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An Oncolytic Adenovirus Selective for Retinoblastoma Tumor Suppressor Protein Pathway-defective Tumors: Dependence on E1A, the *E2F-1* Promoter, and Viral Replication for Selectivity and Efficacy

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ABSTRACT

The use of oncolytic adenoviruses as a cancer therapeutic is dependent on the lytic properties of the viral life cycle, and the molecular differences between tumor cells and nontumor cells. One strategy for achieving safe and efficacious adenoviral therapies is to control expression of viral early gene(s) required for replication with tumor-selective promoter(s), particularly those active in a broad range of cancer cells. The retinoblastoma tumor suppressor protein (Rb) pathway is dysregulated in a majority of human cancers. The human *E2F-1* promoter has been shown to be selectively activated/derepressed in tumor cells with a defect in the Rb pathway. Ar6pAE2fE3F and Ar6pAE2fF are oncolytic adenoviral vectors (with and without the viral *E3* region, respectively) that use the tumor-selective *E2F-1* promoter to limit expression of the viral *E1A* transcription unit, and, thus, replication, to tumor cells. We demonstrate that the antitumor activity of Ar6pAE2fF *in vitro* and *in vivo* is dependent on the *E2F-1* promoter driving E1A expression in Rb pathway-defective cells, and furthermore, that its oncolytic activity is enhanced by viral replication. Selective oncolysis by Ar6pAE2fF was dependent on the presence of functional E2F binding sites in the *E2F-1* promoter, thus linking antitumor viral activity to the Rb pathway. Potent antitumor efficacy was demonstrated with Ar6pAE2fF and Ar6pAE2fE3F in a xenograft model following intratumoral administration. Ar6pAE2fF and Ar6pAE2fE3F were compared with Ad*dl*1520, which is reported to be molecularly identical to an E1B-55K deleted vector currently in clinical trials. These vectors were compared in *in vitro* cytotoxicity and virus production assays, after systemic delivery in an *in vivo* E1A-related hepatotoxicity model, and in a mouse xenograft tumor model after intratumoral administration. Our results support the use of oncolytic adenoviruses using tumor-selective promoter(s) that are activated or derepressed in tumor cells by virtue of a particular defective pathway, such as the Rb pathway.

INTRODUCTION

Oncolytic adenoviruses are an innovative class of promising cancer therapeutics (1, 2). A potential advantage of oncolytic adenoviral vectors over conventional antitumor agents is that viral replication in the tumor will amplify the input virus dose, leading to spread of the virus throughout the tumor. This may allow relatively low, nontoxic doses to be highly effective in the elimination of tumor cells.

One strategy for achieving the desired tumor selectivity is to introduce loss-of-function mutations in viral genes that are essential for viral replication in normal cells but not tumor cells. This strategy is illustrated by Ad*dl*1520 (3, 4), a chimeric Ad5/2 virus that has a deletion in the *E1B* gene coding for the M_r 55,000 protein. This virus is reported to be molecularly identical to one that is being evaluated in clinical trails (5).

Another strategy to achieve tumor-specific adenoviral replication is to use tumor-selective promoter(s) to control the expression of early viral gene(s) essential for replication, such as *E1A* (6–13). Tumor-selective promoters allow for expression of viral genes preferentially in tumor cells; thus, the virus should only replicate and kill those cells. The choice of promoters is important for selectivity and tightness of regulation.

Rb[2] is a nuclear phosphoprotein critical for cell-cycle regulation (14, 15). The Rb family of proteins include Rb, p107, and p130, each of which contains a "pocket domain" responsible for interactions with key regulatory proteins. This family binds to a host of partners including E2F, MDM2, c-Jun, and cyclins D, E, and A (16). These associations and therefore the function of Rb are controlled in part by phosphorylation of Rb by kinases such as cyclin D/cdk4, cyclin D/cdk6, and cyclin D/cdk2 (17), the frequency of which increases as the cell enters S phase. This then leads to changes in Rb-associated proteins and loss of repressive Rb functions. Given the key role of Rb and its pathway members for controlling progression through the cell cycle, it seems likely that this pathway would be disrupted to allow uncontrolled proliferation of cancer cells. In fact, in a majority of tumor types, Rb itself and/or the cell cycle regulatory pathway is dysregulated (18–21). As such, Rb pathway-defective tumors represent a good target for oncolytic viral therapy (22–26).

One of the most well-studied Rb-associated proteins is the group of transcription factors known as the E2F family. E2F-1 through E2F-6 can act as transcriptional activators or repressors (15). During late G_1 and S phase E2F can exist in a heterodimer with a member of the DP family (DP-1 through DP-3), and can bind to E2F-binding motifs in the promoter of target genes, activating transcription (15). E2F/DP can also bind to unphosphorylated Rb present during the G_1 or G_0 phase; when this complex binds to a promoter, it can repress transcription. During tumorigenesis, an effect of the pervasive Rb pathway changes is the loss of Rb binding to E2F, and this leads to an apparent increase in transcriptionally active or "free" E2F in tumor cells. The abundance of free E2F in turn results in high-level expression of E2F-responsive genes in tumor cells, including the *E2F-1* gene itself (27, 28). The hypothesis then is that the increase in free E2F results in an even greater activation of the E2F-1 promoter in tumor cells with an Rb pathway defect than in normal proliferating cells (29). Thus, the promoter for the human *E2F-1* gene is an excellent candidate for tumor selective expression of key viral genes controlling viral replication.

The concept of *E2F-1* promoter tumor selectivity was tested by operably linking the *E2F-1* promoter to the *Escherichia coli* β-gal gene in a replication-defective adenovirus, Ad.E2F-βgal (29). Rat

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[2] The abbreviations used are: Rb, retinoblastoma tumor suppressor protein; Ad, adenovirus serotype 5; ALT, alanine transferase; AST, aspartate transferase; β-gal, β-galactosidase; DB, direct bilirubin; FBS, fetal bovine serum; ITR, inverted terminal repeat; LD_{50}, lethal dose 50%; ψ, packaging signal/*E1A* enhancer; pA, polyadenylation; pfu, plaque forming unit(s); PrEC, prostate epithelial cell; ppc, viral particles per cell; RT-PCR, reverse transcription-PCR; RSV, Rous Sarcoma Virus; SAEC, small airway epithelial cell; vp, viral particle; wt, wild-type; cdk, cyclin-dependent kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium.

livers were efficiently transduced with Ad.E2F-βgal after femoral vein administration, yet no expression of the β-gal gene was detected, as expected. Surprisingly, the β-gal gene was not expressed in regenerating livers after partial hepatectomy. In contrast, high levels of β-gal were expressed in rat glioblastoma tumors injected with the Ad.E2F-β-gal adenoviral vector. These results support the hypothesis that Rb pathway-defective tumor cells have higher levels of transcriptionally active E2F-1 than do quiescent or proliferating normal cells; this in turn results in tumor selective activation and derepression of the *E2F-1* promoter. Thus, the *E2F-1* promoter was less active in normal quiescent and normal proliferating cells than in tumor cells, and the tumor-selective activity of the E2F vector may be recapitulated in the context of an oncolytic adenovirus.

Here we describe a pair of oncolytic vectors, Ar6pAE2fF (E2F vector) and Ar6pAE2fE3F (E2F-E3 vector), and explore the mechanism of replication selectivity in Rb pathway-defective cells. Both vectors use the tumor-selective *E2F-1* promoter to limit expression of the E1A transcription unit to tumor cells. E1A expression, cytotoxicity, and virus production *in vitro* were higher in Rb pathway-defective cells than in normal cells, relative to nonselective virus controls. Oncolytic activity and selectivity was dependent on an Rb pathway defect in the cell, on the *E2F-1* promoter containing intact E2F binding sites, and was enhanced by viral replication. Potent antitumor efficacy was demonstrated with both the E2F and E2F-E3 vectors in a xenograft model after intratumoral administration. Comparisons with Add*l*1520, which is reported to be molecularly identical to an oncolytic vector currently in clinical trials (5), revealed that the E2F and E2F-E3 vectors had higher tumor-cell selectivity *in vitro*, and greater efficacy and less severe acute hepatotoxicity *in vivo*. Our results support the use of oncolytic adenoviruses targeting a pathway commonly dysregulated in cancer.

