Low-Dose Radiation Enhances Survivin-Mediated Virotherapy against Malignant Glioma Stem Cells

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Abstract

To improve the efficacy and selectivity of virotherapy for malignant glioma, we designed a strategy to amplify adenoviral replication in conjunction with radiotherapy using a radioinducible promoter. First, we compared the radiation-inducible activity of FLT-1, vascular endothelial growth factor, DR5, Cox2, and survivin. We then examined the capacity of the optimal promoter to modulate transgene expression followed by E1A activity *in vitro* and *in vivo* in a glioma stem cell model. In the presence of radiation, survivin mRNA activity increased 10-fold. Luciferase transgene expression was dose dependent and optimal at 2 Gy. A novel oncolytic adenovirus, CRAd-Survivin-pk7, showed significant toxicity and replication against a panel of passaged and primary CD133$^+$ glioma stem cells. On delivery of radiation, the toxicity associated with CRAd-Survivin-pk7 increased by 20% to 50% ($P < 0.05$). At the same time, the level of E1A activity increased 3- to 10-fold. *In vivo*, treatment of U373MG CD133$^+$ stem cells with CRAd-Survivin-pk7 and radiation significantly inhibited tumor growth ($P < 0.05$). At the same time, the level of E1A activity was 100-fold increased versus CRAd-Survivin-pk7 alone. Selected genes linked to radioinducible promoters whose expression can be regulated by ionizing radiation may improve the therapeutic ratio of virotherapy. In this study, we have identified a new radioinducible promoter, survivin, which greatly enhances the activity of an oncolytic adenovirus in the presence of low-dose radiotherapy. [Cancer Res 2008;68(14):5778–84]

Introduction

Glioblastoma multiforme (GBM) represents one of the most aggressive forms of primary brain cancer. The 2-year survival rate is 26.5% with radiotherapy plus temozolomide and 10.4% with radiotherapy alone (1). These tumors are morphologically diverse and highly invasive and generally recur close to the original tumor resection secondary to disseminated micrometastases (2, 3). Recently, a fraction of cells resistant to therapy due to activation of the Hedgehog-Gli pathway (4, 5), also known as CD133$^+$ cancer stem cells, have been identified in the context of glial tumors (6). These cells are believed to be responsible for glioma recurrences and decreased survival (5). CD133$^+$ glioma stem cells are resistant to current modes of therapy, including radiation- and temozolomide-based chemotherapy, and are present at a higher fraction in recurrent gliomas (7). These cells also up-regulate antiapoptotic proteins such as Bcl-2, Bcl-XL, cIAP-1, and survivin (6), indicating that they evade normal cellular regulation. Novel therapies are therefore being pursued to improve prognosis and increase the length of survival of patients with GBM by selectively targeting these highly resistant stem cells.

One potential therapy involves the use of oncolytic viral vectors. Recent preclinical studies suggest potential efficacy of these viruses and clinical studies in patients have confirmed the safety profile of these agents (8–11). However, a major limitation of these vectors has been poor replication and poor transduction of neighboring tumor cells following intracranial injection. To bypass these problems, a new generation of vectors is being developed, which selectively target tumor cells and which allow for increased viral replication. This can be achieved by use of tumor-specific promoters (TSP) or modifications of viral capsids to enhance viral transduction. Our group has previously identified the survivin promoter as a TSP (12, 13) and shown that an adenoviral vector that uses the survivin promoter and binds to heparan sulfate proteoglycans (CRAd-Survivin-pk7) can greatly enhance antitumor efficacy in an experimental glioma model (14).

In the present study, we sought to (*a*) evaluate a series of tumor-associated promoters with respect to radioinducible properties, (*b*) examine the role of radiotherapy in conjunction with oncolytic virotherapy, and (*c*) test the capacity of our novel oncolytic adenovirus, CRAd-S-pk7, to effectively target CD133$^+$ glioma stem cells. The rationale for this work is based on the fact that very few radioinducible promoters have been identified to date and the possibility of enhancing viral replication via radiation-induced promoter modulation could significantly enhance the potential efficacy of virotherapy, especially in the context of glioma stem cells. To the best of our knowledge, this is the first study to show that the survivin promoter is a novel radioinducible promoter that can increase viral replication and enhance the cytopathic effect observed with oncolytic vectors *in vivo*. Moreover, we also show that CRAd-S-pk7 targets CD133$^+$ cells *in vitro* and *in vivo*, which is relevant in light of the fact that these cells confer resistance to chemotherapy and radiotherapy in glioma patients and are responsible for recurrence of these malignancies.

Materials and Methods

Cell lines. The human glioma U373MG cancer cell line was obtained from the American Type Culture Collection. U118MG was provided by Dr. Joanne Douglas (University of Alabama, Birmingham, AL). These cells were grown in MEM with 10% FCS, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 100× antibiotic-antimycotic solution (Invitrogen). GBM samples were obtained directly from patients undergoing a resection in accordance with a protocol approved by the Institutional Review Board at

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the University of Chicago. The diagnosis of a grade 4 astrocytoma was confirmed by an attending neuropathologist.

