Cancer Therapy with a Replicating Oncolytic Adenovirus Targeting the Hypoxic Microenvironment of Tumors

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ABSTRACT

Hypoxia plays a critical role in driving tumor malignancy and is associated with poor patient survival in many human cancers. Novel therapies targeting hypoxic tumor cells are urgently needed, because these cells hinder tumor eradication. Here we demonstrate than an anticancer strategy based on intratumoral delivery of a novel type of oncolytic adenovirus targeting tumor hypoxia is therapeutically efficient and can augment standard chemotherapy. We used a conditionally replicative adenovirus (HYPR-Ad) to specifically kill hypoxic tumor cells. Viral infection and conditional replication occurred efficiently in hypoxic/hypoxia-inducible factor-active cells in culture and *in vivo*, prevented tumor formation, and reduced the growth of established tumors. Combining HYPR-Ad with chemotherapy effective against normoxic cells resulted in strongly enhanced antitumor efficacy. These studies demonstrate that targeting the hypoxic microenvironment of tumors rather than an intrinsic gene expression defect is a viable and novel antitumor therapeutic strategy that can be used in combination with existing treatment regimens. The replication and oncolytic potential of this virus was made dependent on hypoxic/hypoxia-inducible factor, a transcription factor activated in the tumor hypoxic microenvironment, broadening its therapeutic use to solid tumors of any genetic make-up or tissue of origin.

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INTRODUCTION

New treatments targeting hypoxic tumor cells are greatly needed. Most solid tumors have regions of hypoxia, low oxygen (O_2), and the cells in these areas pose a major challenge for therapy (1). Hypoxia leads to the production of cell survival factors such as glycolytic enzymes and proangiogenic factors and is associated with a resistance of cancer cells to conventional radio- and chemotherapies, the selection of tumor clones with defective apoptotic pathways, and a predisposition to cell invasion and metastasis (1–4). Variable O_2 conditions ranging from 0% to 5% with a median level of 1.3% have been measured in tumors (1). Until recently, it was believed that in tumors hypoxia was mostly confined to the necrotic center (5). Recent advances suggest that microhypoxic regions are present throughout the tumor mass including the leading edge of the tumor (6–8). These regions contain viable hypoxic tumor cells that strongly contribute to tumor expansion (9, 10). These findings have spurred a quest for hypoxia-targeted therapeutic strategies.

One approach to specifically target hypoxic tumor cells exploits the hypoxia-inducible factor (HIF). HIF is a heterodimeric transcription factor regulating the physiologic reaction to hypoxia by binding to hypoxia-response elements (HRE) in target genes (11, 12). These genes encode glucose transporters, all of the glycolytic pathway enzymes, proangiogenic factors such as vascular endothelial growth factor (VEGF) and its receptor VEGFR1, and proteins involved in cell migration such as met (3) and CXCR4 (4). The activation of the HIF pathway enables cancer cells to survive and proliferate in a hypoxic environment and contributes to a more aggressive phenotype. There are three HIF-α subunits of which the protein levels and activity are regulated by hypoxia whereas the β-subunit is constitutively expressed. The expression and activity of the subunits are also regulated by many tumor-specific genetic alterations, such as inactivation of the von Hippel-Lindau (pVHL), PTEN, and p53 tumor suppressors and dysregulation of the phosphatidylinositol 3-kinase/AKT and mitogen-activated protein kinase signal transduction pathways (11). HIF-1α is overexpressed in numerous cancer types and their metastases (6). Therefore, the HIF/HRE system of gene regulation, which is active under hypoxia or as a result of genetic alterations during cell transformation, is particularly attractive to specifically target therapeutic gene expression to solid tumors.

Numerous genetically engineered replication-competent adenoviruses and herpes simplex virus-1 (HSV-1) have been designed to selectively kill tumor cells (13–17). The expectation is that after an initial infection, cycles of viral amplification, tumor cell lysis, viral spread, and infection of additional tumor cells will lead to tumor eradication. Such adenoviruses and HSV-1 vectors have displayed an increase in the differential lysis of tumor *versus* normal cells in culture and antitumor activity in cancer models. Most importantly, several of these

viruses have entered clinical trials where their overall safety in cancer patients and modest antitumor activity have been demonstrated (13, 17).

To exploit HIF activation in tumors for therapeutic purposes, oncolytic replicative adenoviruses with an *E1A* gene driven by a HIF-responsive promoter were engineered. *In vitro* studies demonstrated that these viruses could specifically kill tumor cells that are hypoxic (18) or constitutively HIF expressing due to genetic inactivation of von Hippel-Lindau (19). Whereas these studies were encouraging, they yet did not address the daunting obstacle of whether such viruses could be used successfully *in vivo* to prevent and reduce the growth of genetically and biologically heterogeneous tumors with uneven susceptibility to viral infection and extreme variations in microenvironmental O_2 pressure. Clearly, it remained uncertain whether the variable O_2 conditions and presence of chronic *versus* acute hypoxic states in a tumor would be sufficient to allow robust activation of viral replication and conversely if extreme hypoxia (anoxia) would hinder or prevent viral replication. Also, it was unknown if normoxic cells surrounding hypoxic cell pockets would function as a barrier to viral spread throughout the tumor mass. Furthermore, it was untested whether combining these viruses with a chemotherapeutic agent targeting the normoxic tumor fraction would result in a greater therapeutic effect rather than blocking viral replication. Here we undertook studies to specifically address these issues.

We demonstrate that infection and replication of adenoviruses occur efficiently under hypoxic conditions and, therefore, represent a feasible approach to kill hypoxic tumor cells *in vivo*. We show that a hypoxia/HIF-dependent oncolytic adenovirus displayed conditional replication under hypoxic/HIF-active conditions and was able to prevent tumor formation and reduce the growth of established tumors. Moreover, the combination of this virus with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) chemotherapy, which is effective against normoxic cells, resulted in augmented antitumor efficacy. These data support the use of HIF-dependent replicative adenoviruses as a novel antitumor therapy for hypoxic/HIF-active tumor cells and their addition to a multimodal tumor treatment program, regardless of tumor type or genetic background.

