analyzed for the E2F-1 promoter by restriction digestion and PCR using the primers 7S (5'-TCG-TGG-CTC-TTT-GGC-GGC-AA-3') and 8S (5'-ACC-GAA-GGC-TCT-CTG-CTC-GC-3'), which span the junction between the E2F-1 promoter and the E1A gene as shown in Fig. 1. After three rounds of plaque purification on 293 cells, one clone was processed for large-scale AdE2F-1RC preparation and purified by two-step ultra-centrifugation on cesium chloride gradients. Serial dilutions of AdE2F-1RC were plated on 293 cells for plaque assay, and the titer was expressed as pfu/ml. Ad5d309 (d309) is a typical wild-type virus but has the 14.7K and RID genes in the E3 region deleted. The E1/E3-deleted
AdRSVik and AdLucZ viruses described elsewhere (41) are replication defective and contain RSV promoter-driven herpes simplex virus-thymidine kinase gene and cytomegalovirus-driven LucZ 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, NHDF, HDLF HMEC, and WI38) essentially by the protocol described by Zumbansen and Stoffel (43).

To measure E1A protein levels, nonproliferating NHFL, HDLF, and WI38 cells were infected with different viruses at MOIs of 2000 to achieve at least 10% infectivity, predetermined with the replication-defective AdLucZ virus. Later (48 h), cell extracts were prepared by scraping the cells into lysis buffer (2% SDS, 20 mM Tris-HCl, pH 6.8) and staining the DNA with an insulin syringe. Total protein in the nuclear and cell extracts was measured using the BCA protein assay kit (Pierce, Rockford, IL).

Protein (20 μg) was separated on 10% SDS-polyacrylamide gel and electrophoresed onto polyvinylidene difluoride (NEN 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 (Amersham 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 (NEN 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 WI38 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 Assay kit (Promega, Madison, WI) based on the dehydrogenase enzyme activity chemiluminescence reagent substrate according to the manufacturer’s instructions (NEN Life Sciences, Boston, MA).

**In Vitro Cytotaphic Assay.** REN, HeLa, A549, and SKOV3 cells were plated at a density of 2–3 × 10^4 in six-well plates and 24 h later infected with dl309 or AdE2F-1RC at various MOIs. Normal cells (W138, IMR90, NHDF, NHFL, 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, NHFL, and SAEC). The assay was terminated once dl309-infected cells showed significant CPE (~90–95%). All of the cells were fixed in acetone:methanol (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 scraped 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: 

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VRI = \frac{A_{490nm} \text{ of infected cells}}{A_{490nm} \text{ of uninfected cells}} \times 100%.
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**Tumor Cell Express High Levels of E2F-1 Protein.** One consequence of a deregulated pRb-E2F 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 nonproliferating normal fibroblasts (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, pSVβ-gal plasmid. In REN cells, with low levels of E2F-1, the promoter showed 8% of the control SV40 promoter activity. In log phase A549 cells the E2F-1 promoter activity was 23% of control. In H661 cells, with a higher E2F-1 protein expression than A549 cells, the E2F-1 promoter activity was 45% of control. The E2F-1 promoter element demonstrated the capability of directing the transcription of the luciferase gene, and its activity correlated well with E2F-1 protein levels. It was not possible to measure the promoter activity in nonproliferating normal cells, as they could not be easily transfected with the plasmids.

E2F-1 Promoter Has Negligible Activity in Normal Quiescent Cells. To test the E2F-1 promoter activity in an adenoviral context, we infected nonproliferating normal fibroblasts with dl309 at MOI of 2000. After infection (48 h), E1A protein was measured by semiquantitative immunoblot analysis, and this is shown in Fig. 2A. Clearly, dl309 containing the intact E1A promoter expressed E1A protein and this is shown in Fig. 2B. Forty-eight h later, E1A protein was detected by immunoblot analysis. To test the anticancer effects of AdE2F-1RC virus, different cancer cells in log phase of growth were infected with AdRSV, 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 infected nonproliferating normal fibroblasts with dl309 (wild-type adenovirus), or AdE2F-1RC to various MOIs, and cell viability was measured because of the leakiness of the E2F-1 promoter. We would require a construct with no E1A 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 (WI38) cells were infected with AdRSV, 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, AdRSV.

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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 rate of 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, NHDF, 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:dl309 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 NHDF cells with low or 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 11)–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 cytolytic 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 (SKVO3) 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

Fig. 4. AdE2F-1RC virus replication is severely restricted in nonproliferating normal cells but not in tumor cells. Seven quiescent human cell lines (Lanes 1–7) and four tumor cell lines (Lanes 8–11) were infected with dl309 or AdE2F-1RC. Normal HMEC cells were infected at MOI of 1000 and all other cells at MOI of 2000. Tumor cells were infected at various MOIs, chosen to infect ~10% of cells. Forty-eight h after infection cells were scraped into 1.0 ml culture medium, subjected to three cycles of freeze/thaw and assayed for pfu on 293 cells. Viral replication is reported as a ratio of pfu/ml for AdE2F-1RC to dl309 per 48 h. AdE2F-1RC replicated in normal cells 2–5 logs less well than dl309. In contrast, AdE2F-1RC replicated almost as well as dl309 in four tumor cell lines. Results are of triplicate assays.
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, \textit{in vivo} of tumors (data not shown).