MATERIALS AND METHODS

Cells. AE1–2A is an adenovirus complementing cell line (30) and was cultured in Richter's medium containing 5% heat-inactivated FBS. SAECs, PrECs, and lung human microvascular endothelial cells were all obtained from Clonetics/BioWhittaker (Walkersville, MD). The tumor cell lines used were obtained from American Type Culture Collection (Manassas, VA). WI-38 cells (ATCC CCL-75) are normal diploid embryonic lung fibroblasts. WI-38 VA-13 cells (termed "VA-13" cells in this report; ATCC CCL 75.1) are SV40-transformed WI-38 cells. These cells were cultured in MEM with Earle's Balanced Salt Solution, adjusted to contain 2 mM glutamine, 1.5 g/liter (w/v) sodium bicarbonate, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 10% FBS. For proliferating cell culture conditions, the cells were grown to 40–50% confluency when they were infected with the viral vectors. For quiescent cell culture conditions, cells were grown to 100% confluency followed by starvation in serum-free medium for 24 h before infection. Cell lines with Rb pathway defects were used. These cell lines were H460 (NCI-H460; ATCC HTB-177) and H1299 (NCI-H1299; ATCC CRL-5803), two human non-small cell lung carcinoma cell lines, which are $p16^-$ (31); PANC-1 (ATCC CRL-1469) a human epithelioid carcinoma of the pancreas, which is $p16^-$ (32); HT-29 (ATCC HTB-38) a colorectal adenocarcinoma line, which is $p16^-$ (33, 34); and Hep 3B (Hep 3B2.1–7; ATCC HB-8064) a hepatocellular carcinoma line, which is Rb^- (35, 36). The H460 cells were cultured in RPMI 1640 containing 10% FBS, 4.5 g/liter glucose, 2 mM glutamine, 10 mM HEPES, and 1.5 g/liter (w/v) sodium bicarbonate. The H1299 cells were cultured in RPMI 1640 containing 10% FBS. The PANC-1 cells were cultured in DMEM containing 10% FBS. The HT-29 were cultured in McCoy's 5A medium containing 10% FBS. The Hep3B were cultured in Eagle's minimum essential media containing 10% FBS. Tissue culture reagents were obtained from Life Technologies, Inc. (Rockville, MD). FBS was purchased from Hyclone (Logan, UT).

Plasmids and Viruses. All of the vectors used in this report and their designations are described in Table 1. Ar6pAE2fF (E2F vector) is an oncolytic virus based on Add*l*327, an Ad type 5 serotype harboring a deletion of the XbaI fragment D in the E3 region (bp 28,592–30,470; Fig. 1; Table 1; Ref. 37). The E2F vector was constructed as follows: a shuttle plasmid containing the first 8098 bp of the Ad genome was modified by replacing the packaging signal and the *E1A* promoter region (bp 104–551) with a multiple cloning site to generate pDL6. An SV40 early pA sequence from pSVSPORT1 (Life Technologies, Inc., Rockville, MD) was then inserted into the multiple cloning site to generate pDL6pA. A 273-bp fragment of the human *E2F-1* promoter from pGL2-AN (29, 38) was cloned into the MCS between the pA and *E1A* region to generate pDL6pAE2f. A second shuttle plasmid was constructed containing 8 kb of the right end of the virus. The packaging signal was inserted upstream of the right ITR to generate pDR2F. Introduction of these modified regions into the plasmids containing the Ad genome was performed by homologous recombination in *E. coli* strain BJ5183 (39, 40).

Ar6pAE2fE3F (the E2F-E3 vector) is identical to the E2F vector except for a restoration of all of the *E3* region genes, except the 14.7 K gene (Fig. 1; Table 1). The first 6 amino acids of the 14.7 K protein coding sequence is replaced by 6 amino acids encoded by a bacteriophage P1 loxP site, the first of which is an ATG codon; it is unknown if this chimeric protein is expressed or functional.

Ar6pAF (the promoterless vector) is identical to the E2F vector except that there is no promoter driving expression of the *E1A* region (Table 1). Ar6pARsvF (the RSV vector) is identical to the E2F vector except that the *E2F-1* promoter has been replaced with a constitutive promoter comprised of the RSV long terminal repeat and the Ad5 spliced major late tripartite leader sequence (Ref. 41; Table 1). Ar6pAE2fdF (the E2F mutant vector) is identical to the E2F vector except that the *E2F-1* promoter contains mutations that inactivate the E2F binding sites (Table 1; Ref. 38). Ar11pAE2fF (the replication-defective E2F vector) is identical to the E2F vector except that it contains a deletion in the *E2A* region (bp 22,447–24,033 as numbered in the Ad5 sequence), rendering the virus replication-defective (Ref. 30; data not shown).

To generate infectious vector particles, the plasmids containing the corresponding adenoviral genomes were linearized by restriction enzyme digestion to release the ITRs. Linear DNA was transfected by LipofectAMINE Plus (Life Technologies, Inc.) into AE1–2A cells (30). After 7 days, crude viral lysates were generated by lysing the cells with five freeze-thaw cycles and centrifugation to remove cell debris. Crude viral lysates were then placed on fresh AE1–2a cells. This was repeated until viral cytopathic effect was observed. Viruses were then purified on CsCl gradients and particle titers determined as described previously (42, 43).

The replication-defective adenovirus Add*l*312 is deleted in the *E1A* region (bp 448-1349 in Ad5; Ref. 44). Add*l*1520 is a chimera of Ad2 and Ad5 adenoviruses, containing a deletion in the viral E1b-55K gene (3, 4).

Northern Analysis. Total RNA was isolated using RNAzol B (Tel-TEST, Friendswood, TX). Northern analysis was performed with 20 μg of RNA resolved on a 6% formaldehyde-0.8% agarose gel. The RNA was transferred

Table 1 *Description of adenoviral vectors used*

Vector name	Vector designation	*E1A* promoter	*E1A*	*E1B*	Replication
Ar6pAE2fF	E2F	*E2F-1*	wt	wt	Competent
Ar6pAE2fE3F	E2F-E3	*E2F-1*	wt	wt	Competent
Ar6pARsvF	RSV	RSV	wt	wt	Competent
Ar6pAF	Promoterless	None	wt	wt	Competent
Ar11pAE2fF	Replication-defective E2F	*E2F-1*	wt	wt	Defective
Ar6pAE2fdF	Mutant E2F	*E2F-1*, mutant	wt	wt	Competent
Add*l*1520	Add*l*1520	wt	wt	Δ*E1B-55K*	Competent
Add*l*312	Add*l*312	wt	Deleted	wt	Defective
Add*l*327	Add*l*327	wt	wt	wt	Competent

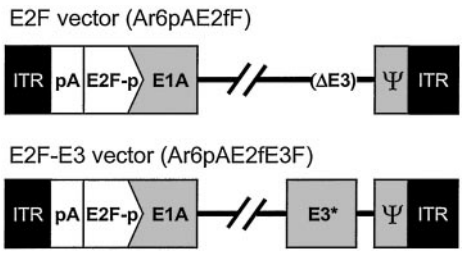


Fig. 1. Schematic representation of the E2F vector and the E2F-E3 vector genomes. The E2F vector is based on the Add*l*327 backbone (37). The packaging signal (Ψ) has been moved to the right end of the genome, the native *E1A* promoter replaced with the *E2F-1* promoter (E2F-p), and the SV40 early pA signal inserted between the left ITR and the *E2F-1* promoter. The (ΔE3) represents the deletion in Add*l*327 of the *E3* region. The E2F-E3 vector is identical to the E2F vector with the addition of a wt *E3* region except for the 14.7K gene (∗), which contains a replacement of the first six amino acids. This schematic is not to scale.

to a Hybond-N+ nylon membrane (Amersham Life Science, Buckinghamshire, United Kingdom) and hybridized overnight with a radiolabeled 350-bp DNA fragment from the *E2F-1* 3′ untranslated region located in exon 6. Radiolabeling was performed by incorporation of [^{32}P]dCTP by random priming. Blots were washed to 0.1× SSC/0.01% SDS at 68°C. The E2F-1 message was detected as a 2.5-kb transcript (45, 46). Equivalent amounts of RNA were observed on the membrane by methylene blue staining after transfer (data not shown). The membrane was also hybridized with a probe for glyceraldehyde phosphate dehydrogenase mRNA (Ambion, Austin, TX), which was radiolabeled and used as an internal control for sample loading.