Culture of primary glioblastoma cells. Specimens were washed in sterile saline at 4°C and processed immediately after resection by dissociation with mechanical disruption through a 40-μm strainer (Fisher Scientific). Erythrocytes were lysed using NH_4Cl. Trypan blue staining confirmed >80% viability after the procedure. Tumor cells were resuspended in neurobasal medium (Invitrogen) containing 20 ng/mL of recombinant basic fibroblast growth factor (Invitrogen), 20 ng/mL of epidermal growth factor (Invitrogen), N2 supplement (Invitrogen), B27 supplement (Invitrogen), and 10% heat-inactivated fetal bovine serum (Atlanta Biochemicals). The cultured cells were maintained for two passages.

Magnetic separation of $CD133^+$ glioma stem cells. For magnetic separation of CD133 glioma stem cells, the samples were dissociated and resuspended in PBS containing 0.5% bovine serum albumin. $CD133^+$ cells were isolated using the Miltenyi Biotec CD133 isolation kit. Positive magnetic cell separation (MACS) was done using several MACS columns in series. The purity of isolated cells was determined by staining with CD133/2-APC (Miltenyi Biotec) or isotype control antibody following analysis on a BD FACSCalibur (BD Biosciences). Sterile aliquots of $CD133^+$ and $CD133^-$ cells were resuspended in complete medium mentioned above and maintained for experiments.

Viruses. The AdCMV-Luc, AdVEGF, AdDR5-Luc, and AdSurvivin-Luc are replication-incompetent adenoviruses. AdCMV-Luc and AdSurvivin-Luc have been characterized previously (13, 15). These vectors contain luciferase genes replacing the E1A region under control of human promoters as follows: 0.51 kb of cytomegalovirus (CMV; ref. 15), 2.6 kb of vascular endothelial growth factor (VEGF) promoter region (16), 1.2 kb of DR5 (17), and 0.26 kb of survivin (18), respectively. The replication-deficient adenoviral vectors were constructed based on homologous recombination between pCMV, pVEGF, pDR5, or pSurvivin shuttle vectors and pVK500 adenoviral backbone that contain the entire adenoviral genome with deletion at E1A region. After rescuing in HEK293 cells, viral particles were purified by double CsCl gradient ultracentrifugation followed by titration of viral particles. Physical particle concentration (viral particles per milliliter) was determined by measuring absorbance at 260 nm.

The competent vector CRAd-S-pk7 was generated by our group and has been described earlier (14). Briefly, the CRAd virus was constructed using a shuttle vector pSc/Survivin/PA, which carries a human survivin promoter and Ad5pk7-based adenoviral wild-type 5 backbone containing polylysine modification in the fiber knob (CRAd-S-pk7) by homologous recombination in *Escherichia coli*. AdWT replication-competent vector was used as a negative control (19). All viruses were selected from single plaque on 911 cells, expanded in A549 cells, and then purified by double CsCl gradient ultracentrifugation (20).

Cellular response to radiation. For optimal dose determination, 5×10^4 U373MG cells were plated in six-well plates. The cells were then infected with either AdCMV-Luc or AdSurvivin-Luc (1,000 viral particles per cell). After 1-h adsorption, virus-containing medium was replaced with fresh growth medium. After 24 h, cells were irradiated [X-ray therapy (XRT)] with doses of 0, 2, 4, 8, and 12 Gy using a γ-irradiator (MG324, Philips). Twenty-four hours later, cells were analyzed by luciferase assay. For *in vitro* experiments, 1×10^3 of unsorted or $CD133^+$ cells (U373MG, GBM1, or GBM2) were grown in 96-well flat-bottomed plates (Costar, Corning, Inc.). Twenty-four hours later, the cells were infected with AdWT and CRAd-S-pk7 or mock infected (1,000 viral particles per cell) as described above. After 24 h, the cells were exposed to radiation at 2 Gy followed by addition of fresh growth medium. Twenty-four hours after XRT, virus-induced toxicity was measured by CytoTox Cytotoxicity Assay kit (Promega) as per the manufacturers' instruction. A fraction of the cells were subjected to quantitative PCR (qPCR) analysis for E1A expression.

Quantitative reverse transcription-PCR and PCR. Total RNA (5 μg) was isolated by RNeasy kit (Qiagen) and used for cDNA synthesis by oligo(dT) and SuperScript II RNase H reverse transcriptase (Invitrogen). Quantitative real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems) was performed with Opticon 2 detection system (Bio-Rad) according to the manufacturer's protocol. Primers used in the reverse transcription-PCR (RT-PCR) assays were described before (8, 21, 22). Briefly, the following E1A primers were used: 5′-AACCAGTTGCCGTGAGAGTTG-3′ (forward) and 5′-CTCGTTAAGCAAGTCCTCGATACAT-3′ (reverse). All the assays were carried out in triplicates and three independent experiments

