 ICOVIR-5 Shows E2F1 Addiction and Potent Antiglioma Effect In vivo

Marta M. Alonso, 1 Manel Cascallo, 3 Canelaria Gomez-Manzano, 1 Hong Jiang, 1 B. Nebiyou Bekele, 2 Anna Perez-Gimenez, 2 Frederick F. Lang, 1 Yuji Piao, 1 Ramon Alemany, 3 and Juan Fueyo 1

1 Brain Tumor Center and 1 Department of Biostatistics, University of Texas M. D. Anderson Cancer Center, Houston, Texas and 3 Translational Research Laboratory, Institut Català d’Oncologia Barcelona, Barcelona, Spain

Abstract

During 2007, ~200,000 people in the United States will be diagnosed with brain tumors. Gliomas account for 77% of primary malignant brain tumors, and the prognosis has hardly changed in the past 20 years, with only 30% of patients with malignant glioma surviving 5 years after diagnosis. Oncolytic adenoviruses are promising therapies for the treatment of gliomas. Here, we report the antiglioma activity of the tumor-selective ICOVIR-5 adenovirus, which encompasses an early 1A adenoviral (E1A) deletion in the retinoblastoma (Rb) protein-binding region, substitution of the E1A promoter for E2F-responsive elements, and an RGD-4C peptide motif inserted into the adeno viral fiber to enhance adenoviral tropism. Mechanistic studies showed a dramatic addiction of ICOVIR-5 to the E2F1 oncogene in vitro and in vivo. This addiction was mediated by the occupancy of the ectopic adeno viral E2F1-responsive elements by the endogenous E2F1 protein resulting in high level of E1A expression in cancer cells and potent antiglioma effect. Importantly, we showed for the first time the ability of oncolytic adenoviruses to enhance E2F transcriptional activity in vivo, and we provided direct evidence of the interaction of the E2F1 protein with native and ectopic adenovirus promoters. Restoration of Rb function led to the association of Rb/E2F1 repressor complexes with ICOVIR-5 ectopic E2F1 promoter and subsequent down-modulation of E1A, dramatically impairing adenoviral replication. In xenografted mice, intratumoral injection of ICOVIR-5 resulted in a significant improvement of the median survival (P < 0.0001), and furthermore, led to 37% of long-term survivors free of disease. The antiglioma activity of ICOVIR-5 suggests that it has the potential to be an effective agent in the treatment of gliomas. [Cancer Res 2007;67(17):8255–63]

Introduction

Glioblastoma multiforme, the most common primary brain tumor, is clinically and pathologically malignant. Current treatment strategies involve surgery, chemotherapy, and radiotherapy, alone and in combination, but the prognosis for patients with these tumors has not changed dramatically in the past 20 years (1). Targeted therapies directed against the fundamental genetic abnormalities of cancer offer the promise of more rational and effective treatments. However, with the exception of a few agents being studied in early-stage clinical trials (2), current antiglioma therapies do not target specific genetic abnormalities in cancer cells. One major target is the genetic alterations affecting the p16INK4a, cyclin-dependent kinase 4, and cyclin D1 proteins that govern the phosphorylation of the retinoblastoma (Rb) protein leading to E2F activation (3). Indeed, the resultant inactivation of this pathway and consequential excess of “free” E2F may well be a condition for glioblastoma development (4).