Quantitative PCR. For quantitative RT-PCR analysis for E1A expression, RNA was isolated using RNAzol B (Tel-TEST). For *in vitro* experiments, RNA was isolated at 4 h after infection, before the onset of viral DNA replication, from cells that were infected with 100 ppc. To precisely control the time for E1A transcription initiation, cells were incubated with viruses for 1 h at 4°C, washed to remove unbound virus, then incubated for 4 h at 37°C to allow for viral internalization. For animal studies, RNA was isolated from liver tissue. First-strand cDNA was generated from 50 or 100 ng of RNA in the following conditions: 1× TaqMan reverse transcription Buffer, 5.5 mM $MgCl_2$, 3.8 mM deoxynucleotide triphosphate mixture, 2.5 μM random hexamers, 1 unit/μl RNase inhibitor, and 2.5 units/μl of Multiscribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) in a total reaction volume of 35 or 70 μl. The reactions were incubated for 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. E1A expression levels were measured by a real-time PCR assay with the following primers: *E1A* forward primer, 5′-AGCTGTGACTCCGGTCCTTCT-3′; *E1A* reverse primer, 5′-GCTCGTTAAGCAAGTCCTCGA-3′; *E1A* probe, 5′-carboxyfluorescein-TGGTCCCGCTGTGCCCCATTAAA-6-carboxytetramethylrhodamine-3′. Amplification was performed in 50 μl under the following conditions: 20 μl of sample cDNA, 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM forward primer, 900 nM reverse primer, and 100 nM *E1A* probe. Thermal cycling conditions were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The 18S rRNA levels were determined with Predeveloped TaqMan Assay Reagents (Applied Biosystems) as an endogenous control. Amplification was performed according to the manufacturer's specifications.

The expression level of E1A for each vector was normalized to viral DNA copy number to allow comparison among cell lines that differ in viral transduction efficiency. This was done by measuring the adenoviral hexon gene copy number per cell using a quantitative PCR assay (47). For the *in vitro* experiments, transduction efficiency was measured in cells infected with 100 ppc of Ar6pARsvF vector, as described above. Because the capsids of all of the viruses were identical, the transduction efficiency of each vector on a particular cell line was assumed to be similar to that of Ar6pARsvF. For the animal experiments, DNA was isolated from liver tissue, as described (47).

Cytotoxicity Assays. Cells were seeded in 96-well dishes in 90-μl volume 1 day before adenoviral infection. The next day, adenoviruses were diluted serially in the appropriate growth medium and 10 μl of each dilution added to the wells. Cells were exposed to virus for 7–10 days, after which an MTS cytotoxicity assay (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI) was performed according to the manufacturer's instructions. Absorbance values are expressed as a percentage of uninfected control and plotted *versus* vector dose. A sigmoidal dose-response curve was fit to the data and a LD_{50} value calculated for each replicate, using GraphPad Prism software, version 3.0. Two to five independent experiments were performed, each in triplicate, representing 6–15 dose-response curves per vector.

Virus Production Assay. A modified tissue-culture infectious dose-50% assay was used to determine the level of virus production, in pfu/ml, as described previously (6).

Animal Studies. All of the animals were cared for and maintained in accordance with applicable United States Animal Welfare regulations under an approved Institutional Animal Care and Use Protocol in the Genetic Therapy Inc. animal facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

For evaluation of E1A-mediated hepatotoxicity, 5-week-old male C.B-17/lcr-SCID mice (Harlan Sprague Dawley, Indianapolis, IN) were used. A dose of 6.25×10^{11} particles/kg of each test vector was administered i.v. on study day 1 into the lateral tail vein ($n = 10$/group) at a dose volume of 10 ml/kg. A control group of animals ($n = 10$) was injected with an equivalent dose volume of HBSS. Serum was collected by orbital sinus bleeds on study day 4 ($n = 10$/group) and study day 15 ($n = 5$/group), and submitted to Anilytics, Inc. (Gaithersburg, MD) for selected clinical chemistry analyses: ALT, AST, and DB. At the study day 4 and 15 necropsies, livers were collected, and portions were frozen on dry ice for DNA analysis, frozen in liquid nitrogen for RNA analyses, or fixed in zinc-formalin for microscopic evaluation.

For xenograft tumor studies, Hep3B cells (1×10^7 cells in 100 μl of HBSS) were implanted s.c. on the right flank of 6–8 week-old female nude mice (Hsd:Athymic Nude-*nu*; Harlan, Indianapolis, IN). Tumor volumes were recorded twice weekly using the formula length × width^2 × π/6. For the study described in Fig. 8*A*, a cohort of 130 mice (range of tumor volume, 92.9–228.4 mm^3) were selected and evenly distributed by tumor volume into six dose groups. Mice ($n = 20$–25/group) were injected intratumorally with each test vector or HBSS at 2×10^9 vp/dose at a final concentration of 6.67×10^{10} vp/ml.

For the study described in Fig. 8*B*, a study cohort of 90 mice were selected and evenly distributed by tumor volume into six dose groups. Fifteen mice/group were injected intratumorally on 5 consecutive days with HBSS diluent control, Add*l*1520, or the E2F-E3 vector at the doses of vector indicated in Fig. 8*B*, in a 30 μl volume. Mean tumor volumes were calculated for all of the treatment groups as long as each group remained intact. Animals were sacrificed when tumors exceeded a volume of 2000 mm^3 or if the physical condition of the animal warranted intervention.

Immunohistochemistry. Livers were collected at the study day 4 necropsy, fixed in Z-fix (Anatech Ltd., Battle Creek, MI), embedded in paraffin, sectioned, and mounted on poly-L-lysine coated slides. Detection of E1A protein was performed by incubating the sections with a primary rabbit antiadenovirus type 2 E1A antibody; (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with a biotinylated goat-antirabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized by incubating the slides in ABC complex (ABC Elite Standard kit; Vector Laboratories) followed by incubation with a 3,3′-diaminobenzidine chromogen (Research Genetics, Huntsville, AL). The intensity of nuclear and cytoplasmic E1A immunoreactivity was ranked by a board-certified veterinary pathologist as none < minimal < slight < moderate. Qualitative differences in E1A expression were also noted.

Statistical Analysis. All of the quantitative data were tested for normality and equal variance (SigmaStat 2.03). One-way ANOVA or a paired two-tailed Student's *t* test was used to determine whether statistically significant differences between groups were present. The level of significance was set at $P < 0.05$ for all of the tests.

RESULTS

Vector Construction. To achieve tumor-selective transcriptional control of E1A in the E2F vector, three modifications to the viral genome were made (Fig. 1): (*a*) the *E1A* promoter was replaced with a 270-bp fragment of the human *E2F-1* promoter; (*b*) the ψ was moved to a position 5′ of the right ITR; and (*c*) the potential for read-through transcription from cryptic start sites in the left ITR was

minimized by placing a pA upstream of the *E2F-1* promoter (see "Materials and Methods" for locations). These changes were made to insulate the *E1A* transcription unit from the nonselective effects of any endogenous viral transcription and enhancer elements remaining in the left ITR. The human *E2F-1* promoter was operably linked to *E1A* to restrict expression of E1A to Rb pathway-defective tumor cells (29, 38). The E2F-E3 vector is identical to the E2F vector except that the entire *E3* region was restored, although the *14.7K* gene coding sequence contains a replacement of the first six amino acids (Fig. 1).

In our experiments to analyze the oncolytic activities of the E2F vector, a panel of viruses was used, including Add*l*327 (wt) and Add*l*312 (E1A-deleted; Table 1). In addition, the following vectors were constructed that were identical to the E2F vector, except for one key element, to help elucidate aspects of its oncolytic activities. Ar6pARsvF (the RSV vector) contains the constitutive RSV promoter driving E1A. Ar6pAF (the promoterless vector) contains a deletion of the *E2F-1* promoter. Ar11pAE2fF (replication-defective E2F vector) has a deletion in another early region essential for replication, *E2A*, and Ar6pAE2fdF (the mutant E2F vector) contains loss-of-function mutations in the E2F binding sites within the *E2F-1* promoter.

***In Vitro* Characterization of Isogenic Cells with and without Rb Pathway Defect.** To analyze the oncolytic activity of the E2F vector and its dependence on Rb pathway defects, we used the WI-38 and VA-13 isogenic cell lines. WI-38 cells are normal human fibroblasts that are passage-limited in cell culture. WI-38 VA-13 (termed VA-13) cells are derived from WI-38 and are transformed with SV40, and are, therefore, immortal. Because of the expression of the SV40 large T antigen, the tumor suppressor proteins pRb and p53 are inactivated (48, 49). To confirm dysregulation of the Rb pathway and up-regulation of the endogenous *E2F-1* gene, Northern blot analysis was performed (Fig. 2*A*). VA-13 cells expressed high levels of E2F-1 under both proliferating and quiescent culture conditions, whereas WI-38 cells expressed either very little under proliferating conditions or undetectable amounts under quiescent conditions. Therefore, this isogenic pair of cell lines was used, along with other normal and tumor cells, to determine selectivity of adenoviral-mediated E1A expression and cytotoxicity.