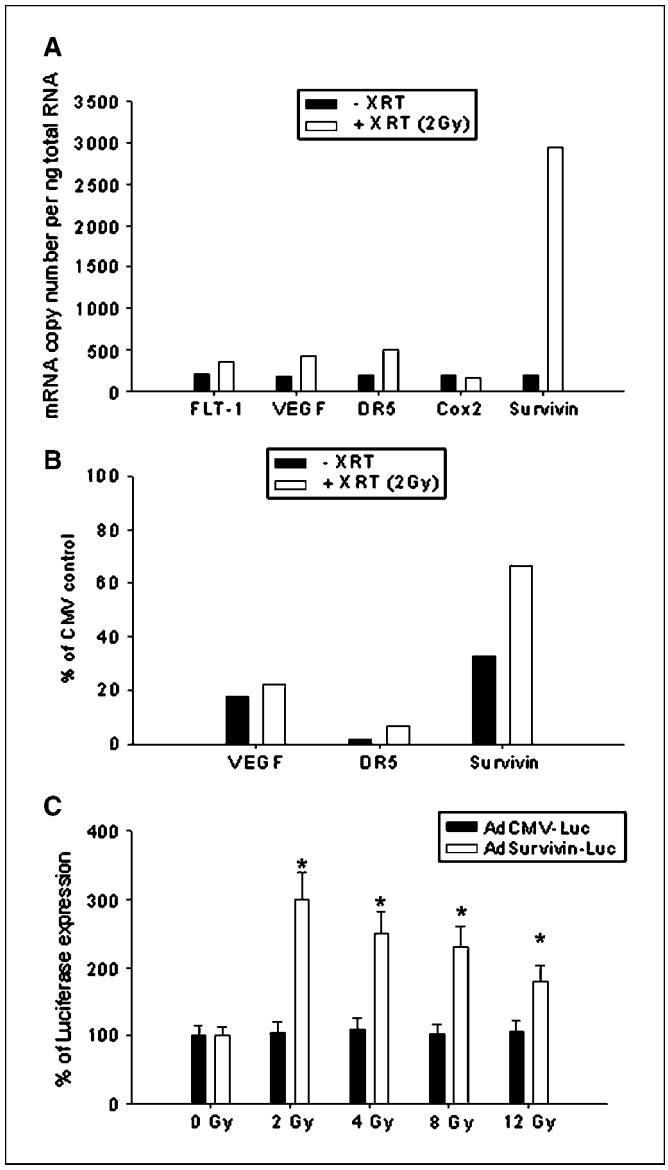

Figure 1. Real-time PCR detection of promoter activity after radiation exposure. *A,* five different human promoters (FLT-1, DR5, VEGF, Cox2, and survivin) were assessed for expression of mRNA in U118MG cells 2 h after exposure to 2 Gy radiation using quantitative RT-PCR. The levels of mRNA expression in treated and untreated cells were normalized using GAPDH expression and presented as a bar diagram. Experiments were performed thrice in triplicates each. *B,* detection of luciferase expression using replication-deficient adenovirus before and after radiation. Adenoviral constructs containing DR5, survivin, or VEGF promoter driving luciferase expression were used for infection of U118MG cells. AdVEGF-Luc and AdDR5-Luc showed an increase of 5% to 7% in luciferase expression in response to radiation. AdSurvivin-Luc, however, showed an increase from 30% to 70% in response to radiation. *C,* dose-dependent transcriptional activity of survivin promoter measured by luciferase assay. U373MG cells were infected with adenoviral constructs (1,000 viral particles per cell) containing the *luciferase* gene under either control of survivin or CMV promoters. After virus infection (2 h), cells were exposed to 2, 4, 6, 8, or 12 Gy radiation and grown for an additional 24 h. Total luciferase activity was measured and values were normalized to amounts of total protein. Experiments were performed twice in triplicates. *Columns,* mean; *bars,* SD. *, $P < 0.05$, compared with nonradiated control.