Oncolytic adenoviruses are promising therapies for the treatment of gliomas. However, untargeted viral replication and the paucity of coxsackie-adenovirus receptors (CAR) on tumor cells are the major hurdles for adenovirus-based treatment. Currently, there are three main strategies used to improve the potency and selectivity of oncolytic adenoviruses. The first involves the modification of the interaction between adenoviral proteins and tumor suppressor genes or oncogenes (5). Another strategy is based on the modification of the adenoviral tropism to render cancer cells susceptible to adenoviral infection. The third strategy involves the insertion of ectopic regulatory elements to restrict the expression of early adenoviral genes to cancer cells. On the basis of studies of the transforming regions of the early 1A adenoviral (E1A) protein (6, 7), oncolytic adenoviruses targeting the Rb pathway were developed (8, 9). Because cancer cells are characterized by a paucity of the CAR (10), E1A-mutant adenoviruses were modified so that they could infect tumor cells via CAR-independent mechanisms. In the best-examined strategy, increased infectivity was achieved through the addition of the RGD-4C motif within the viral fiber knob of the adenoviral construct (11, 12). Although RGD-modified E1A-mutant adenoviruses showed enhanced infectivity and an augmented anticancer effect (12), the excessive levels of E1A in normal cells would risk E1A-mediated toxicity (13, 14). Among the strategies that could be used to control the level of expression of E1A in normal cells is the insertion of cell cycle–dependent promoters, such as E2F1, as the main regulators of E1A expression (15–17). We hypothesized that an oncolytic adenovirus harboring all three modifications will display an improved selectivity while retaining a robust and effective antiglioma effect. However, currently, there are no reports of the preclinical characterization of oncolytic adenoviruses that incorporate all three modifications.

In the present study, we analyzed the antiglioma effect of a new retargeted oncolytic adenovirus (ICOVIR-5) in which both the level and function of E1A are controlled at the transduction, transcription, and protein/protein interaction levels. ICOVIR-5 infection induced the up-regulation of E2F1 transcriptional activity and occupation of the ectopic E2F1-responsive elements by the endogenous E2F1 protein in vitro. In stark contrast, adenoviral activation was suppressed by Rb in normal cells. Intratumoral
administration of ICOVIR-5 resulted in a significant increase in the median survival of tumor-bearing animals ($P < 0.001$) and prolonged long-term survival.

**Materials and Methods**

**Cell lines and culture conditions.** The glioma cell lines U251 MG and U87 MG were obtained from the American Type Culture Collection. Cell lines were maintained in DMEM/F12 (1:1, v/v) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO$_2$ at 37°C. Normal human astrocytes (NHA) were purchased from Clonetics/BioWhittaker and maintained according to the manufacturer's instructions.

**Adenovirus construction and infection.** Construction of wild-type (WT) adenovirus (Ad300; ref. 18), WT-RGD (19), Δ-24 (8), and Δ-24-RGD (11, 12) has previously been described. Adenoviral constructs inactivated containing the E2F promoter and E1A was subcloned into pGEM-3Z (Promega). Finally, pShuttle-DM-E2F-D24 was recombined by homologous fragment containing the E2F-E1A modified with the Kozak sequence and was used to plasmid the E1A translation start site using oligonucleotides with the Kozak sequence. The KpnI fragment containing the E2F-E1A modified with the Kozak sequence was returned to pShuttle-DM-E2F-D24 to obtain pShuttle-DM-E2F-KD24. Finally, pShuttle-DM-E2F-D24 was recombined by homologous recombination with pVS503 that contains complete Ad5 genome with RGD-modified fiber.

** Luciferase assays.** Cells (3 × 10$^4$ per well in 24-well dishes) were transfected with 250 ng of the E2F1 reporter construct (21) using FuGENE 6 transfection reagent (Roche Diagnostics Corp.). One hour after transfection, cells were mock infected or treated with the indicated viral constructs or Ad-β-galactosidase (Ad-β-Gal; an adenovirus that carries the cDNA of the β-galactosidase used as a specificity control). Cells were harvested 24 h after infection, and the reporter activity was measured using the Dual Luciferase assay (Promega). Transfections were normalized for efficiency using pRL-CMV (Promega) and expressed as folds of induction relative to that of mock-treated cells (assigned an arbitrary value of 1).

**Chromatin immunoprecipitation assays.** The chromatin immunoprecipitation assay was done by use of the chromatin immunoprecipitation assay kit (Upstate Biotechnology) by following the manufacturer's instructions. E2F1 (KH-95), pRB (BD Biosciences), or mouse immunoglobulin G (IgG) antibodies (Santa Cruz Biotechnology) were used to immunoprecipitate the cross-linked chromatin. The following primers were used in the PCR (33 cycles) to amplify a 272-bp fragment in the E2F1 promoter and the adjacent viral genome: 5'-TGTCTGTCACCCCACTTACGAC-3' and 5'-GCCGCTTTATGCTTACAACTTT-3'. E2 primers were designed to amplify a 52-bp fragment in the E2 promoter containing two binding sites for E2F1: 5'-TCGCCACAAAAACGCGGAAATTTAAA-3' and 5'-TTAAACCTTTTCCCCCGCATTTGCACT-3'.