E1A Expression in Rb Pathway-functional and -defective Cell Lines. We correlated E1A expression levels with the Rb pathway defect in the WI-38 and VA-13 cells. E1A mRNA levels were quantitated by real-time RT-PCR at 4 h after infection, before the onset of viral replication. VA-13 cells infected with the E2F vector displayed higher levels of E1A mRNA per viral genome than WI-38 cells (Fig. 3). The replication-defective E2F vector also expressed higher levels of E1A per viral genome in VA-13 cells than in WI-38.

The selective expression of E1A by the E2F vector and the replication-defective E2F vector was dependent on the presence of the

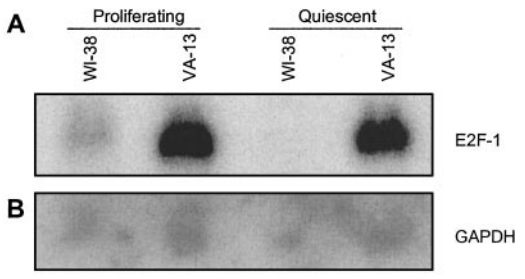


Fig. 2. Endogenous *E2F-1* gene expression in the WI-38 and VA-13 isogenic cell line pair. Cells were cultured in conditions favoring either proliferation or quiescence, as described in "Materials and Methods." Total RNA was isolated and subjected to Northern blot analysis with an *E2F-1* specific probe (*A*). Equivalent loading of RNA samples on the gel was demonstrated by rehybridization with a probe for glyceraldehyde phosphate dehydrogenase (*GAPDH*; *B*).

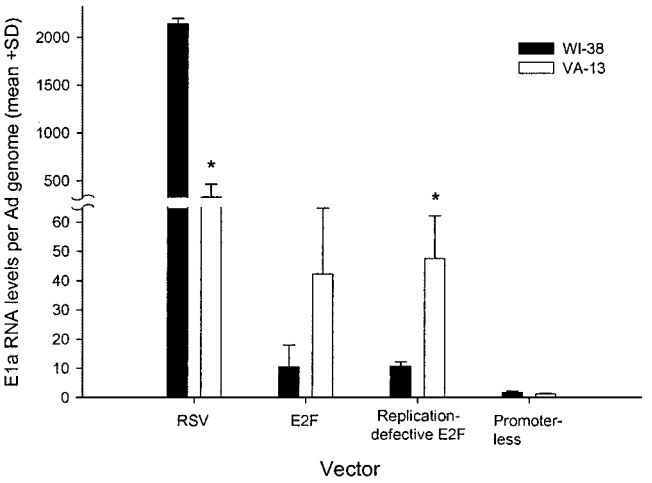


Fig. 3. Adenoviral E1A expression in WI-38 and VA-13 cells. Cells were infected at 1000 vp/cell with the indicated adenoviral vectors. Four h later, viral copy number and E1A transcript levels were quantitated by a real-time PCR assay. E1A expression is expressed on a per Ad genome basis to control for transduction differences between the two cell cultures. Values shown are mean for triplicate samples; *bars*, ± SD. *, $P < 0.05$ for indicated vector on VA-13 *versus* WI-38 cells, by *t* test.

E2F-1 promoter because the promoterless vector-infected cells did not have appreciable levels of E1A in either VA-13 or WI-38 cells (Fig. 3). In contrast, the constitutive RSV promoter in the RSV vector expressed high levels of E1A per viral genome in both cell lines. The reason for the higher E1A transcript levels in the WI-38 cells was not clear. We conclude that the *E2F-1* promoter mediates higher E1A transcript levels in infected VA-13 cells and that this E1A expression is dependent on the presence of the *E2F-1* promoter.

We next analyzed E1A expression in a broad panel of human tumor cell lines having at least one defect in the Rb pathway (Hep3B, PANC-1, H1299, and H460) compared with primary human nontumor cell cultures that lack such defects (SAEC and primary human hepatocytes). The E2F vector selectively expressed E1A in the tumor cell lines compared with the nontumor cell cultures, and this selectivity was found to be dependent on the *E2F-1* promoter (data not shown).

***In Vitro* Cytotoxicity.** We evaluated the mechanism of cytotoxicity of the Rb pathway-dependent E2F vector *in vitro*. We first analyzed the cell-killing ability of the E2F vector on the WI-38 and VA-13 isogenic cell line pair. To elucidate the mechanism of E2F vector-mediated cell killing, we also tested the RSV vector, the promoterless vector, the replication-defective E2F vector, the mutant E2F vector, and Add*l*312 (an *E1A*-deleted replication-defective control). We used a standard MTS cytotoxicity assay, which reflects the number of living cells at a specific time after infection relative to the uninfected control. The cytotoxicity of each vector relative to the others is illustrated by the representative dose response curves shown in Fig. 4 and the mean LD_{50} values calculated from all of the experiments (Table 2). By using this panel of vectors, we addressed three issues: (*a*) the relationship between Rb pathway status and cytotoxicity of the E2F vector; (*b*) the dependence of selective E1A expression on the *E2F-1* promoter and on E2F binding to the *E2F-1* promoter; and (*c*) the importance of viral replication in the oncolytic activity of the E2F vector.

The cell-killing activity of the E2F vector was compared on the WI-38 and VA13 cell pair. Oncolytic activity was found to be dependent on the cell type, and correlated with the E2F-1 (Fig. 2*A*) and E1A (Fig. 3) expression levels. On VA-13 cells, the dose-response curve for the E2F vector was similar to the nonselective RSV vector (Fig. 4). This similarity in potency is also evident in the LD_{50} values (Table 2). However, on the WI-38 normal cells, the E2F vector

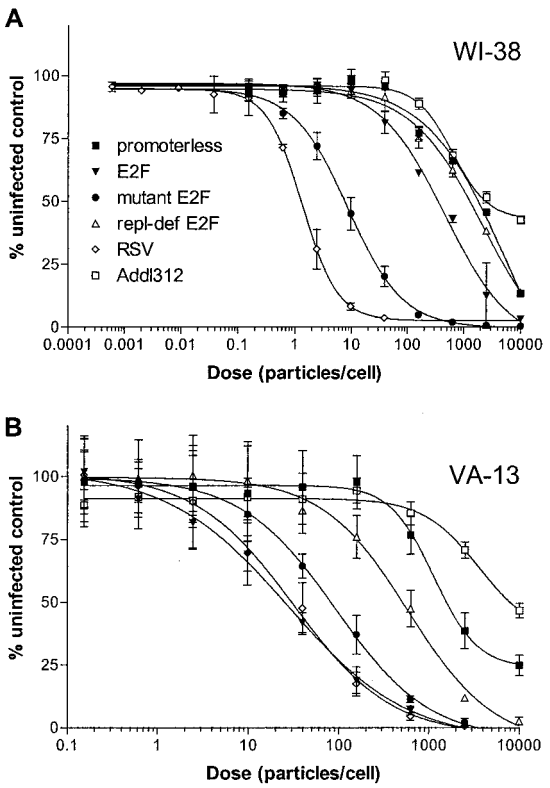


Fig. 4. Dose response curves from a representative MTS cytotoxicity experiment. WI-38 (*A*) and VA-13 (*B*) cells were infected with a dilution series of the indicated vectors. After 7 (VA-13) or 10 (WI-38) days, an MTS cytotoxicity assay was performed. The viability of each infected culture is expressed as a percentage of uninfected controls. A sigmoidal dose-response curve was then fit to the data, and LD_{50} values were calculated by GraphPad Prism version 3.0 software (Table 2). Each data point is the mean of three replicate curves; *bars*, ±SD.

Table 2 *Adenoviral cytotoxicity on the WI-38 and VA-13 isogenic cell line pair*

Mean LD_{50} values for the indicated vectors calculated from the MTS cytotoxicity assay. Experiment was performed five times, in triplicate except for the mutant E2F vector, which was performed twice in triplicate on WI-38 and three times in triplicate on VA-13. LD_{50} values for Add*l*312 are estimates since at the doses used here, this virus does not kill efficiently enough to generate reliable dose-response curves.