MATERIALS AND METHODS

Cells and Viruses. Human embryonic kidney (HEK) 293 and normal human foreskin fibroblasts (Hs68) were purchased from the American Type Culture Collection (Rockville, MD). LN229, U251MG, and U87MG human malignant glioma cells were described previously (20). U251MG-T2 is a subculture of U251MG that we rendered more tumorigenic (tumor establishment is higher and growth is faster) in *nu/nu* mice by passaging it twice as a s.c. xenograft. LN229-HRE-AP cells were constructed by stably transfecting an *alkaline phosphatase* reporter gene under the regulation of the V6R hypoxia/HIF-responsive promoter in LN229 cells (21). Cells were maintained in DMEM containing 10% fetal calf serum under normoxia (21% O_2). Hypoxia (1% O_2) was generated in a water-jacketed CO_2 incubator with a built in oxygen control system (Thermo-Forma, Marietta, OH) by displacing O_2 with infusion of N_2 from an external high-pressure liquid nitrogen tank. HYPR-Ad#1 is a conditionally replicative adenovirus containing the *E1A* gene under the regulation of a hypoxia/HIF-responsive promoter composed of six copies of the HRE from the VEGF gene (V6R; ref. 18). AdLacZ is replication deficient, lacks the *E1* gene region, and expresses LacZ. dl309-Ad is replication-competent and contains a wild-type *E1* gene region (22). Purified, high-titer viral stocks were generated at the University of North Carolina Vector Core Facility (Chapel Hill, NC).

Viral Replication and Cytopathic Effect Assays. For viral replication assays, 1×10^5 cells per well were seeded in 12-well plates, infected with HYPR-Ad#1 at a multiplicity of infection (MOI) of 1.0 (HEK293 and LN229) or 100 (Hs68) in triplicate, and then incubated under normoxia or hypoxia. At the indicated time points, visual evidence of cytopathic effect (cell lysis/detachment) was noted, and the media and cells (by scraping with a rubber policeman) were independently collected. Virus was harvested from these samples by three freeze/thaw cycles followed by microcentrifugation for 5 minutes at $16{,}000 \times g$. Virus containing supernatant was titered using the Adeno-X-Rapid Titer kit (BD Biosciences, Palo Alto, CA). For cytopathic effect assays, 1.5×10^5 U87MG cells per well were seeded in 6-well plates and then infected under normoxia for 3 hours in DMEM 2.5% fetal calf serum at MOI 10 with AdLacZ, dl309-Ad, HYPR-Ad#1, or were left uninfected. Subsequently, virus was aspirated, DMEM 10% fetal calf serum was added, and the cells were incubated under normoxia or hypoxia until 100% cytopathic effect was visually observed.

Tumor Studies and Analysis. For *in vivo* viral replication studies, 1×10^7 LN229-HRE-AP cells were implanted s.c. in the flanks of *nu/nu* mice (Athymic NCr-nu, National Cancer Institute, Bethesda, MD) individually marked with tattoos (23). Tumors were established to an average size of 65 mm^3 (28 days after implantation) and then treated intratumorally for 5 days with 1.0×10^8 plaque-forming units (pfu)/day of AdLacZ, HYPR-Ad#1, or PBS. On days 10 and 30 from the start of virus injection, mice from each group were injected i.p. with 60 mg/kg pimonidazole hydrochloride, a 2-nitroimidazole hypoxia marker (Chemicon, Temecula, CA). Ninety minutes later the tumors were harvested, weighed, stored in 10% buffered formalin overnight at room temperature, and transferred to 70% EtOH at 4°C. The tumors were embedded in paraffin and sectioned. Deparaffinized sections were immunostained for pimonidazole adducts with a Hypoxyprobe-1 antibody following manufacturer instructions (Chemicon). Immunohistochemical detection of adenovirus hexon protein was performed using a protocol similar to Hypoxyprobe-1 with the following changes: (1) 0.01% Pronase for 20 minutes at 4°C, (2) primary antibody for adenovirus hexon is Mb805 (Chemicon) diluted 1:1000 for 1 hour at 35°C, and (3) secondary antibody treatment for 15 minutes at 35°C. For tumor prevention studies, LN229-HRE-AP cells were preinfected in culture under normoxia at MOI 1 with HYPR-Ad#1 for 3 hours in DMEM 2.5% fetal calf serum or were left uninfected. Subsequently virus was aspirated, DMEM 10% FCS was added, and the cells were incubated overnight under normoxia. The cells were harvested by trypsinization and 1×10^7 cells were injected s.c. in both the left and right flank of *nu/nu* mice (HYPR-Ad#1 infected: $n = 9$ mice, uninfected: $n = 8$ mice). *In situ* detection of alkaline phosphatase activity was performed by fixing 5-mm frozen tumor tissue sections with

0.5% glutaraldehyde in PBS for 10 minutes, washing with PBS, incubating for 10 minutes at room temperature with the 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) enzyme substrate (BCIP: 0.15 mg/mL; NBT: 0.30 mg/mL; Sigma, St. Louis, MO), and then washing with PBS. For tumor growth reduction studies, LN229 (1×10^7) and U87MG (5×10^6) cells were implanted s.c. in the flanks of *nu/nu* mice. Tumors were established to an average size of 70 mm^3 (LN229, 29 days after implantation) or 15 mm^3 (U87MG, 11 days after implantation) and then treated intratumorally for 5 days with 0.7 to 1.0×10^8 pfu/day of HYPR-Ad#1, dl309-Ad, or PBS (100 μL total injection volume). For HYPR-Ad#1 + BCNU (DNA alkylating agent) chemotherapy studies, U251MG-T2 (2×10^7) and LN229 (1×10^7) cells were implanted s.c. in the flanks of *nu/nu* mice, and tumors were established to an average size of 55 mm^3 (U251MG-T2, 22 days after implantation) or 60 mm^3 (LN229, 35 days after implantation). For U251MG-T2 tumor treatment, BCNU (30 mg/kg, Sigma) was administered i.p. on days 22, 26, and 30 and HYPR-Ad#1 (1×10^8 pfu) was administered intratumorally on days 28, 29, 32, 33, and 34. For LN229 tumor treatment, BCNU was administered i.p. on days 35 (30 mg/kg) and 39 (25 mg/kg), and HYPR-Ad#1 (1×10^8 pfu) was administered intratumorally on days 35 to 39. Tumor size was monitored with calipers and tumor volume was calculated [(length $\times$ width^2)/2].