**Cell cycle analysis.** The cell cycle phase distribution was analyzed by measuring the DNA content, as previously described (22).

**Infection with exogenous wild-type Rb or p21.** The AdCMV-pA (empty adenovirus), Rb, or p21 adenoviruses used in this study and their infectivity have previously been described (22, 23). Cell viability was monitored daily and was quantified using the trypan blue exclusion test.

**Cell viability assay.** Cristal violet and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiments were done to quantify cell viability, as previously described (12).

**Viral replication assays.** We seeded human glioma and NHA cells at 10$^4$ per well in six-well plates, which, 20 h later, were infected with the indicated adenovirus at a multiplicity of infection (MOI) of 1. Three days after infection, we scraped the cells into culture medium and lysed them with three cycles of freezing and thawing. We used the tissue culture infection dose replication assay (TCID$_{50}$ method) to determine the final viral titration. Briefly, the cell lysates were clarified by centrifugation and the supernatants were serially diluted in medium for infecting A549 cells in 96-well plates. We analyzed the cells for cytopathic effect 10 days after infection. Final titers were determined as plaque-forming units (pfu) using the validation method developed by Quantum Biotechnology.

**Immunoblotting assay.** Samples containing identical amounts of protein (20 μg) were subjected to SDS-Tris-glycine gel electrophoresis. Membranes were incubated with the following antibodies: E2F1, E1A (Santa Cruz Biotechnology), fiber (NeoMarkers, Lab Vision Corp.), and α-tubulin (Sigma). The membranes were developed according to the enhanced chemiluminescence protocol (Amersham Bioscience).

**TaqMan analysis.** Quantitative PCR analysis was done on a Chromo 4 sequence detection system (Bio-Rad) as described elsewhere (15). For the detection of E2F1 and E1A mRNA transcripts, we used primers cited elsewhere (15, 24). The cycling conditions for PCR were as follows: 10 min at 95°C for 1 cycle, 15 s at 95°C, and 1 min at 60°C for 40 cycles. To determine relative gene expression, the comparative threshold cycle (Ct) method was used (24).

**Bioluminescence imaging.** Cells were transfected with 250 ng of E2F1 reporter plasmid (21) using FuGENE 6 transfection reagent (Roche). Where indicated, cells were cotreated with AdpRb. Cells were harvested 48 h after treatment and implanted in the brains of athymic mice. We anesthetized the mice with isoflurane 48 h later and imaged for E2F-Luc–induced luciferase expression after the i.p. injection of D-luciferin (4 mg/g of body weight) using the IVIS imaging system (Xenogen). Acquisition parameters were as follows: exposure time, 5 min; binning, 4; no filter; f/stop, 1; field of view, 10 cm.

**Animal studies.** Nude (nu/nu) mice were obtained from the breeding facility at the University of Texas M.D. Anderson Cancer Center. U87 MG human glioma cells (5 × 10$^6$) were engrafted into the caudate nucleus of athymic mice using a previously described guide-needle system (25). On days 3, 5, and 7 after the implantation of tumor cells, animals were treated with 5-μl intratumoral injections of the indicated adenovirus (all 3 × 10$^9$ pfu/ mouse). All animal studies were done in the veterinary facilities of The University of Texas M.D. Anderson Cancer Center in accordance with institutional, state, and federal laws and ethical guidelines for experimental animal care.

**Immunohistochemical analysis.** Tumor xenografts and paraffin-embedded sections of the mouse tumors were treated with either goat anti-hexon antibody (Chemicon) or goat anti-E1A (Santa Cruz Biotechnology; ref. 26). For immunohistochemical staining, Vectastain ABC kits (Vector Laboratories) were used according to the manufacturer's instructions.