	VA 13		WI-38	
Ad vector (Table 1)	Mean LD_{50}	SD	Mean LD_{50}	SD
E2F vector	43.7	13.7	699.8	641.6
RSV vector	34.0	6.7	1.5[a]	1.1
Add*l*312	~7000	na[b]	~4000	na
Promoterless vector	1242.5[a]	578.5	1341.0	646.5
Replication-defective E2F vector	629.1[a]	206.4	1630.8	731.1
Mutant E2F vector	113.0[a]	34.5	13.5[a]	5.7

[a] na, not calculated since dose response curves for Add*l*312 do not return LD_{50} values.
[b] $P < 0.05$ *versus* the E2F vector by one-way ANOVA. Statistical comparisons with Add*l*312 not performed because LD_{50} values are approximations.

dose-response curve was more similar to that of the replication-defective Add*l*312.

We next determined whether the selective cytotoxicity seen with the E2F vector was dependent on the *E2F-1* promoter. On both VA-13 and WI-38 cells, the dose response curves and the LD_{50} values for the promoterless vector were similar to Add*l*312 (Fig. 4; Table 2). However, the E2F vector LD_{50} on VA-13, but importantly not WI-38, was significantly lower compared with the promoterless vector (Table 2). These data suggest that the *E2F-1* promoter is critical for the selective killing of VA-13 cells by the E2F vector.

We next asked whether the Rb pathway dependence of the oncolytic activity of the E2F vector was dependent on the two E2F binding sites in the *E2F-1* promoter. It has been shown previously that mutating both of the E2F sites results in constitutive expression of a reporter gene driven by the *E2F-1* promoter (38). On both VA-13 and WI-38 cells, the potency of the mutant E2F vector was more similar to the RSV vector than to Add*l*312 (Fig. 4). The LD_{50} values for the E2F mutant were significantly higher on VA-13 cells and significantly lower on WI-38 cells than the E2F vector (Table 2; $P < 0.05$). This suggests that inactivation of the E2F binding sites results in derepression of *E1A* gene transcription and increased cytotoxicity in normal cells like WI-38. This is consistent with the concept that pRb-E2F complexes bind to and actively repress the *E2F-1* promoter in normal cells with a functional Rb pathway.

We next explored the role of viral replication in the oncolytic activity of the E2F vector *in vitro*. There are at least two possible mechanisms for the adenoviral cytotoxicity observed in this *in vitro* cell-based assay system. First, cytotoxicity may be the result of E1A-dependent viral replication and spread. Alternatively, cytotoxicity may be because of E1A expression or other adenoviral genes transactivated by E1A directly without concomitant viral replication and spread (50, 51). To address these two possible mechanisms, LD_{50} values were compared. The LD_{50} value for the replication-defective E2F vector was significantly higher ($P < 0.05$) than the E2F vector on VA-13 cells (Fig. 4; Table 2). In contrast, the LD_{50} values for the E2F vector and for the replication-defective E2F vector on WI-38 cells were not significantly different (Fig. 4; Table 2). These data suggest that for maximum and selective cytotoxicity, replication of the oncolytic adenoviral vector is necessary.

We also measured the cell-killing activity of the E2F vector on a panel of Rb pathway-defective tumor cell lines (see "Materials and Methods" for specific Rb pathway defects) and primary cell cultures (Fig. 5). The LD_{50} dose for each vector relative to that of the wt control (Add*l*327) was determined by the formula: LD_{50} Add*l*327/LD_{50} oncolytic vector. Relative LD_{50} values of 1 indicate that cell killing is identical to that obtained with Add*l*327, whereas relative values approaching 0 indicate little or no cell killing compared with Add*l*327. In all five of the tumor cell lines (Hep3B, PANC-1, H1299, HT29, and H460), relative LD_{50} values calculated for the E2F vector ranged from 0.45 to 1.55, indicating that the E2F vector was similar to Add*l*327 in its ability to kill tumor cells (Fig. 5). In contrast, in all three of the nontumor primary cell cultures, relative LD_{50} values for the E2F vector ranged from 0.025 to 0.071, indicating relatively low cell killing by the E2F vector. These data, comparing vector-mediated killing of the WI-38 *versus* VA-13 cells, and human primary *versus*

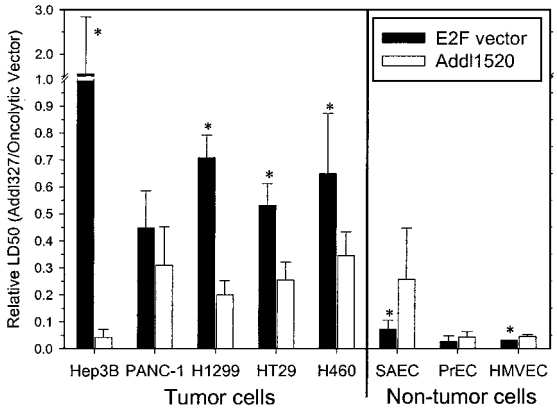


Fig. 5. Relative LD_{50} values for oncolytic vectors on tumor cell lines and normal cell cultures. The LD_{50} dose for each vector relative to that of the wt promoter control (Add*l*327) was determined by the formula: LD_{50} Add*l*327/LD_{50} oncolytic vector. Relative LD_{50} values of 1 indicate that cell killing is identical to that obtained with Add*l*327, whereas relative values approaching 0 indicate little or no cell killing compared with Add*l*327. In this way, differences in Ad infectivity and other aspects of the Ad life-cycle can be normalized across cell lines. *, $P < 0.05$ *versus* Add*l*1520 by *t* test.

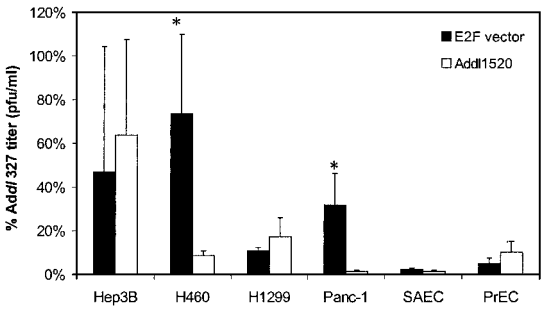


Fig. 6. Virus production by the E2F vector and Add*l*1520 in tumor cell lines and normal cell cultures. Measurements were performed by tissue-culture infectious dose-50% assay on AE1–2A cells and expressed as pfu/ml as a percentage of the Add*l*327 pfu/ml. All primary infections were performed with 1 ppc and harvested 3 days later. Values represent means of three replicates; *bars*, +SD. ∗, $P < 0.05$ *versus* Add*l*1520.

tumor cells, suggest that the relative cell killing efficiency of the E2F vector is greater on Rb pathway-defective cells than on cells with an intact Rb pathway.

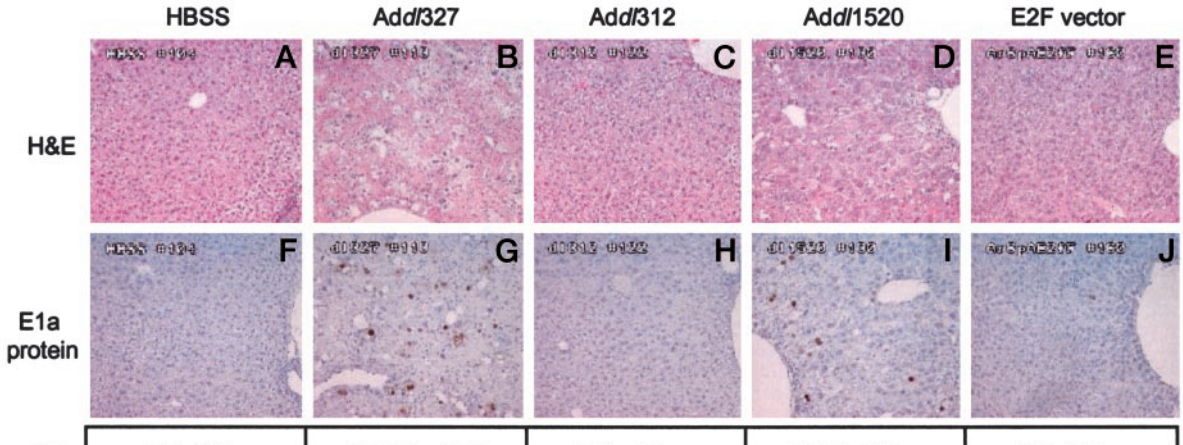
The effect of restoring the E3 region in the E2F vector was evaluated in Hep3B and H460 cells. We tested a version of the E2F vector, the E2F-E3 vector, which has a wt *E3* region except for the *14.7K* gene (Fig. 1; Table 1). We saw no difference in the dose-response between the E2f and E2F-E3 vectors at 3, 5, and 7 days after infection in the MTS assay (data not shown).