\textbf{Effect of a Single Dose Treatment of AdE2F-1RC on Tumor Growth.} To determine the \textit{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 \( 3 \times 10^9 \) pfu of viral vectors given previously) of vectors or medium alone was administered intratumorally into very small tumors (30–35 mm\(^3\)). In another similar experiment the tumors were allowed to grow to a larger size (\( \sim 900 \) mm\(^3\)) 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 \( 10^9 \) pfu of AdE2F-1RC and dl309 were significantly smaller from the medium alone-treated groups by

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**Figure 5.** AdE2F-1RC virus exhibits a potent CPE in tumor cells but not in normal cells. \( A \), monolayers of nonproliferating normal cells (WI38, IMR90, NHLF, SAEC, and HMEC) were infected with either dl309 or AdE2F-1RC virus at MOI of 2000 except for HMEC (1000). Cells were monitored for CPE and compared with uninfected cells. CPE was almost complete with dl309 within 8–12 days, and cells were fixed and stained with 1% crystal violet, and documented as photographs. dl309 caused extensive CPE in all normal cells tested. In contrast, AdE2F-1RC virus induced no CPE in any of the normal cell lines; \( B \), subconfluent tumor cells (REN, A549, and HeLa) were infected at MOI of 5 (A549 and HeLa), and 0.1 and 1.0 (REN). dl309 caused extensive CPE in all tumor cells. Interestingly, AdE2F-1RC also caused an equivalent CPE in all tumor cells with a dose-dependent CPE in REN cells.
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less, we were concerned that E1A expression was not completely replicate in growth-arrested CCD32-Luc lung fibroblasts. Nonethe-

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the flanks of SCID mice injected with 4

/10 6 A549

A

B

cells were injected with 10 9 pfu of AdE2F-1RC or dl309 intratumorally, 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

dl309 intratumorally, three times every other day.

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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 EIB 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, corre-

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 informat-

data not shown). Although we formally did not test viral replication in proliferating normal cells, Heise et al. (20) have demon-

strated that their dl922-947 E1A-mutant virus (unable to bind pRb) induced S phase, and replicated in proliferating epithelial and endo-

thelial 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 EIA promoter upstream of their respective heterologous promoters. In CV890, the α-fetoprotein promoter drives the 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 EIB 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.

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 EIA 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

DISCUSSION

Our study is the first report of an RSAd, in which the “promoter-

based regulation of EIA,” approach is used to target the deregulated G1 to S phase in tumor cells. We demonstrated that AdE2F-1RC replicative 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.

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 1 × 10 9 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

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A549 Tumor Weights

B

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SKOV3 Tumor Weights

Day

Tumor Weight (g)

0

5

10

15

20

25

30

35

0

0.002

0.004

0.006

0.008

0.01

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0

5

10

15

20

25

30

35

0

0.2

0.4

0.6

0.8

1

1.2

1.4

1.6

***

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lapses with the transactivation of the E2F-1 promoter by E2F-1 protein and it appears not be affected by other genetic lesions, particularly pRb. For example, the virus demonstrated equivalent potency in SKOV3 (pRb−/p53−), A549 (pRb−/p53−), REN (pRb+/p53−), and HeLa (pRb+/p53−) cells. The potency effects of AdE2F-1RC may be additionally enhanced by the differential regulation of the E2 promoter in quiescent normal and tumor cells. Although E2F-1 binding sites are present in the promoters of E1A and E2 (Ref. 46; essential for DNA synthesis) genes, these promoters, because of the free E2F-1 binding, 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, ONX-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, ONX-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 these tumors 60 days after the first treatment, tumor growth continued to regress compared with the control group (data not shown). Why cells that had not responded to the initial treatment and continued to grow for a long time would respond to a second treatment is unclear. Moreover, tumor growth inhibition was maintained over a prolonged time period although not totally eradicated. Our data agrees with that of Harrison et al. (49), who demonstrated that the growth of A549 tumors could be effectively inhibited by treatment with dl309. However, tumors could never be completely eradicated. Surprisingly, they were able to isolate dl309 virus from tumors 100 days after the first injection. Additional tumor growth retardation was achieved when the E1B 19 kDa gene was deleted, but again tumors did not disappear completely (50). They attributed tumor persistence to mouse cells growing within the tumors and forming barriers that prevented human adenoviral replication and spread.

The overall therapeutic index for a treatment is determined by the safety and the efficacy of the treatment. A major safety concern for the transition of AdE2F-1RC into the clinic as a therapeutic agent is its replication in normal tissues. A common difficulty in testing replication-selective viruses is that they there are no good laboratory animal models. However, the clinical experience with replicating vectors has indicated them to be generally nontoxic and nonimmunogenic (51, 52). Although not directly comparable, Parr et al. (40) developed a replication-incompetent AdE2F-1β-gal vector in which the β-galactosidase gene was placed under the control of the E2F-1 promoter. 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 chemosensitization 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 AdΔA24 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 multicopy 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, ONX-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, ONX-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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