RESULTS

Adenovirus Replication Occurs Efficiently Under Hypoxia. We examined whether hypoxia alters the efficiency of viral replication compared with normoxia. This concern is based on findings that the proliferation of many cell lines is reduced after exposure to hypoxia, whereas the induction of cell cycle progression is necessary for adenovirus replication. Whereas the adenovirus E1A protein promotes reentry of quiescent cells into the cell cycle, it was unclear whether E1A could overcome hypoxia-induced cell cycle arrest and permit viral replication. For these studies, HEK 293, Hs68, and LN229 cells were infected with AdLacZ (replication-deficient) and dl309-Ad (replication-competent) and then incubated under normoxia *versus* hypoxia. Virus from the cell lysate and conditioned media were collected and quantified on the indicated days postinfection. Both replication-deficient (AdLacZ) and replication-competent (dl309-Ad) adenoviruses can be grown in 293 cells, because this cell line contains a stably integrated adenovirus E1 gene region. There was no significant difference in the amount of virus collected from the cell lysate (Fig. 1*A*) or conditioned media (Fig. 1*B*) of AdLacZ or dl309-Ad infected 293 cells maintained under normoxia *versus* hypoxia for 2 days ($P > 0.113$). As expected, AdLacZ did not replicate in Hs68 (Fig. 1*C* and *D*) or LN229 cells (Fig. 1*E* and *F*) and, therefore served as a negative control and a visual marker that the hypoxic and viral infection conditions are not cytotoxic (evidenced by the lack of cytopathic effect, not shown). Similar amounts of dl309-Ad were measured in the cell lysate of infected Hs68 cells grown under normoxia *versus* hypoxia on days 2, 4, and 7 ($P > 0.096$, Fig. 1*C*) and in the conditioned media on days 2 and 4 ($P > 0.447$, Fig. 1*D*), whereas a 40% increase was detected on day 7 ($P = 0.041$, Fig. 1*D*). A significant increase in dl309-Ad levels under hypoxia was also evident in the cell lysate (Fig. 1*E*) and conditioned media (Fig. 1*F*) of infected LN229 cells on day 4 ($P = 0.041$ and 0.026, respectively). These results demonstrate that adenovirus replication can occur efficiently in hypoxic normal or tumor cells.

Effective and Selective Replication of a Hypoxia-Dependent Adenovirus (HYPR-Ad#1) in Cultured Cells. We examined whether HYPR-Ad#1, a hypoxia/HIF-dependent oncolytic virus, could selectively replicate and generate viral progeny efficiently in hypoxic cells. As controls and for comparison, we used AdLacZ and dl309-Ad (Fig. 1). We previously generated HYPR-Ad#1 by placing the *E1A* viral replication gene under the regulation of an exogenous hypoxia/HIF-responsive promoter (Fig. 2*A*; ref. 18). Whereas we showed that the virus could conditionally lyse hypoxic cell *in vitro*, its ability to selectively and efficiently replicate and generate progeny virus under hypoxia has not been examined previously. HYPR-Ad#1-infected cells were incubated under normoxia or hypoxia, and virus from the cell lysates and conditioned media were collected and quantified on the indicated days after infection. Normal human fibroblasts (Hs68) were chosen so that HYPR-Ad#1 replication could be assessed under HIF protein levels in nontransformed cells exposed to hypoxia compared with normoxia. Hs68 cells contain a hypoxia-activated HIF-1α pathway with undetectable levels of HIF-1α under normoxia (18). In tumor cells, HIF-1 levels and activity can be altered in response to genetic alterations that occur during cellular transformation (11). Throughout the 13-day time course, a significant increase ($P < 0.0073$) in HYPR-Ad#1 collected from hypoxic *versus* normoxic Hs68 cell lysates was measured (Fig. 2*B*). On days 3, 6, 8, 10, and 13 there was a 1.5-, 4.4-, 8.6-, 5.6-, and 6.5-fold increase in HYPR-Ad#1 detected in cell lysates of infected Hs68 cells maintained under hypoxia *versus* normoxia. This generally correlated with the hypoxia-dependent expression of viral E1A (Fig. 2*B*) and cellular HIF-1α (18) in these cells. There was a disparity in the levels of E1A and viral titer on day 13 in lysates of Hs68 cells maintained under hypoxia. We have observed that as monolayer cells infected with replication-competent adenoviruses approach 100% cytopathic effect that the levels of E1A dramatically drop (18). On day 13, ~90% of the Hs68 cells infected with HYPR-Ad#1 and maintained under hypoxia are undergoing cytopathic effect (18). During the cytopathic effect process, cells first detach from the dish and remain alive as floating cells for several days before undergoing cytolysis, a process that results in release of viral particles into the medium. These cells were included in our analysis and most likely explain the disparity. Low levels of HYPR-Ad#1 were detectable in the cell lysate of infected Hs68 cells maintained under normoxia and most likely result from the initial infection of these cells rather than viral replication, because this amount of virus showed little variation during the time course. This is consistent with the tight regulation of the hypoxia/HIF-responsive promoter under normoxia, as evidenced by lack of E1A expression under normoxia (Fig. 2*B*). As expected, the conditioned media of these cells also showed a significant increase in the amount of virus on days 10 and 13 (6.8-fold increase, $P = 0.0003$ and 13.0-fold increase, $P = 0.0001$, respectively, Fig. 2*C*).