**Statistical analysis.** For the in vitro experiments, statistical analyses were done with the two-tailed Student t test. Data are expressed as mean ± SD or 95% confidence intervals (95% CI). The in vivo cytotoxic effect of ICOVIR-5 on human glioma xenografts was assessed by plotting survival curves according to the Kaplan-Meier method. Survival in different treatment groups was compared using the log-rank test. Pairwise comparisons of the difference in the proportion of surviving mice were done with the Fisher exact test.

**Results**

**E1A expression is mediated by E2F in ICOVIR-5–infected cells.** ICOVIR-5 is a third-generation retargeted oncolytic adenovirus in which both the level and function of a mutant E1A are controlled at the transcription (integrin-mediated adenoviral infection), transcription (E2F1 regulated), and protein/protein interaction (unable to bind Rb) levels. The structure of a similar construct (ICOVIR-2) has previously been described (20). We hypothesized that ICOVIR-5 will interact with free E2F1 in cancer cells and with Rb/E2F1 repressor complexes in normal quiescent cells. To assess the transcriptional activity of E2F1 in cancer and normal cells, we transfected U87 MG cells, U251 MG cells, or arrested NHAs with an E2F-Luc reporter construct (21). E2F1-mediated luciferase activity was 12- and 14-fold higher in glioma cells, respectively, than in arrested NHAs, in which the E2F1 activity was hardly detectable ($P < 0.001$; data not shown). Because adenoviral infection results in increased E2F1 activity, we next examined the
responsive of the E2F1 promoter to adenovirus infection. Glioma cells infected with any of the three adenoviruses showed a significant (10-fold) increase in luciferase activity in comparison with mock-infected cells (P < 0.001). Similar results (9.5- and 6.8-fold increases in cells infected with WT-RGD and Δ-24-RGD adenovirus, respectively) were obtained in growth-arrested NHAs (Fig. 1A). However, ICOVIR-5 infection in NHAs transfected with E2F-Luc did not induce a significant increase in the activity of the E2F promoter (1.4-fold increase in comparison with adenovirus control; P > 0.05).

Twenty-four hours after infection with ICOVIR-5, chromatin immunoprecipitation assays revealed the physical interaction of the E2F1 protein and the ectopic E2F1-responsive promoter encompassed in the adenoviral genome in glioma cells (Fig. 1B). In addition, immunoprecipitation with the Rb protein did not reveal the association of Rb/E2F1 complexes with ICOVIR-5, strongly suggesting that ICOVIR-5 promoters are occupied by free E2F1 protein in cancer cells. The opposite was seen in normal cells. Thus, chromatin immunoprecipitation assays showed that the Rb protein was recruited to the recombinant E2F-responsive elements of ICOVIR-5 in serum-starved NHAs, suggesting the association of Rb/E2F1 repressor complexes with the ectopic E2F1-responsive elements in the adenovirus.

Cell cycle profiles of ICOVIR-5–infected human glioma and normal cells. Our model predicted that ICOVIR-5 replication depends on the interaction of ICOVIR-5 with free E2F1. Because the free transcriptional activity of E2F1 is observed during the transition from the late G1 to the S phase (27, 28), we analyzed the cell cycle profile of ICOVIR-5–infected glioma and normal cells. Infection with WT-RGD, Δ-24-RGD, and ICOVIR-5 showed a similar proportion of cells in the S phase (57%, 43%, and 35%, respectively, in U87 MG cells; 50%, 52%, and 43%, respectively, in U251 MG cells). Beyond 24 h, the cell cycle profile was so disrupted that the S-phase population could not be accurately quantified. In contrast, the percentage of ICOVIR-5–infected NHAs in the S phase was significantly lower (8%; P < 0.001) than the percentage of WT-RGD–infected cells (36%) or Δ-24-RGD–infected cells (15%; Fig. 1C). These data suggested that ICOVIR-5 infection renders cell cycle profiles potentially favorable to viral replication in cancer but not in normal cells. To test this hypothesis, we examined whether the transcriptional activity of E2F1, the levels of E2F1 mRNA, and the percentage of cells in the S phase were associated with differential expression levels of E1A in ICOVIR-5–infected cancer cells and arrested NHAs (Fig. 1C and D). Whereas E2F1 mRNA and E1A protein were highly expressed in normal cells and
cancer cells infected with control adenoviruses, their expression was hardly detectable in ICOVIR-5–infected NHAs. Therefore, normal cells seem to be resistant to ICOVIR-5 by maintaining tight control of cell cycle progression, thereby preventing E2F1-mediated activation of E1A transcription.