We also compared the relative killing ability of the E2F vector and Add*l*1520 on this panel of cell lines (Fig. 5). Add*l*1520 is reported to be molecularly identical to an oncolytic adenovirus currently in clinical trials for a number of cancer indications (5). The E2F vector was more effective at killing four of five Rb pathway-defective tumor cell lines than Add*l*1520. Three of these cell lines (Hep3B, H1299, and HT29) were also p53-defective (31, 33–36). In addition, the E2F vector had less cytotoxicity in two of the three nontumor cell cultures tested than Add*l*1520 and similar cytotoxicity in the third culture. These data demonstrate that the E2F vector has higher potency and selectivity compared with Add*l*1520.

Virus Production. We tested the infectious titers of the E2F vector after infection of tumor and normal cells *in vitro*. Virus production by the E2F vector was 11–74% of the levels observed for Add*l*327 in the four Rb pathway-defective tumor cell lines (Fig. 6). In contrast, virus production by the E2F vector in two primary nontumor cell cultures was much lower, at 2–5% of the levels produced by Add*l*327. Thus, production of progeny virus by the E2F vector was more similar to Add*l*327 in the Rb pathway-defective tumor cell lines than in the primary nontumor cell cultures. In two of the four tumor cell lines, H460 and Panc-1, the relative virus production by the E2F vector was significantly greater than that for Add*l*1520 ($P < 0.05$). Because Panc-1 cells are $p53^-$ and $p16^-$, these data suggest that Add*l*1520 may be more attenuated than the E2F vector, even in cells in which both p53 and Rb pathways are altered.

***In Vivo* Characterization of *E2F-1* Promoter Activity in SCID Mice.** The ability to culture normal cells *in vitro* in conditions that closely mimic quiescent cells (G_0/G_1) *in vivo* is a challenge. To better assess the control of E1A transcription in a quiescent nontumor cell, we used an *in vivo* system that relies on E1A-related hepatotoxicity after systemic administration of viral vectors in SCID mice. At a single i.v. dose of 6.25×10^{11} particles/kg, the *E1A*-deleted Add*l*312 does not result in significant hepatotoxicity, as measured by morbidity, body weight loss (data not shown), serum levels of liver enzymes, liver histopathology, and E1A expression in liver (Fig. 7). At this same dose, pronounced effects can be seen for the E1A-expressing vector Add*l*327 (Fig. 7). Thus, we used these molecular and toxicological parameters as end points for the activity of heterologous tumor-selective promoters regulating E1A expression.

For the E2F vector-treated group, there were no deaths or even any significant differences in the mean body weight with that of the Add*l*312- or HBSS-treatment groups at any time during the study (data not shown). In contrast, the Add*l*327 and Add*l*1520-treated groups lost weight after treatment (data not shown). The Add*l*327-



	HBSS	Add*l*327	Add*l*312	Add*l*1520	E2F vector
ALT	87* ±100	10230* ±1600	86* ±144	4548* ±648	914 ±540
DB	0 ±0	2.83* ±0.65	0 ±0	2.17* ±1.35	0.01 ±0.03
DNA copies	not done	441* ±93	1* ±0.3	17 ±17	29 ±15
E1A mRNA	not done	2370* ±1438	not done	2787 ±1932	69 ±18

Fig. 7. Molecular, histological, and toxicological effects in SCID mice treated i.v. with adenoviral vectors. Mice were treated on study day 1 with HBSS alone or 6.25×10^{11} particles/kg of the indicated vectors. Livers were collected on study day 4 and stained with H&E (*A–E*) or immunostained for E1A (*F* and *G*). Also shown are study day 4 serum levels of ALT, DB, viral DNA copies per cell, and E1A transcript levels per Ad genome in the liver. ∗, $P < 0.05$ *versus* the E2F vector by *t* test.

treated animals had to be sacrificed at study day 4 because of their moribund condition. Add*l*1520-treated animals regained their body weight and were indistinguishable from HBSS-treated control animals by study day 15. Serum levels of ALT, an indicator of hepatocellular injury, in the E2F vector treatment group were significantly lower than those of the positive control, Add*l*327 group, and the Add*l*1520 group on study day 4 ($P < 0.05$; Fig. 7). Similar results were seen with AST (data not shown). Serum levels of DB, a measure of biliary injury, in the E2F vector treatment group were also significantly lower than in the positive control Add*l*327 group and the Add*l*1520 group on study day 4 ($P < 0.05$; Fig. 7). With both the E2F vector and Add*l*1520 treatments, the serum levels of AST, ALT, and DB were lower on study day 15 (data not shown), although still above levels in the vehicle-treated group.

The effect of restoring E3 to the E2F vector was evaluated both in SCID mice and in the immunocompetent mouse strain, C57BL/6. In SCID mice after a single i.v. injection of 6.25×10^{11} vp/kg, no difference was observed in the study day 4 or 15 serum ALT, AST, or DB levels between mice treated with the E2F vector or the E2F-E3 vector, although at a higher dose of 2.5×10^{12} vp/kg there was some increase in serum levels of AST and ALT with the E2F-E3 vector. No differences were observed in the serum chemistries of C57BL/6 mice treated with a single i.v. dose of 5×10^{12} vp/kg of the E2F and E2F-E3 vectors (data not shown).

Histopathological analysis of the livers was also performed. The E2F vector treatment group (Fig. 7*E*) showed less severe histopathology in the liver than the Add*l*327 (Fig. 7*B*) and Add*l*1520 (Fig. 7*D*) groups. With both the E2F vector and Add*l*1520 treatment, the morphological changes that were observed were less severe at the study day 15 necropsy than the study day 4 necropsy (data not shown).

We next correlated the attenuated hepatotoxicity of the E2F vector with viral DNA copy number and E1A expression at the mRNA and protein levels in the liver. The quantity of viral DNA present per liver cell on study day 4 was quantitated by real-time PCR designed to detect adenoviral hexon gene DNA. Although equivalent doses of each virus were administered, the E2F vector and Add*l*312 groups had significantly lower viral copy numbers per cell than the Add*l*327 group by study day 4 (Fig. 7). These data suggest either differences in viral persistence or differences in viral DNA replication. There is evidence that murine cells can support adenoviral DNA replication (52).

Interestingly, despite the difference in the toxicological parameters, levels of viral DNA per cell for Add*l*1520-treated mice were indistinguishable from the E2F vector treatment group. Therefore, we analyzed E1A mRNA levels in the liver by quantitative RT-PCR. Mice treated with the E2F vector showed significantly lower levels of E1A mRNA levels per Ad genome than both the Add*l*327 and Add*l*1520 treatment groups on study day 4 (Fig. 7). This was also observed at the protein level. E1A immunoreactivity in the livers of mice treated with the E2F vector was lower than in the Add*l*327- and Add*l*1520-treated mice (Fig. 7*J*). The E2F vector group had no E1A immunoreactivity in the cytoplasm and minimal nuclear E1A immunoreactivity (5 of 5 mice). In contrast, Add*l*327-treated mice showed minimal to slight E1A immunoreactivity in the cytoplasm (9 of 10 mice) and minimal to moderate immunoreactivity in the nucleus (10 of 10 mice). Add*l*1520-treated mice showed minimal cytoplasmic staining (2 of 5 mice) and minimal to moderate nuclear E1A immunoreactivity (5 of 5 mice). These data support the concept that E1A itself may elicit toxicity and suggest that the *E2F-1* promoter in the E2F vector was minimally active in mouse liver at the doses evaluated.

Antitumor Efficacy *in Vivo*. A Hep3B s.c. xenograft model of hepatocellular carcinoma was used to assess antitumor efficacy after intratumoral injection of the E2F vector (Fig. 8*A*) and the E2F-E3 vector (Fig. 8*B*). Administration of 2×10^9 vp/dose was performed intratumorally on 5 consecutive days (study days 1–5). The E2F vector and the E2F-E3 vector both significantly inhibited tumor growth relative to the vehicle control and Add*l*312 by study day 8 ($P < 0.001$). There was no difference in efficacy between the E2F and E2F-E3 vectors at the doses used in this model. This was consistent with the lack of difference in Hep3B cytotoxicity in the MTS assay *in vitro* between the E2F and E2F-E3 vectors (data not shown).