Next we examined whether HYPR-Ad#1 can also replicate in a hypoxia-dependent fashion in tumor cells (LN229

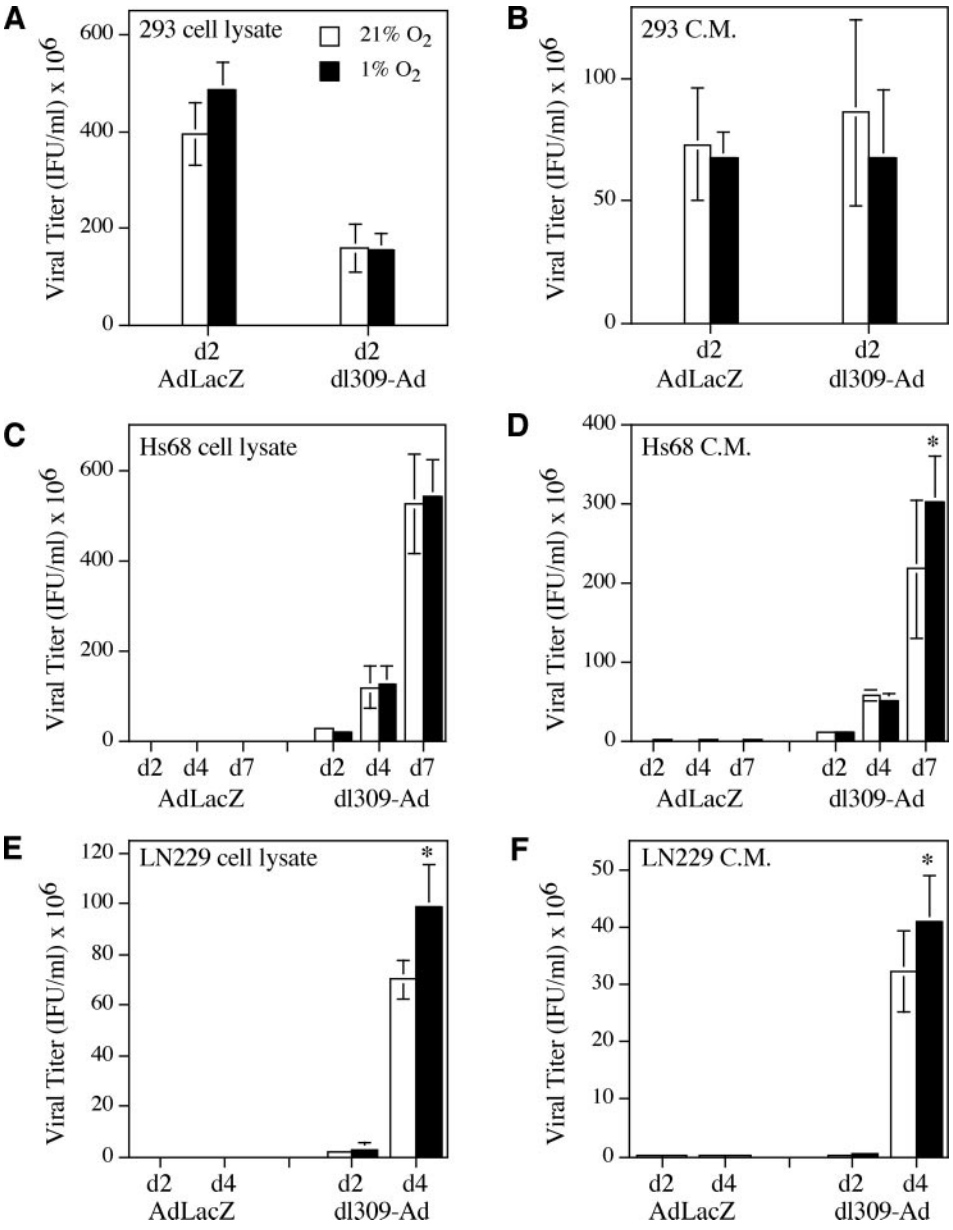

Fig. 1 Adenovirus replication occurs efficiently under hypoxia. HEK 293 (*A* and *B*), Hs68 (*C* and *D*), and LN229 (*E* and *F*) cells were infected with AdLacZ or dl309-Ad at MOI 1.0 (HEK 293 and LN229) or 100 (Hs68) and then incubated under normoxia (21% O_2, *white*) or hypoxia (1% O 2, *black*). At the indicated time points, virus was harvested from the cells (*A, C,* and *E*) and conditioned media (*CM*; *B, D,* and *F*) and titered [infectious forming units (*IFU*)/mL]. The levels of AdLacZ in infected Hs68 and LN229 cells were below the level of detection for this assay, consistent with it being a replication-deficient virus; *bars,* ±SD. *Stars* indicate a statistically significant (Student's *t* test) increase in virus production under hypoxia (*D*: $P = 0.041$; *E*: $P = 0.041$, *F*: $P = 0.026$).

glioblastoma). These cells contain low levels of HIF-1α under normoxia that can be activated by hypoxia, demonstrating a functional hypoxia-activated HIF pathway (18). Consistent with viral replication initiated by low levels of E1A in normoxic LN229 cells, we observed a measurable increase in virus production under normoxia during the time course (Fig. 2*D* and *E*). A significant increase in HYPR-Ad#1 was first detectable on day 4 in cells maintained under hypoxia *versus* normoxia (10-fold increase, $P = 1.1 \times 10^{-8}$; Fig. 2*D*). This significant increase under hypoxia continued through day 7 (3.5-fold increase, $P = 0.0003$) and day 9 (4.2-fold increase, $P = 5 \times 10^{-5}$). In the conditioned media derived from these cells (Fig. 2*E*), a significant increase in virus production was detectable throughout the time course under hypoxia *versus* normoxia with induction levels of 18- ($P = 0.0007$), 4.5- ($P = 1.4 \times 10^{-5}$), 8.4- ($P = 2.6 \times 10^{-5}$), and 4.5- ($P = 2 \times 10^{-5}$) fold on days 2, 4, 7, and 9, respectively. Lower levels of HYPR-Ad#1 were detected under hypoxia in LN229 cells compared with Hs68 cells. This difference most likely results from the different MOIs used (MOI 1 *versus* 100, respectively), because the adenovirus preferentially infects tumor cells (24). These data demonstrate that HYPR-Ad#1 conditionally replicates in a hypoxia/HIF-dependent manner in both normal and tumor cells in culture.