ICOVIR-5 displays a robust replication phenotype and antiglioma effect in vitro. Qualitative (crystal violet) and quantitative (MTT) dose-dependent assays showed that ICOVIR-5 infection resulted in a cytopathic effect on U87 MG and U251 MG glioma cells. In particular, crystal violet staining showed that ICOVIR-5 infection resulted in noticeable cell death in both cell lines at an MOI of 1 (Fig. 2A). MTT assays showed that the LC50 of ICOVIR-5 in both glioma cell lines ranged between MOIs of 1 and 5. Importantly, higher doses (5–10 MOIs) were required in cells infected with Δ-24 (Fig. 2B), suggesting that the fiber modification enhances ICOVIR-5 infectivity and, therefore, the capability of the vector to replicate in and kill cancer cells in comparison with adenovirus expressing wild-type fiber.

To ascertain whether the cytopathic effect was due to effective replication, we carried out TCID50 assays in U87 MG and U251 MG glioma cells. ICOVIR-5 replicated efficiently in both cell lines (8.3 × 10^7 and 9.1 × 10^8 pfu/mL, respectively), producing slightly inferior viral titers to those of Δ-24-RGD (8.7 × 10^8 and 8.9 × 10^9 pfu/mL, respectively; Fig. 2C). Importantly, ICOVIR-5 not only showed levels of replication similar to those of the adenovirus control in CAR-positive cells (U251 MG) but also displayed a more efficient replication phenotype than the wild-type adenovirus in glioma cells expressing low levels of CAR.

**Figure 2. Antiglioma effect in vitro.** A and B, examination of the cytopathic effect. Cells were plated and, 24 h later, were infected with oncolytic adenoviruses at doses ranging from 0.1 to 10 MOIs. The dose-dependent cytopathic effect was assessed by crystal violet (A) or MTT (B) assays. Representative crystal violet assay result. MTT analyses were done thrice in triplicate wells. UV-inactivated adenovirus and simulated adenovirus infections were used as negative controls. C, analysis of the replication phenotype. Glioma cell lines were plated and infected with oncolytic adenoviruses at 1 MOI and cell lysates were used to infect A549 cells 72 h postinfection. Viral titers were determined by the TCID50 method and expressed as plaque-forming units. Three independent assays were done for each glioma cell line. D, expression of early and late adenoviral genes. For the immunoblot analysis of E1A and fiber expression, cells were plated and, 24 h later, infected with the different adenoviruses. Then, cells were harvested 36 h after the infection and protein and mRNA were extracted. Representative result from immunoblot analyses for E1A and fiber proteins. α-Tubulin is shown as the loading control.
The results of the replication assays were consistent with the levels of expression of early and late adenoviral genes, as assessed by Western blot analyses of E1A and fiber proteins (Fig. 2D).

**Restoration of the Rb pathway abrogates ICOVIR-5 anticancer effect.** We previously showed that the restoration of Rb function in glioma cells substantially decreased the cytopathic effects of D-24 and D-24-RGD adenoviruses (8, 12). To determine whether ICOVIR-5-mediated cell death also depends on the cell cycle regulatory function of the Rb protein, we treated glioma cells with replication-deficient adenoviral vectors expressing either Rb or p21 (or Ad5CMV-pA) and infected the cells with ICOVIR-5 or D-24-RGD. U251 MG and U87 MG glioma cell cultures pretreated with Rb followed by ICOVIR-5 infection showed less cell death (5 ± 2.3% and 8 ± 3.4%, respectively) than did cultures infected with Ad5CMV-pA (91.4 ± 1.9% and 90.2 ± 2.3%; P < 0.001; Fig. 3A). We next examined the ability of the cyclin-dependent kinase inhibitor p21, a regulator of Rb function, to reduce the effect of ICOVIR-5 on the viability of wild-type Rb cells. p21 pretreatment almost completely protected cells from the effect of ICOVIR-5, as reflected by 90.1 ± 2.1% and 87.4 ± 3.8% increases in the viability of U87 MG and U251 MG cells, respectively (P < 0.001; Fig. 3A).