We also evaluated the mechanism of the antitumor activity of the E2F vector in this Hep3B xenograft model. By study day 8, the promoterless vector was significantly less efficacious than the E2F vector ($P < 0.01$; Fig. 8*A*). This result illustrates the importance of the *E2F-1* promoter in the antitumor activities of these oncolytic vectors. We also evaluated the role of viral replication. By study day 12, the replication-defective E2F vector was significantly less efficacious than the E2F vector ($P < 0.05$; Fig. 8*A*). This result highlights the benefit of viral replication in sustaining the oncolytic activities of the E2F vector and the E2F-E3 vector. These data suggest that expressing *E1A* and other early genes by themselves is not sufficient to generate a prolonged antitumor response, and that viral replication is required for maximal antitumor efficacy.

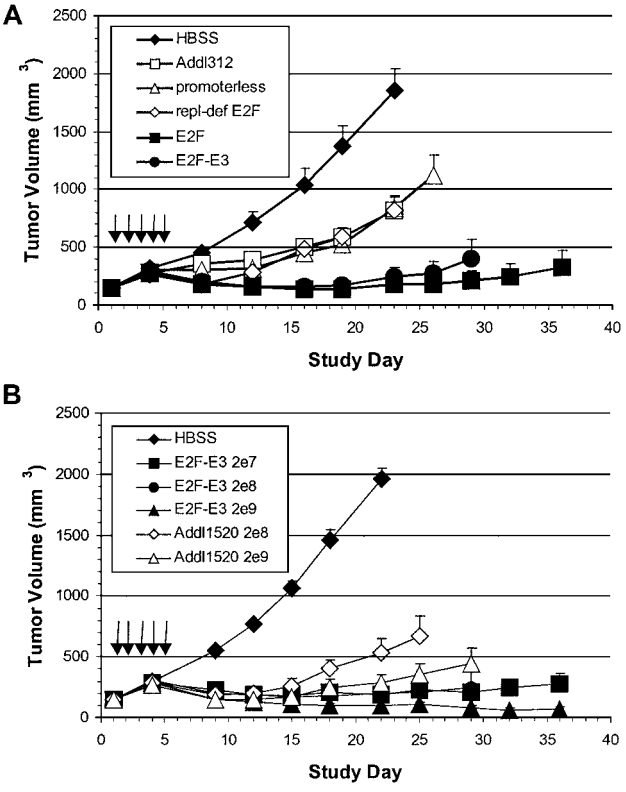


Fig. 8. Antitumor efficacy in a Hep3B xenograft model. Tumors were established by injecting Hep3B cells s.c. into the right flank of nude mice. Groups were treated with five consecutive daily (study days 1–5) intratumoral injections (indicated by *arrows*). Data are expressed as mean tumor volume over time; *bars*, ±SE. Data from a group is displayed until any mice within the group required sacrifice. *A*, comparative antitumor efficacy of indicated vectors. Mice were treated with HBSS or 2×10^9 particles/dose/day of vector. The E2F vector and the E2F-E3 vector were significantly more efficacious than Add*l*312, the promoterless vector, the replication-defective E2F vector, and HBSS on study day 16 and thereafter ($P < 0.05$). At no point during the study was there a statistical difference between the E2F vector and the E2F-E3 vector. *B*, relative efficacy of the E2F-E3 vector and Add*l*1520 in treatment of Hep3B tumors after five consecutive daily intratumoral injections (indicated by *arrows*). The E2F-E3 vector and Add*l*15120 were administered at the indicated doses. At the 2×10^8 vp/dose, the E2F-E3 vector and Add*l*1520 were statistically different on study days 18, 22, and 25 ($P < 0.01$). Likewise, a statistical difference was demonstrated between the E2F-E3 vector and Add*l*1520 on study days 22, 25, and 29 at the 2×10^9 vp/dose ($P < 0.05$); *bars*, ±SD.

The Hep3B xenograft model was also used to assess the dose response and relative efficacy of the E2F-E3 vector and Add*l*1520 (Fig. 8*B*). Because we saw no difference in antitumor efficacy between the E2F vector and the E2F-E3 vector *in vitro* (data not shown) or *in vivo* (Fig. 8*A*), we only compared the E2F-E3 vector with Add*l*1520. Treatment was performed intratumorally on 5 consecutive days (study days 1–5). The doses of vector were 2×10^8 vp/dose and 2×10^9 vp/dose for the E2F-E3 vector and Add*l*1520. An additional dose of 2×10^7 vp/dose was also administered for the E2F-E3 vector (Fig. 8*B*). The E2F-E3 vector treatment significantly inhibited tumor growth compared with the HBSS treatment at all three of the doses by study day 9 ($P < 0.001$; Fig. 8*B*). The E2F-E3 vector also caused significantly greater tumor growth inhibition relative to Add*l*1520 at the two doses in common (2×10^8 and 2×10^9 vp/dose; $P < 0.05$). In addition, the E2F-E3 vector at a 100-fold lower dose was at least as efficacious as Add*l*1520 treatment. This indicates that the *E2F-1* promoter-driven vectors have sustained antitumor effect and that the relative antitumor efficacy of the E2F-E3 vector is higher than that of Add*l*1520 in this tumor model.

DISCUSSION

A broad range of cancers are known to have Rb pathway defects (18, 19) that result in loss of proper regulation of transcriptionally active E2F (19, 27). Because E2F up-regulates many genes involved in proliferation, the cells undergo uncontrolled growth, a hallmark of cancer. The *E2F-1* gene itself contains E2F-binding sites in its promoter, and the result is transcriptional auto-activation. Thus, the choice of the *E2F-1* promoter to regulate adenoviral E1A expression, and subsequently the viral life cycle, should enable broad application to cancer indications by this class of oncolytic viruses. We describe two oncolytic Ad vectors, the E2F vector and the E2F-E3 vector (Table 1; Fig. 1), in which the *E2F-1* promoter is used to drive E1A expression and, thus, viral replication. Other viral genome changes were made to reduce nonspecific activation of E1A expression and, therefore, replication, including moving the Ad packaging signal/enhancer region and inserting a pA signal as an insulator sequence (Fig. 1). We show that the *E2F-1* promoter controls the oncolytic activities of the E2F vector in a tumor-selective fashion, consistent with the results of Parr *et al.* (29). We demonstrate that the E2F vector has tumor-selective E1A expression and virus production *in vitro*, leading to selective tumor cell killing that was dependent on an Rb pathway defect, an intact *E2F-1* promoter, and the ability to replicate. The E2F vector was also demonstrated to have reduced hepatotoxicity after systemic administration and more potent antitumor efficacy by intratumoral treatment than that of Add*l*1520.

The first issue we addressed was whether the selectivity of the *E2F-1* promoter, which was described previously (29, 38), was retained in the context of an oncolytic adenovirus. The changes in the viral backbone that were made (described above) resulted in a reduction in background E1A expression that was because of endogenous viral sequences (data not shown); therefore, these backbone changes allowed for regulation of E1A expression and viral replication dependent only on the inserted promoter. To analyze selectivity of the E2F vector, a matched isogenic pair of cell lines was used that differ in their Rb pathway status. WI-38 cells are normal fibroblasts; VA-13 cells are WI-38 cells that have been transformed with SV40, disrupting the Rb pathway and resulting in the up-regulation of E2F-1 (49). Northern blot analysis confirmed that the expression of E2F-1 was up-regulated in VA-13 cells (Fig. 2). Using this pair of cell lines, we examined the E1A transcription pattern of the E2F vector. We demonstrated that in VA-13 cells, the E2F vector expressed higher levels of E1A RNA (Fig. 3) than in WI-38 cells (Fig. 4; Table 2). A vector with a constitutive promoter, the RSV vector, displayed high E1A mRNA levels in VA-13 and WI-38 cells, regardless of Rb pathway status. The promoterless vector, on the other hand, had dramatically reduced E1A expression in both cell cultures, similar to Add*l*312. Thus, E1A expression by the E2F vector was dependent on the *E2F-1* promoter, and recapitulated the endogenous *E2F-1* gene expression pattern (Fig. 2*A*) and the selectivity described previously (29, 38).