HYPR-Ad#1 Specifically Replicates in Hypoxic Regions of Tumors. We next explored whether HYPR-Ad#1 conditionally replicates in hypoxic regions of tumor xenografts as seen in culture. LN229 tumor xenografts were injected with

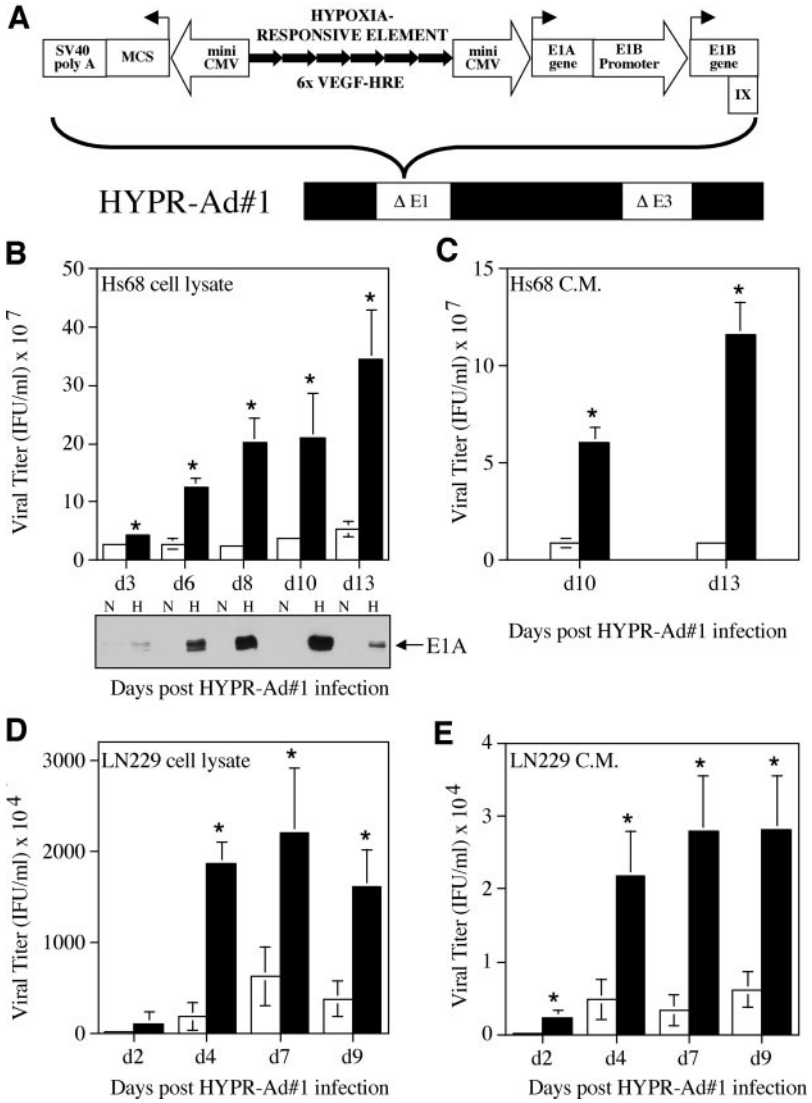

Fig. 2 HYPR-Ad#1 conditionally replicates under hypoxia in culture. *A,* schematic of HYPRAd#1. To generate an adenovirus that selectively replicates in hypoxic/HIF-active cells, a modified adenovirus type 5 E1 gene region was cloned into a replication-deficient Ad5 genome lacking the E1 and E3 genomic regions. The E1A viral replication gene is under the control of an exogenous hypoxia/HIF-responsive promoter, V6R, composed of six copies of the HRE of the VEGF gene (*6x VEGF-HRE*) and a mini CMV promoter (18). The hypoxia-HIF–responsive promoter within HYPR-Ad#1 is bidirectional and has a multiple cloning site (*MCS*) for the future incorporation of an adjuvant therapy gene is shown. *B–E,* hypoxia-dependent replication of HYPR-Ad#1 in culture. Hs68 (*B* and *C*) and LN229 (*D* and *E*) cells were infected with HYPR-Ad#1 at MOI 1 (*LN229*) or 100 (*Hs68*) and then incubated under normoxia (21% O_2, *white*) or hypoxia (1% O_2, *black*). At the indicated time points, virus was harvested from the cells (*B* and *D*) and conditioned media (*CM*; *C* and *E*) and titered. *Inset,* Western blot for Ad E1A expression in cells under the same conditions (*B*). The 1x gene encodes a structural protein of the virion capsid and was also reconstituted and was reported previously (18). *Bars,* ±SD. *Stars* indicate a statistically significant (Student's *t* test) increase in virus production under hypoxia ($P < 0.0073$).

AdLacZ, HYPR-Ad#1, or PBS. Ten and 30 days later, mice were injected with pimonidazole, a marker used to detect areas of hypoxia (<1% O_2) *in vivo* (25). Harvested tumors were then analyzed by immunohistochemistry for hypoxia and adenovirus hexon coat protein expression, a marker of newly synthesized virus (26). In tumors of all three of the groups, pimonidazole staining evidenced microhypoxic regions (Fig. 3 and data not shown). PBS and AdLacZ treated tumors were negative for adenovirus hexon expression (data not shown), whereas pockets of adenovirus hexon-positive cells were evident in HYPR-Ad#1 treated tumors at both 10 and 30 days after viral treatment (Fig. 3). Superimposing consecutive adenovirus hexon and pimonidazole-stained sections revealed that 6 of 6 (10 days) and 13 of 13 (30 days) hexon-positive regions from HYPR-Ad#1 tumors overlapped with pimonidazole-positive areas (Fig. 3). These data demonstrate that HYPR-Ad#1 conditionally replicates in hypoxic regions of tumor xenografts, consistent with *in vitro* results.