We carried out TCID50 assays to correlate cell death with viral replication, which showed a dramatic decrease in the replication capability of ICOVIR-5 in cells overexpressing Rb or p21 (Fig. 3B). Interestingly, the rescue of glioma cells from the cytopathic effect of D-24-RGD in response to the exogenous expression of Rb and p21 was significantly lower than the rescue of cells from the cytopathic effect of ICOVIR-5 (P < 0.01). Accordingly, the ICOVIR-5 titers were lower than the D-24-RGD titers in these cells, suggesting that ICOVIR-5 is more sensitive to the status of the Rb pathway than adenoviruses encompassing exclusively CR2 mutations in E1A but not the E2F-mediated regulation of E1A (15).

**Recruitment of Rb/E2F1 repressor complexes to the E2F-ectopic promoter of ICOVIR results in the down-modulation of E1A expression.** To ascertain whether the activation of the Rb pathway results in low levels of E1A in cells infected with ICOVIR-5, we evaluated the expression levels of E1A in ICOVIR-5–infected cells. In cultures pretreated with Rb, we detected very low levels of E1A mRNA transcripts (1.5 ± 1-fold and 2.1 ± 1.2-fold in U87 MG and U251 MG cells, respectively) than in cultures infected with Ad5CMV-pA. Similar results were seen in cultured cells pretreated with p21 (2.5 ± 1-fold and 3.4 ± 1.2-fold in U87 MG and U251 MG cells, respectively; Fig. 3C).
We next determined whether the Rb-mediated restriction of E1A expression was dependent on the association of newly formed Rb/E2F repressor complexes with ICOVIR-5 promoters. For this study, U87 MG and U251 MG glioma cells were infected with the AdpRb or the Ad5CMV-pA adenovirus at an MOI of 100 and were infected 72 h later with ICOVIR-5 at an MOI of 10. We then assessed whether ectopic Rb might associate with E2F1-responsive promoters. This showed that the E2F promoter sequences encompassed in the genome of ICOVIR-5 were precipitated with a Rb-specific antibody, but this did not occur in the absence of Rb pretreatment (Fig. 3D). These results support the model whereby Rb expression directly counteracts the E2F1-mediated augmentation of gene transcription in the context of ICOVIR-5 infection.

We next examined whether the higher dependence of ICOVIR-5 than that of Δ-24-RGD on an E2F1 free status could also result in an increase in the therapeutic index of Δ-24-RGD in tumor over normal tissue. In this experiment, 3 days after serum starvation, we infected NHAs with ICOVIR-5 at MOIs of 0.1 to 10 and then cytotoxicity was evaluated 7 days later (Fig. 4A). This showed that ICOVIR-5 elicited a 20 ± 5.2% decrease in cell viability at an MOI of 10, whereas Δ-24-RGD elicited a 50% decrease in cell viability at an MOI of only 5 (Fig. 4A).

To determine the therapeutic index (i.e., viral replication in tumor cells/viral replication in normal cells), we compared adenovirus replication in serum-starved glioma cells and NHAs. As expected, Δ-24-RGD displayed an attenuated replication phenotype in NHAs, but more importantly, ICOVIR-5 was unable to replicate in this cell line. In contrast, the Ad300 and WT-RGD adenoviruses replicated efficiently in both gliomas and NHAs (Figs. 4B and 2C). In addition, the expression of E1A mRNA and protein was reduced (2 ± 0.3-fold) in arrested NHAs treated with ICOVIR-5 in comparison with NHAs treated with Ad300 (14.4 ± 3.3-fold), WT-RGD (17.5 ± 5-fold), Δ-24 (5 ± 1.4-fold), or Δ-24-RGD (8.5 ± 2.9-fold; Fig. 4C). Confirming the severely impaired replication capability of ICOVIR-5, fiber expression was absent in ICOVIR-5–infected samples (Fig. 4D).