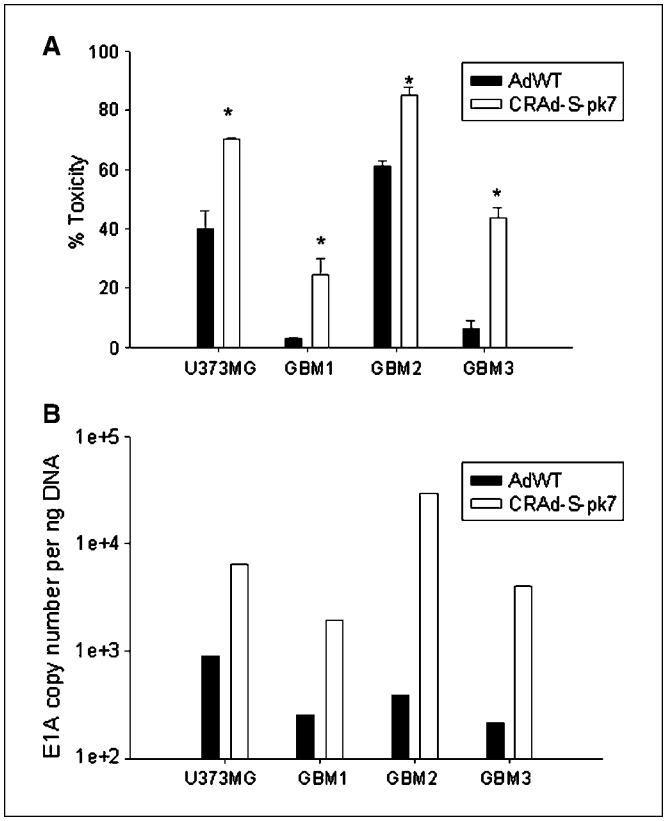

Figure 2. Cytotoxicity and replication of CRAd-S-pk7 in unsorted brain tumor samples. Brain tumor samples were infected with either AdWT or CRAd-S-pk7 and (*A*) were assayed for cytotoxicity 72 h after infection using CytoTox Cytotoxicity Assay kit or (*B*) E1A copy numbers were measured by qPCR. In *A*, the results were normalized to untreated cells and are presented as percentage toxicity. Each experiment was performed in six replicates. In *B*, E1A copy number is expressed per ng DNA. Measurements were performed in triplicates. *Columns,* mean; *bars,* SD. *, $P < 0.05$.

were performed to verify the reproducibility of the results. The results were normalized against human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in each of the samples. For E1A replication experiments, total DNA was extracted from infected/noninfected cells by DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocol. Gene expression was quantified by qPCR using SYBR Green PCR Master Mix and primers recognizing E1A region. For each experiment, a known amount of *AdWT* template DNA (10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 copies/μL) was used as a standard curve to quantify the *E1A* copy numbers of the experimental samples. The total volume of the reaction was 10 μL. The primers used for amplification of E1A have been described above (23). DNA amplification was carried out using Opticon 2 system (Bio-Rad). All samples were run in triplicate. The relative quantity of target DNAs was quantified against an internal control.

Detection of luciferase expression. Firefly luciferase enzyme activity was measured using the standard single luciferase assay (Promega) as per the manufacturer's instruction. To compensate for the differences in cell numbers in different samples, the protein concentration of the lysates was measured using the Bradford protein assay (Bio-Rad). Luciferase expression is presented as normalized to cellular protein concentration.

***In vivo* tumor formation and treatment.** For *in vivo* experiments, 8- to 10-wk-old nude mice (n = 6/group; The Jackson Laboratory) were s.c. implanted with 3×10^5 of U373MG CD133$^+$ human glioma cells in the right leg. Treatment was started 8 d later when tumor had been established in all mice (100–150 mm^3). Tumor-bearing mice were randomly assigned a treatment group (CRAd-S-pk7, CRAd-S-pk7 + XRT, AdWT, AdWT + XRT, XRT alone, or mock treatment) and injected with 1,000 viral particles per cell of CRAd-S-pk7, AdWT, or sterile PBS for mock controls. Twenty-four hours after virus injection, irradiation of the leg bearing the tumor was done using 2 Gy with a γ-irradiator (model 143-45; JL Shepherd Associates). Mice were examined daily for evaluation of tumor growth. Tumors were measured with calipers in three mutually perpendicular diameters (x, y, and z) and the volume (V) was calculated as $V = (x \times y \times z) / 2$. The data are representative of three independent experiments.

Statistical analysis. Results are expressed as mean ± SD. Student's t test was used according to the distribution of experimental values. P values of <0.05 were accepted as significant differences between groups. In the animal studies, tumor volumes were expressed as median ± quartile deviation. To test for significant differences in tumor size between treatment groups, one-way ANOVA was conducted.

Results

Survivin is a radioinducible promoter. To examine the specific expression of possible candidates for promoter-inducible elements, we evaluated the level of mRNA expression before and after radiation exposure. As seen in Fig. 1*A*, the mRNA levels of all these genes were up-regulated on exposure to radiation. However, whereas the levels of expression of FLT-1, DR5, VEGF, and Cox2 increased 2- to 3-fold, the survivin gene was up-regulated to ~10-fold, indicating a strong radiation-inducible promoter.

To further investigate the ability of the survivin promoter to drive a luciferase gene in an adenoviral background, we used replication-deficient adenoviral constructs and examined luciferase expression. We infected U118MG cell lines and, 24 h later, irradiated these cells with 2 Gy of radiation. Luciferase assay was performed 24 h later and the results are shown as percentage of CMV promoter-induced luciferase expression. As shown in Fig. 1*B*, AdVEGF-Luc and AdDR5-Luc showed an increase of 5% to 7% in luciferase expression in response to radiation. AdSurvivin-Luc, however, showed an increase from 30% to 70% in response to radiation.

To find the optimal radiation dose for the activation of the survivin promoter, we conducted a dose-response assay. U373MG cells were infected with AdSurvivin-Luc and irradiated with a single dose of 2, 4, 8, and 12 Gy. Luciferase assay was performed 24 h later.