HYPR-Ad#1 Prevents Tumor Formation. As a first step to evaluate the antitumor properties of HYPR-Ad#1, we examined whether it could act as a tumor prevention agent when introduced into tumor cells before implantation into animals. This study design allowed us to specifically evaluate the antitumor potency of the virus while circumventing problems associated with variable distribution of virus after intratumoral injection. A subclone of LN229 cells stably transfected with a hypoxia-inducible *alkaline phosphatase* reporter gene (LN229-HRE-AP) was chosen for these studies so that hypoxic cells in tumor xenografts could be identified by alkaline phosphatase staining. Cells were preinfected in culture with HYPR-Ad#1 (MOI 1.0) or were left uninfected (mock). The next day, cells from both groups (HYPR-Ad#1 and mock-infected) were harvested, injected s.c. in the flanks of *nu/nu* mice, and tumor formation monitored. As a control, an aliquot of the infected cells was maintained in culture so that the specific lytic ability of the virus under hypoxia could be verified. As expected, hypoxic-infected tumor cells underwent 100% cytopathic effect, whereas those maintained under normoxia became 100% confluent with no visual evidence of cell lysis/detachment (Supple-

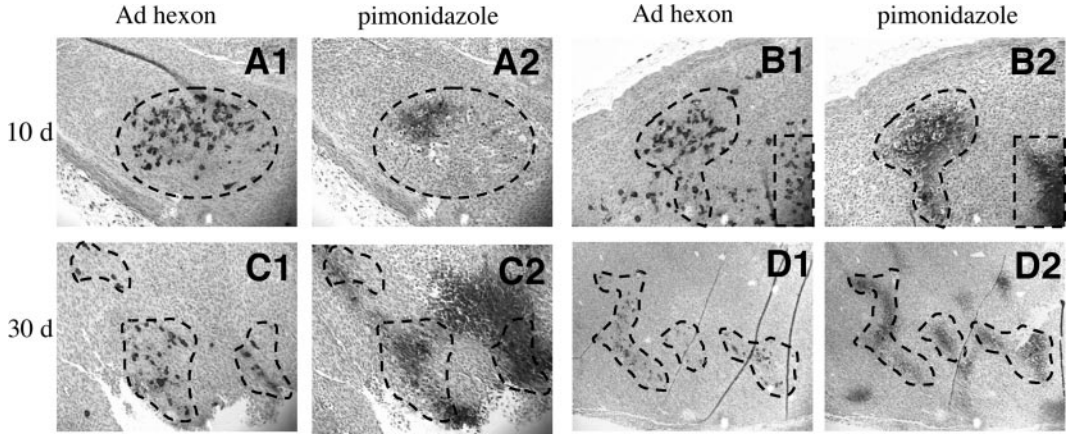

Fig. 3 Hypoxia-dependent replication of HYPR-Ad#1 in tumor xenografts. LN229 tumor xenografts were treated intratumorally with HYPR-Ad#1. On days 10 (*A* and *B*) and 30 (*C* and *D*) from the start of virus injection, mice were injected with pimonidazole, a marker used to detect areas of hypoxia ($<1\%$ O_2) *in vivo*. Tumors were analyzed by immunohistochemistry for adenovirus hexon coat protein expression (*A1-D1*) and pimonidazole (*A2-D2*) labeling. ×50 (*D*) and ×100 (*A–C*) magnification. Note overlay between areas (····) that show HYPR-Ad#1 presence and areas that evidence hypoxia in serially stained tumor sections.

mentary Fig. 1). In the *in vivo* experiment, all (8 of 8) of the mice in the mock-infected group developed tumors within 3 to 4 weeks of tumor cell implantation and reached an average size of 1296 mm^3 at 76 days when they had to be terminated due to Institutional Animal Care and Use Committee regulations. In contrast, 7 of 9 mice in the HYPR-Ad#1 group were tumor-free for 76 days, and the remaining 2 mice formed tumors that were 30-fold smaller than the control group (average size of 43 mm^3, $P < 0.0001$, Fig. 4*A* and Supplementary Table 1). Tumor growth in the HYPR-Ad#1 group was observed for another 30 days with 4 of 9 mice displaying tumor growth (average size: 46 mm^3, range: 4–180 mm^3). We then examined whether there was a qualitative difference in the number of HIF-expressing cells between control and HYPR-Ad#1-infected tumors by assaying for alkaline phosphatase activity (Fig. 4*B,* black precipitate). A reduction in alkaline phosphatase-expressing cells was seen in infected tumors compared with controls, consistent with the specific killing of hypoxic/HIF-active tumor cells by HYPR-Ad#1.

HYPR-Ad#1 Reduces Growth of Established Tumors. The capacity of HYPR-Ad#1 and controls dl309-Ad and PBS to reduce the growth of established tumors was examined using three aggressive experimental xenograft tumor models that are representative of the genetic and biological heterogeneity of human tumors (Figs. 5 and 6; ref. 20). Established s.c. LN229 glioma tumor xenografts were treated with HYPR-Ad#1, dl309-Ad, or PBS. Tumor growth was monitored for 8 weeks when animals had to be sacrificed due to the large size of the PBS-treated tumors. Treatment with HYPR-Ad#1 and dl309 adenoviruses greatly reduced tumor growth (Fig. 5*A*) and weight (Fig. 5*B*) compared with controls. Four of 4 PBS treated tumors displayed growth progression, whereas in the dl309-Ad treatment group, 1 of 4 tumors exhibited growth progression and 3 of 4 underwent regression to barely palpable size (Supplementary Table 2). The response to HYPR-Ad#1 treatment was variable with the tumors showing slow growth progression (3 of 5), stabilization (1 of 5), and regression (1 of 5; Supplementary Table 2). At termination the mean weight of tumors injected