**ICOVIR-5 infection enhances the E2F1 activity in vivo.** First, we aimed to detect the transcriptional activity of E2F1 within a glioma xenograft in vivo. For this purpose, we implanted U87 MG cells transfected with the E2F-Luc construct in the brains of nude mice and then measured E2F1 activity with the use of bioluminescence imaging. We detected E2F1-mediated luciferase expression in vivo in tumors (1.6 × 10^5 light units; Fig. 5A). Importantly, the intratumoral injection of ICOVIR-5 produced strong luciferase expression (Fig. 5A) throughout the tumor but no signal was detected outside the brain. In addition, the pretreatment of U87-E2F-Luc cells with an empty adenoviral vector followed by ICOVIR-5 injection resulted in luciferase levels in the tumors that were not significantly different from levels in tumors treated with ICOVIR-5 (data not shown). In contrast, the pretreatment of U87-E2F-Luc cells with Rb protein resulted in less luciferase expression.
Remarkably, the Rb protein efficiently repressed the ICOVIR-5–mediated increase in E2F1 activity, such that luciferase expression remained below the basal level observed in untreated U87-E2F-Luc cells (Fig. 5A). In experiments done in parallel, we assessed whether there was a prolonged interaction of the free cellular E2F1 with the recombinant E2F-responsive elements of ICOVIR-5 in vivo. For this purpose, U87 MG xenografts were treated with ICOVIR-5 intratumorally 3 days after tumor cell implantation. Consistent with our previous findings, we observed that the ICOVIR-5 E2F1 promoter was occupied by the cellular E2F1 in U87 MG glioma xenograft (Fig. 5B). Interestingly, under these conditions, chromatin immunoprecipitation analysis revealed similar robust binding of E2F1 to the E2 promoter in glioma cells infected with ICOVIR-5 and other replication-competent adenoviruses (Fig. 5B). Collectively, these data are the first to show the in vivo ability of oncolytic adenoviruses to enhance E2F transcriptional activity and to provide direct evidence of the interaction of the E2F1 protein with native and ectopic adenovirus promoters in vivo in an experimental cancer model.

**ICOVIR-5 proves a potent antiglioma efficacy in vivo.** Mice bearing U87 MG intracranial xenografts were given intratumorally PBS, ICOVIR-5, or adenoviral controls (3 × 10⁸ pfu/mouse). The median survival for mice receiving PBS was 31.5 days (95% CI, 31–33 days). In contrast, the median survival time in mice receiving ICOVIR-5 was 46.5 days (95% CI, 37 days to NA).
Moreover, whereas all the mice treated with PBS died by day 37, 37% of mice treated with ICOVIR-5 survived more than 90 days (Fig. 6A). Examination of the brains of asymptomatic long-term survivors showed complete tumor regression in all animals but one in which a small tumor remained (data not shown).

Examination of the brains of ICOVIR-5–treated mice that died between 35 and 50 days after treatment showed that their deaths resulted from the mass effect of large tumors. Microscopic examination of the brains of animals with tumors, however, revealed the presence of prominent viral inclusions, which suggested efficient infection and replication (Fig. 6B). Accordingly, the inclusion bodies showed immunohistochemical staining for E1A and hexon proteins. Intratumoral expression of E1A was also confirmed by quantitative reverse transcription-PCR (RT-PCR; Fig. 6C). Immunohistochemical analyses of the brains of the long-term survivors using both anti-E1A and anti-hexon antibodies did not reveal viral particles.

**Discussion**

Deregulation of E2F occurs in virtually all human cancers as a downstream result of mutations in several components of the Rb pathway (29). In this work, we showed that an adenovirus engineered to exploit aberrant E2F in cancer cells and tight regulation of E2F in normal cells allowed for enhanced selectivity while exerting a potent antiglioma effect *in vitro* and *in vivo*.