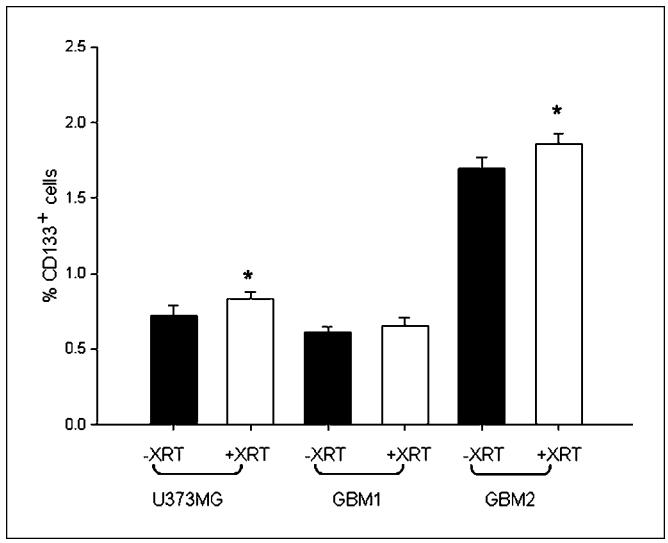

Figure 3. Enrichment of CD133$^+$ cells after radiation *in vitro*. Patient samples GBM1 and GBM2 and the cell line U373MG were exposed to 2 Gy radiation and grown for 48 h along with unexposed controls. CD133$^+$ subpopulation was determined by flow cytometric analysis. *Columns,* mean; *bars,* SD. *, $P < 0.05$.

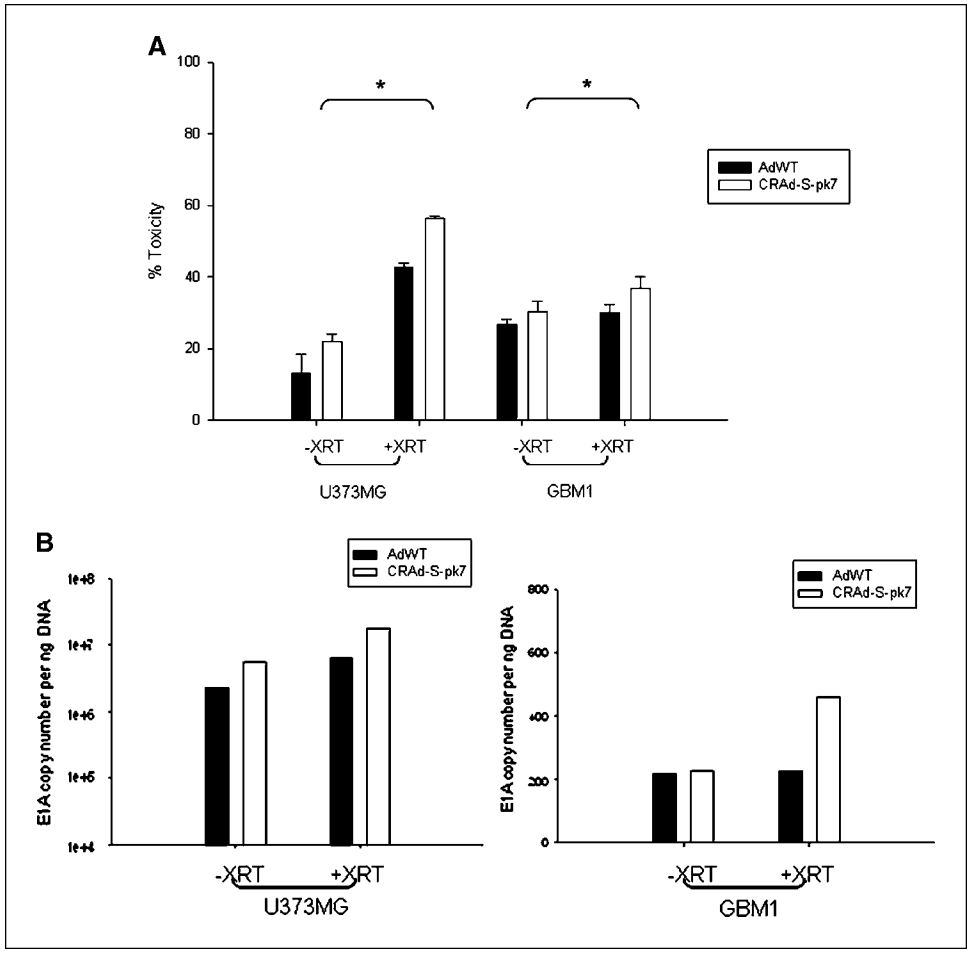

Figure 4. Survivin induced killing of CD133$^-$ cells *in vitro* in response to radiation. Cells were infected with CRAd-S-pk7 or AdWT and, 24 h later, exposed to 2 Gy of radiation. They were then grown for a further 24 h in complete medium. *A,* cytotoxicity was assayed by LDH release and is presented as percent toxicity and normalized to untreated cells. *Columns,* mean; *bars,* SD. *, $P < 0.05$. *B,* viral replication was determined by E1A copy number using qPCR and presented as copies per ng total DNA. Experiments were performed twice in triplicates.

As seen at Fig. 1*C*, the maximum response to radiation exposure was observed at dose 2 Gy (~ 3-fold). At higher radiation doses, the response diminished to 2- to 1.5-fold. We therefore chose 2 Gy for further studies.