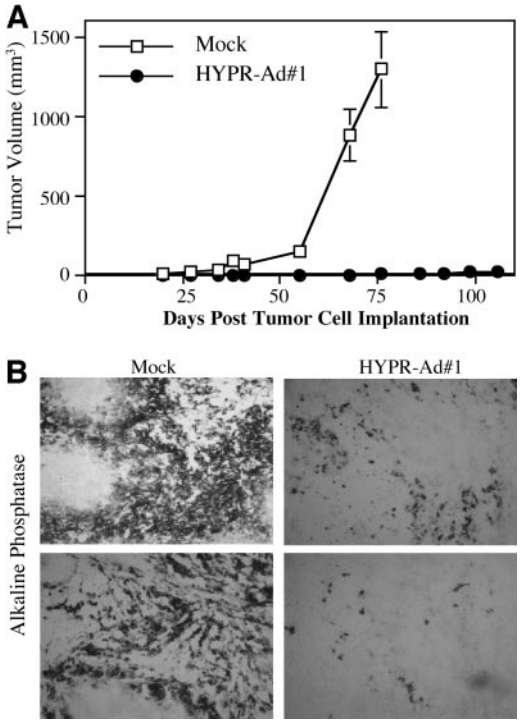

Fig. 4 HYPR-Ad#1 prevents tumor formation. LN229 cells containing a stably integrated *alkaline phosphatase* reporter gene construct under the regulation of the V6R-HRE promoter (*LN229-HRE-AP*) were pre-infected in culture at MOI 1 with HYPR-Ad#1 or mock infected. Twenty four hours later the cells were collected and injected s.c. in the flanks of *nu/nu* mice. *A.* Tumor formation and growth (*Mock:* $n = 8$ mice, *HYPR-Ad#1*: $n = 9$ mice) were monitored. *Bars,* ±SE are shown. *B.* Hypoxic tumor regions were visualized by assaying for alkaline phosphatase activity *in situ* (*black precipitate*) using the BCIP/NBT enzyme substrate. Photographs of two independent fields for each tumor group are shown. All four pictures were taken at ×50 magnification.

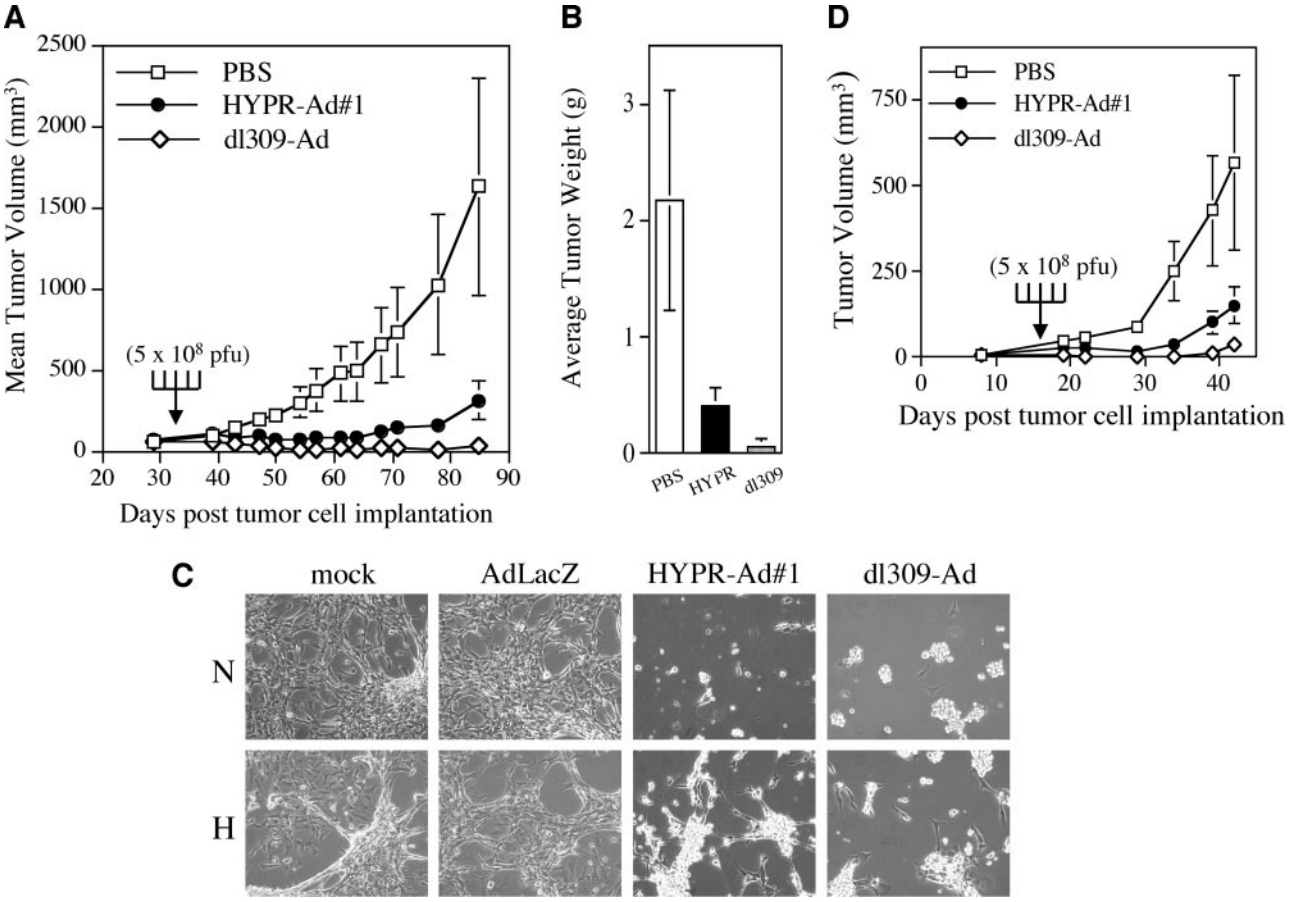

Fig. 5 HYPR-Ad#1 reduces the growth of established human tumor xenografts. *A.* Established s.c. LN229 tumors that have a hypoxia-activated HIF pathway were injected i.t. (*arrow*) with HYPRAd#1 ($n = 5$), dl309-Ad ($n = 4$), or PBS ($n = 4$), and tumor growth was monitored. *B,* the mean weight of harvested LN229 tumors. *C.* U87MG cells were infected at MOI 10 with AdLacZ, HYPR-Ad#1, dl309-Ad, or mock infected, and cell lysis (*CPE*) under normoxia (*N*) *versus* hypoxia (*H*) was monitored. *D*. Established s.c. U87MG tumors that have a constitutively active HIF pathway were injected intratumorally (*arrow*) with HYPR-Ad#1 ($n = 8$), dl309-Ad ($n = 5$), or PBS ($n = 8$), and tumor growth was monitored. For all of the graphs, *bars,* ±SE are shown.

with HYPR-Ad#1 was 5.3-fold smaller than PBS injected tumors and that of dl309-Ad injected tumors was 34-fold smaller than the PBS group. Similar results were observed in an independent repeat experiment (see Fig. 6*A*). These results are consistent with our hypothesis that HYPR-Ad#1 reduces tumor growth by specifically causing cytolysis of infected hypoxic tumor cells, whereas dl309-Ad would show additional efficacy by killing both normoxic and hypoxic cells.