Having established that the transcriptional selectivity of the *E2F-1* promoter was retained in the oncolytic vector context, we addressed whether the *E2F-1* promoter control of E1A expression could mediate selective viral cytotoxicity *in vitro* and *in vivo*. We found that the cytotoxicity *in vitro* correlated with E1A expression levels. The cell killing activity of the E2F vector was selective for Rb pathway-defective cells; the cytotoxicity was significantly higher in VA-13 than in WI-38 cells (Table 2; Figs. 4 and 5). The RSV vector killed both cell lines regardless of Rb pathway status, and the promoterless vector had attenuated cytotoxicity, at levels similar to Add*l*312. Similar trends were observed in the Hep3B xenograft model *in vivo*. The E2F vector had antitumor efficacy that was significantly greater than that of the promoterless vector (Fig. 8*A*). Thus, the *E2F-1* promoter fragment was required for both E1A transcription and for cytotoxicity in cells containing an Rb pathway defect.

Because of SV40 large T antigen expression, VA-13 cells have inactivation of both the Rb and p53 pathways (48, 49), so we could not rule out that disruption of the p53 pathway had a role in the oncolytic activity of the E2F vector. Therefore, we analyzed the mutant E2F vector in which mutation of both E2F binding sites leads to dysregulation of the *E2F-1* promoter (29, 38). Our comparison of the E2F vector with the E2F mutant vector revealed that inactivation of the E2F binding sites resulted in loss of selectivity for VA-13 cells. Relative to the E2F vector, we observed an increase in killing on WI-38 cells and a slight decrease in killing on VA-13 cells by the mutant E2F vector (Fig. 4). These results are consistent with the concept that in normal cells, pRb-E2F complexes repress the *E2F-1* promoter through its E2F binding sites and that in tumor cells, other E2F family members are involved in activation of E2F-responsive genes (15). These data demonstrate a link between the selective oncolysis by the E2F vector *in vitro* and the Rb pathway.

One of the major differences in the oncolytic vector approach compared with traditional gene therapy for cancer is that these vectors replicate and produce progeny virus. This has the advantage of allowing amplification of the input dose and, presumably, the subsequent spread and destruction of the tumor. To address whether viral replication indeed contributes to oncolysis, we created a replication-defective version of the E2F vector. The *E2A* deletion in the replication-defective E2F vector completely inhibits viral replication (data not shown). Unlike replication-defective controls used in other studies, the replication-defective E2F vector still expresses E1A. Because E1A itself can have cytotoxic effects (50, 51), we wanted to test whether E1A expression alone was a determinant in the oncolytic activity of the E2F vector or whether viral replication was also important. In cells infected with the replication-defective E2F vector, E1A was still expressed in a tumor cell-selective way in Rb pathway-defective VA-13 cells (Fig. 3). Abrogating the ability to replicate caused a severe attenuation of its efficacy compared with the E2F vector both in the MTS cytotoxicity assays (Fig. 4) and in antitumor efficacy *in vivo* (Fig. 8*A*). Interestingly, the antitumor activity of the replication-defective E2F vector was slightly greater than the promoterless vector, or the replication-defective E1A-deleted Add*l*312 *in vitro* or *in vivo* on study days 8 and 12. The promoterless vector (Ar6pAF) and the *E1A*-deleted virus (Add*l*312) express little to no E1A, respectively. The low level of cytotoxicity and antitumor activity with the replication-defective E2F vector could be attributed to

E1A expression or the transactivation of other viral genes in the absence of viral replication. Nevertheless, replication is a significant factor in the oncolytic activity of the E2F vector. The LD_{50} of the replication-defective E2F vector was ~14 times higher than the E2F vector (Table 2).

For systemic delivery, one major hurdle is toxicity that may result from infection of normal cells. The *in vitro* analysis of tumor-selective vectors in normal Rb pathway-proficient cells is hampered by the difficulties in establishing cell culture conditions that closely mimic the *in vivo* milieu. For this reason, we have used an *in vivo* assay of E1A-related hepatotoxicity to evaluate the activity of tumor-selective promoters in oncolytic vectors in the context of normal liver cells in SCID mice. It is known that adenoviruses efficiently transduce the mouse liver after i.v. administration and can produce clinical signs of hepatotoxicity within 1 week (53, 54). Some of the hepatotoxic effects of adenovirus can be directly or indirectly attributed to E1A expression. We used molecular and toxicological parameters of hepatotoxicity as end points for the activity of heterologous tumor-selective promoters regulating E1A expression. We found that the *E2F-1* promoter in the E2F vector expressed less E1A than either Add*l*327 or Add*l*1520, both of which have wt *E1A* promoters. We reasoned that the low hepatotoxicity of the E2F vector-treated animals was not because of a failure of the human *E2F-1* promoter to efficiently use the mouse transcriptional machinery. The mouse and human *E2F-1* promoters share a high degree of similarity (55). In addition, the human *E2F-1* promoter is regulated in a similar manner in both human and mouse cells (38).

Add*l*1520 is an oncolytic virus reported to be molecularly identical to one currently in clinical trials (5). Its selectivity is based on the deletion of the viral *E1B-55K* gene, and this has been hypothesized to make it selective for tumors that have a defective p53 pathway. We chose to compare the antitumor activities of our oncolytic vector to Add*l*1520 *in vitro* using cytotoxicity and vector production assays on various tumor and normal cells, and *in vivo* in the Hep3B xenograft model. The relative LD_{50} values for the E2F vector were higher in 4 of 5 tumor cell lines tested and lower in 2 of 3 normal cells (Fig. 5), despite p53 status. Similar results were obtained for the vector production assay, as shown in Fig. 6. Interestingly, in Hep3B and H1299 cells, the E2F vector and Add*l*1520 had similar titers but different LD_{50} values in the MTS assay. This may be because of differences in the assays. For example, the MTS assay is read out at 7 days after infection with a broad range of doses, whereas titering is read out at 3 days after infection with a single dose. In general, the selectivity of the E2F vector as measured by oncolytic activity on tumor cells *versus* normal cells, was greater than that of Add*l*1520. And *in vivo*, the antitumor efficacy of the E2F-E3 vector was higher than that of Add*l*1520 (Fig. 8*B*).

We also compared the toxicological and molecular parameters of E1A-related hepatotoxicity in SCID mice after i.v. administration of the E2F vector or Add*l*1520. On study day 4, both of these oncolytic vectors showed lower copy number per cell in the livers of treated mice than the Add*l*327 control group (Fig. 7). These data, along with reports that murine cells can support adenoviral DNA replication (52), suggest that these differences are because of differences in viral DNA replication. However, despite equal input doses, the E2F vector produced less severe acute hepatotoxicity *in vivo* after systemic administration when compared with Add*l*1520 and Add*l*327 (Fig. 7). The mechanism of action of Add*l*1520 is based not on control of E1A expression but on the concept that E1B-55K-deleted viruses are selective for $p53^-$ cells. Therefore, because there is no control of E1A expression in normal cells, it is not surprising that we observed higher E1A expression and the greater E1A-related hepatotoxicity associated with Add*l*1520.

During preparation of this article, two reports were published describing oncolytic adenoviral vectors that were engineered to be selective for Rb pathway-defective tumors. In one, selectivity for Rb pathway-defective tumors was achieved by a CR2 mutation in E1A and by placing two early gene regions, *E1A* and *E4*, under the control of the E2F-1 promoter (24). In the other, selectivity was achieved by placing the E1A transcription unit under the control of the *E2F-1* promoter (26). Our results are consistent with these studies but add two important points. One is that the CR2 mutation may not be critical for oncolytic adenoviral vectors targeting Rb pathway defects because we and Tsukuda *et al.* (26) demonstrate selectivity with vectors containing a wt E1A coding region. Second, we explore the mechanistic basis of the oncolytic activity on Rb pathway-defective cells, and demonstrate the dependence of oncolysis on the Rb pathway defect, the importance of the E2F-1 promoter, and viral replication.

In conclusion, we have demonstrated that the E2F vector selectively kills a broad range of Rb pathway-defective tumor cells *versus* normal cells. We have shown that the mechanism of this selectivity is based on the presence of the E2F binding sites within the *E2F-1* promoter in the virus and also a disruption of the Rb pathway in the target cell. This characteristic will allow therapeutic broad application of this vector to many cancer types. We also show that the ability of the vectors to replicate is a requirement for full oncolytic activity both *in vitro* and *in vivo*. With systemic delivery, this vector is less toxic than wt and Add*l*1520, additionally indicating its selectivity. Most importantly, we have demonstrated potent antitumor efficacy *in vivo* that is greater than that of Add*l*1520.

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