An oncolytic vector that uses the survivin promoter is effective against glioma. Using primary tumor samples from three GBM patients along with U373MG cell line, we checked whether CRAd-Survivin-pk7 (CRAd-S-pk7) was able to efficiently target these cells and exert its virolytic effect. Figure 2*A* shows that significant cell death occurred in response to treatment with CRAd-S-pk7 in cells from the primary tumor samples as well as the passaged glioma cell line. In all cases, the toxicity was increased by about 25% to 40% compared with AdWT-infected cells ($P < 0.05$). To establish that the cytotoxic effect of the adenovirus was due to replication of the viral genome inside the tumors and not due to any extraneous variable, we looked at the copy number of the viral protein E1A in the cells. Our results show that the virolytic effect exhibited by CRAd-S-pk7 correlates with E1A copies detected in each sample (Fig. 2*B*). This is consistent with toxicity (Fig. 2*A*) as determined by lactate dehydrogenase (LDH) release and shows that human glioma samples preferentially support replication of CRAd-S-pk7 over AdWT (U373MG, 6.7×10^2 versus 903.41 copies; GBM samples, 1.92×10^2 versus 254, 2.93×10^4 versus 386, and 3.9×10^3 versus 212 copies per ng DNA).

CRAd-S-pk7 effectively targets CD133$^+$ glioma stem cells. We next examined the response of brain tumor samples enriched for CD133$^+$ cells in response to CRAd-S-pk7 and radiation. First, we analyzed the response of CD133$^+$ glioma stem cells to radiation. As shown in Fig. 3, the percentage of CD133$^+$ tumor cells in both primary (GBM1 and GBM2) and passaged cell line (U373MG) increased in response to 2 Gy radiation (0.6–0.7%, 1.7–1.9%, and 0.75–0.9%, respectively), suggesting that stem cells have increased proliferative capacity following radiation therapy. The limiting amounts of cells in the GBM3 sample prevented us from doing this assay in this tumor sample.

To further dissect the preferential target of CRAd-S-pk7, we examined its efficacy against CD133$^-$ and CD133$^+$ glioma stem cells. First, CD133$^-$ cells were infected with wild-type virus or CRAd-S-pk7 ± XRT and then assayed for cytotoxicity and replication efficiency. As shown in Fig. 4, we observed an increase in toxicity from 20% to 57% and from 30% to 38% in U373 and GBM1, respectively. There was also a concomitant increase in viral replication of about 5- and 2-fold in CRAd-S-pk7 and GBM1, respectively.

Next, to see whether our CRAd could preferentially target CD133$^+$ cells in conjunction with radiation, CD133$^+$ cells from U373MG, GBM1, and GBM2 were infected with wild-type and CRAd-S-pk7 adenovirus. The cells were then assayed for cytotoxicity and replication efficiency. We observed significantly higher toxicity in cells or tumor tissues infected by CRAd-S-pk7 compared with those that were AdWT infected (U373MG, 22.09 ± 2.05 versus 12.9 ± 5.49; GBM1, 80.41 ± 2.82 versus 46.6 ± 7.58; and GBM2, 41.9 ± 1.85 versus 30.98 ± 1.97; $P < 0.05$) as shown in Fig. 5*A*. The virolytic effect of CRAd-S-pk7 was further enhanced when the

cells were exposed to 2 Gy radiation. The level of toxicity, as measured by LDH release, increased to 38.99 ± 0.76, 96.82 ± 3.11, and 80.41 ± 12.82 ($P < 0.05$) for U373MG, GBM1, and GBM2, respectively (Fig. 5*A*). The absolute increase in toxicity was therefore greater for $CD133^+$ than $CD133^-$ stem cells. These data correlated very well with the increased viral replication in radiated cells particularly those infected with CRAd-S-pk7 (Fig. 5*B*). In fact, radiation exposure induced 1.97-, 43.62-, and 42.63-fold increase of viral replication for U373MG, GBM1, and GBM2, respectively.

Tumors formed from $CD133^+$ enriched cells *in vivo* are targeted by CRAd-S-pk7 and low-dose radiation in a synergistic manner. To examine the hypothesis that survivin-regulated viral toxicity will be increased *in vivo* in response to low-dose radiation, we injected nude mice with 3×10^5 of $CD133^+$ U373MG cells. The cells were injected under the skin to facilitate local radiation and allow for precise tumor measurement. After the tumors reached a volume of 100 mm^3, the mice were randomly divided into six groups and treated as described in Materials and Methods. As shown in Fig. 6*A*, median tumor volumes were plotted over a 6-day period. In response to radiation, CRAd-S-pk7 treatment group achieved the most significant tumor growth inhibition ($P < 0.05$). To ascertain the viral replication in response to radiation, three mice from each group were sacrificed at day 6 after irradiation and the tumors were resected. Viral copy number was ascertained from DNA isolated from these tumors (Fig. 6*B*). The CRAd-S-pk7 + XRT group showed about a 100-fold increase in viral replication compared with CRAd-S-pk7 alone.

Discussion

A major limitation of oncolytic virotherapy consists of poor *in vivo* replication and viral spread. This problem, at least in part, is due to the need for attenuation of viral backbone, which in turn has been shown to decrease viral propagation and spread. Two examples of this phenomenon include ONYX-015 and G207. The attenuation of E1B in the former and the deletion of the γ34.5 (RL1) in the latter are both associated with decreased viral replication (9). As such, clinical studies of ONYX-015 (adenovirus) or G207 (a herpes simplex virus containing the 34.5 deletion) have failed to show efficacy, partly due to the fact that the virus does not replicate with robust efficiency (8, 10). The ability to enhance viral replication, by means of using tumor-selective promoters, is likely to enhance virotherapy. We have previously shown that the survivin promoter may represent an optimal TSP and in this study provide compelling data to suggest that radiation, a mainstay of glioma therapy, further enhances survivin-mediated adenoviral replication and *in vivo* efficacy.