It has been reported that E2F-responsive promoters are active in glioma cells because of an excess of free E2F as a result of loss of pRb/E2F repressor complexes (30–32). Interestingly, the E2F1 promoter encompasses E2F binding sites (21, 32). The ICOVIR model is based on the potential of E2F1 promoter for autoregulation by E2F1 protein. Importantly, our group has previously shown that E2F1 is overexpressed in malignant gliomas and that E2F overexpression is strongly associated with a poor prognosis in patients with high-grade gliomas (33). In light of these results, the correlation between E2F1 free activity and ICOVIR-5–mediated oncolytic effect is significant. Our results proved that in the context of cancer cells, the viral E2F-responsive promoter was occupied by free E2F (Figs. 1B and 5B), leading to increased E1A expression and cell cycle progression, thus generating an auto-feedback loop that reinforced E1A activity and, subsequently, viral replication. Accordingly, arrested NHA astrocytes did not display E2F activity, and further infection with ICOVIR-5 did not result in induction of S phase and led to attenuated E1A expression and an impaired replication phenotype (Figs. 1A–D and 4B). Analyses of the cytopathic effect of ICOVIR-5 in normal and cancer cells showed an exceedingly broad therapeutic index in comparison with Δ24 and Δ24-RGD. In addition, restoration of the Rb pathway in cancer cells resulted in the formation of repressor Rb/E2F complexes with the adenoviral E2F-responsive elements leading to a drastic abrogation of cell death (Fig. 3A). Moreover, restoration of the Rb pathway in glioma cells treated with Δ24-RGD rescued viability in less than 50% of infected cells. Interestingly, we were able to show the E2F1 transcriptional activity and the *in vivo* binding of the tumoral E2F1 to the E2F-responsive elements in ICOVIR-5 infected tumors. To our knowledge, this is the first time that the *in vivo* ability of the virus to enhance E2F transcriptional activity has been shown.

The modification of the fiber HI loop of adenoviruses by the insertion of the RGD-4C motif (34) greatly enhances the adenovirus tropism and infectivity and thus oncolytic potency (11, 12). This seems to be true also in the context of ICOVIR-5. When we compared the potency of ICOVIR-5 with the adenovirus Δ24, ICOVIR-5 exerted a more robust anticancer effect than Δ24 (Figs. 2A–B and 6A). However, one could argue that this
enhancement could come from the excess of E2F activity in glioma cells. Nevertheless, the fact that Δ24-RGD is even more potent than ICOVIR-5 seems to indicate that the E2F-responsive elements provide more selectivity than potency. Interestingly, whereas normal astrocytes are consistently negative for αv integrins, neoplastic astrocytes in vivo and in vitro showed increased expression of RGD-related integrins (35, 36), indicating that integrin retargeting could result in high levels of transduction within the tumor but not in the normal parenchyma. In fact, our data support this hypothesis because we were unable to detect any sign of viral particles in normal brain tissue. Nevertheless, the ultimate test of selectivity can only be obtained in a human clinical trial due to the nonpermissive nature of the mouse model to adenoviral replication.

To date, there is only one other construct (ONYX411) that combines the addition of E2F promoter elements with the CR2 mutation in E1A (15). However, ONXY411, in addition to remaining strictly dependent on CAR expression for infection, differs from ICOVIR-5 in the addition of E2F responsive elements to regulate not only E1A but also E4. These types of constructs may present some mechanistic and technical problems. It is normally difficult to predict the effect of every regulatory element separately. More worrisome is the evidence that addition of transcriptional elements to regulate E4 could lead to genomic instability and defective adenovirus replication (37).

The broad applicability of ICOVIR-5 based on the disruption of the Rb pathway, a hallmark of cancer, indicates that E2F-dependent adenoviruses constitute an attractive therapeutic strategy for many tumors including gliomas. ICOVIR-5 represents an advance in optimizing the potency and selectivity of oncolytic adenoviruses and as such deserves a further clinical testing in patients with malignant gliomas.

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References

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