To date, very few radioinducible promoters have been described in the literature. A RecA promoter was used as a radiation-inducible promoter to increase tumor necrosis factor-α (TNF-α) production in *Clostridium* sp. (24). The Egr-1 promoter has also been used as a radioinducible promoter to deliver TNF-α to tumor cells (25, 26). The Egr-1 promoter was also studied in the context of radioprotective effect of FLT-3 in severe combined immunodeficient mice (27) for *in vitro* studies on gene activation (28) and for gene expression in the context of a hypoxia-inducible promoters (29). However, these genes are neither specifically expressed in tumor cells nor are they up-regulated in gliomas. Our current study suggests that survivin is a radioinducible promoter that not only increases transgene expression but also modulates E1A adenoviral activity. Both the *in vitro* and *in vivo* studies show the enhanced activity of a survivin-driven oncolytic adenovirus in the presence of low-dose radiotherapy, a finding with significant implications for patients who may one day be treated with this virus and subsequently receive radiotherapy.

In addition to showing the radioinducible characteristics of survivin, we also show that a survivin-driven CRAd preferentially targets $CD133^+$ glioma stem cells, a novel finding that was not previously reported. Moreover, we show for the first time that in the presence of radiotherapy, there is enhanced viral replication in $CD133^+$ cells as measured by E1A copy number, a finding that translates to improved tumor growth inhibition. CRAd-S-pk7 retains its ability to kill these cells and exposure to low-dose radiation increases the ability of the virus to target these tumors more effectively. In comparison with unsorted cells, the replication of CRAd-S-pk7 increased 10% to 25% in irradiated $CD133^+$ enriched primary GBM tissues compared with the nonirradiated tissue. This indicates specific targeting of the cells by the oncolytic CRAd-S-pk7. The excellent correlation between virus-induced cell death

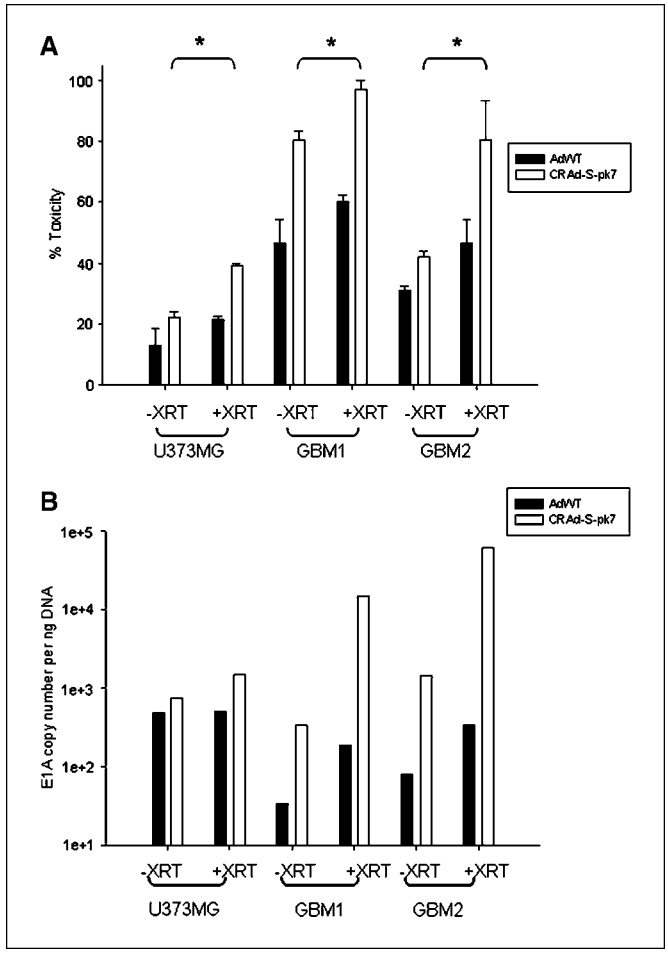

Figure 5. Survivin induced killing of $CD133^+$ cells *in vitro* in response to radiation. *A,* $CD133^+$ enriched tumor cells were infected with either AdWT or CRAd-S-pk7 24 h before exposure to radiation (2 Gy) followed by 24-h growth in complete medium. Cytotoxicity was measured by LDH assay. *Columns,* mean; *bars,* SD. *, $P < 0.05$. Experiments were performed twice with six replicates per condition. *B,* viral replication was determined via E1A copy number measurement using qPCR and presented as copies per ng total DNA. Experiments were performed twice in triplicates.

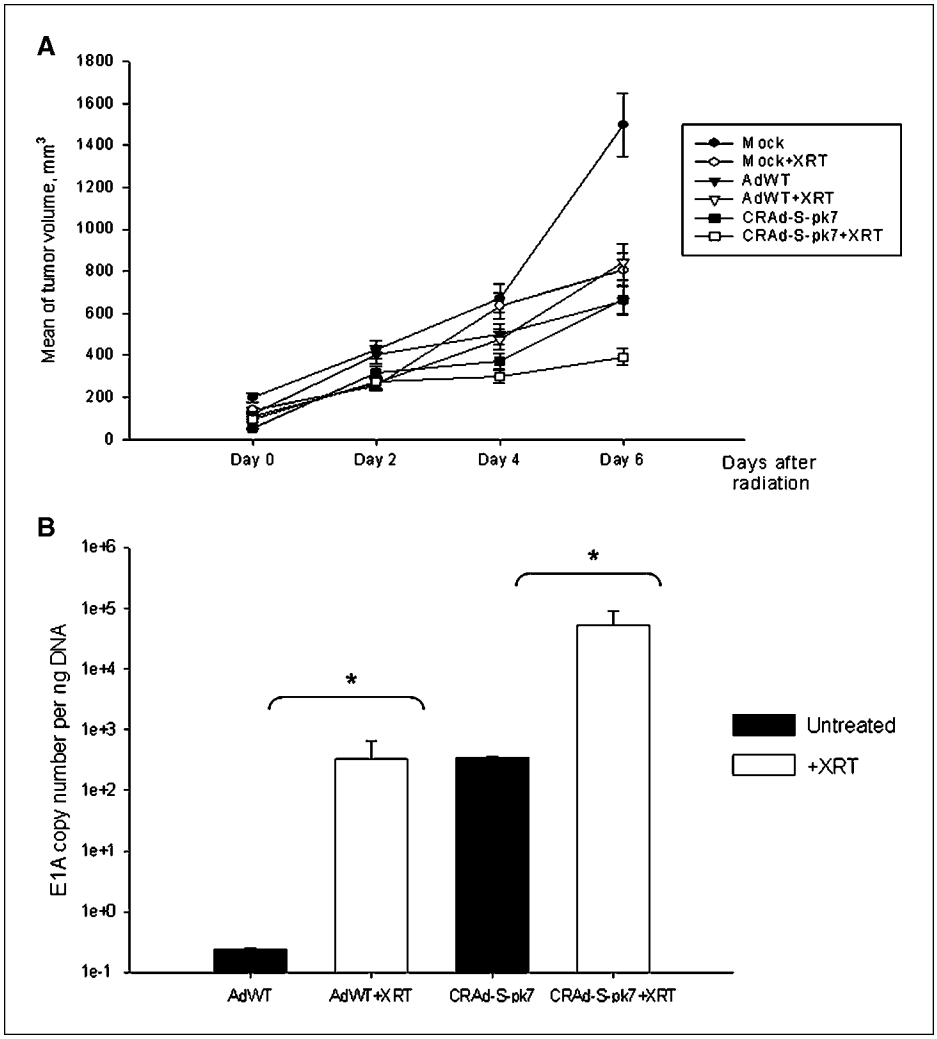

Figure 6. *In vivo* tumor growth rate in nude mice. *A,* $CD133^+$ enriched U373MG cells were injected s.c. in the right hind leg for tumor formation. After 8 d, when the tumor reached 100 mm^3, mice were randomly divided in three groups (mock, AdWT, or CRAd-S-pk7 treatment) and injected with respective virus at 1,000 viral particles per cell or PBS for mock. Twenty-four hours later, each group was further divided in two: one group received single dose of radiation of 2 Gy and the other group was the nonirradiated control. Tumor volumes were measured for 6 d. *Points,* median volumes; *bars,* SD. *B,* viral replication in tumors as measured by E1A qPCR at day 6 after radiation in each group.

and replication of the virus in response to radiation was also observed in a mouse tumor model. We show that mice treated with CRAd-S-pk7 and then exposed to low-dose radiation had significantly smaller tumor size than mice treated with CRAd-S-pk7 alone. Although we acknowledge that one potential limitation of our study was the flank glioma model, it nevertheless allowed us the opportunity to precisely monitor tumor growth in response to radiotherapy and virotherapy *in vivo*. Although studies examining the intracranial model are currently in progress, our current results provide a "proof of principle" with regard to the sensitivity of survivin to radiation and enhanced adenoviral replication and killing both *in vitro* and *in vivo*.

The exact mechanism responsible for increased E1 activity in response to radiation is unclear. However, in studies involving the *Egr-1* gene, the extracellular signal-regulated kinase 1/2 and the stress-activated protein kinase/c-Jun NH_2-terminal kinase pathways were found to be activated (30). Moreover, the p53 and the nuclear factor-κB transcription factors have been consistently found to be activated in response to clinically relevant levels of radiation (31). Although it is possible that the survivin promoter is activated by one of these pathways via the action of these two important transcription factors, further studies are needed to analyze their induction.

In conclusion, we show that exposure to low-dose radiation increases the replication of CRAd-S-pk7 and therefore enhances the cytopathic effect in the context of $CD133^+$ glioma stem cells. $CD133^+$ cells represent the stem cell fraction of gliomas and the ability of CRAd-S-pk7 to target these cells in the presence of radiotherapy is an important step in improving the prognosis of patients with GBM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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