Western blotting of U87MG total cell lysates showed a relatively high level of HIF-1α under normoxia, which could still be increased ~2- to 3-fold upon hypoxia exposure (Supplementary Fig. 2*A*). Given the multiple post-transcriptional levels of HIF regulation (11), we verified that the constitutively elevated levels of HIF-1α found in U87MG cells was biologically active by measuring the overall HIF (HIF-1α, 2α, and 3α) transcriptional activity in these cells using hypoxia/HIF-responsive luciferase reporters (ref. 21; Supplementary Fig. 2*B*). These data show that the U87MG cell line contains a constitutively activated HIF pathway. Consistent with these findings, HYPR-Ad#1 efficiently kills U87MG cells maintained under hypoxia as well as normoxia in culture, similar to the wild-type positive control virus dl309-Ad (Fig. 5*C*). As negative controls, uninfected and AdLacZ-infected cells show no visual evidence of cytopathic effect, demonstrating that the hypoxic and viral infection conditions are not cytotoxic. Treatment of established U87MG tumors with HYPR-Ad#1 and dl309-Ad reduced tumor volume by 3.7- and 15.5-fold, respectively, compared with PBS (Fig. 5*D,* day 42). All of the tumors (8 of 8) in the PBS treatment group exhibited growth progression, whereas HYPR-Ad#1 and dl309-Ad treatment resulted in evidence of growth stabilization or regression in some tumors (2 of 8 and 4 of 5 tumors, respectively; Supplementary Table 2). These data suggest that HYPR-Ad#1 will be effective at reducing the growth of tumors that have a constitutively activated HIF pathway. In summary, these preclinical studies of HYPR-Ad#1 demonstrate that it has antitumor activity against established malignant xenografts.

Combining HYPR-Ad#1 with BCNU Chemotherapy Results in Augmented Antitumor Efficacy. Next we evaluated whether combining HYPR-Ad#1-mediated oncolysis of hypoxic tumor cells with antitumor treatments that are effective

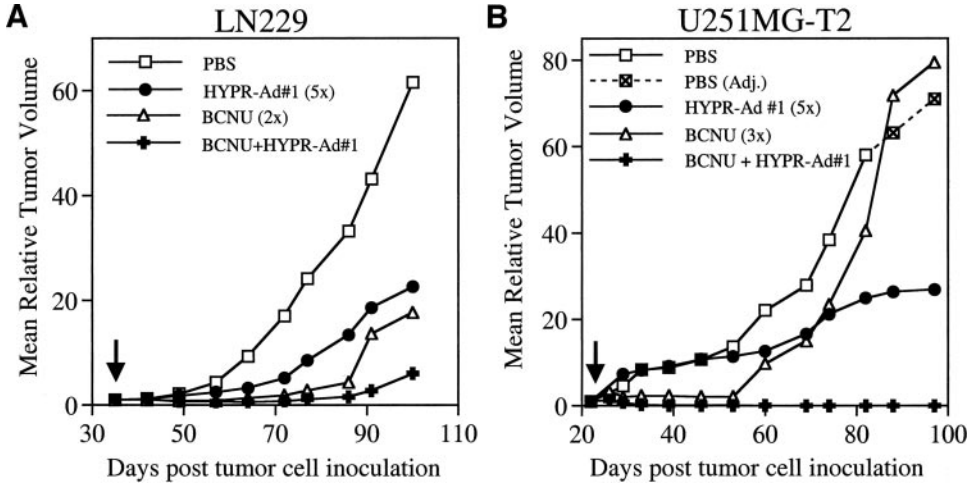

Fig. 6 Combining HYPR-Ad#1 with BCNU chemotherapy results in enhanced antitumor efficacy. *A*. Established s.c. LN229 tumor xenografts were treated intratumorally with HYPR-Ad#1 ($n = 8$; *arrow*), i.p. with BCNU (1×30 mg/kg $+1 \times 25$ mg/kg, $n = 6$), HYPR-Ad#1 + BCNU combined ($n = 8$), or PBS ($n = 6$). *B*. Established s.c. U251MG-T2 tumor xenografts were treated intratumorally with HYPR-Ad#1 ($n = 6$; *arrow*), i.p. with BCNU (3×30 mg/kg, $n = 3$), HYPR-Ad#1 + BCNU combined ($n = 4$), or PBS ($n = 6$). In the PBS group, 1 mouse was sacrificed on day 82 due to Institutional Animal Care and Use Committee regulations regarding tumor burden. The tumor volume of this mouse on day 82 was included in subsequent data points and is reflected by PBS Adjusted (*Adj*). For both studies, tumor volume was normalized to the volume at the start of treatment (*d 22*).

against normoxic cells would lead to a greater therapeutic effect. BCNU chemotherapy was chosen because it is a main clinical chemotherapy treatment for malignant gliomas. The dose and treatment schedule for BCNU was empirically determined. This was due in part to the lack of a standardized BCNU dosing schedule in the literature. The use of BCNU is additionally complicated by its dose-limiting toxicities and rapid progressive loss of anticancer activity at doses lower than the maximum tolerated dose (27, 28). Finally, the sensitivity of our glioma cell lines to the cell killing effects of BCNU was unknown. The *in vivo* therapeutic effect of combining HYPRAd#1 and BCNU treatment was evaluated in two models, LN229 (Fig. 6*A*) and U251MG-T2 (Fig. 6*B*). In the LN229 study, we chose a dose of BCNU that is nontoxic yet exhibits a measurable antitumor effect. Moreover, we selected a suboptimal therapeutic dose of BCNU that does not lead to tumor growth regression by itself, to observe an enhanced therapeutic effect of combined BCNU and HYPR-Ad#1 treatment. Established s.c. LN229 tumor xenografts were treated with HYPR-Ad#1, BCNU (1×30 mg/kg $+1 \times 25$ mg/kg, with a 4-day interval), HYPR-Ad#1 and BCNU combined, or PBS (Fig. 6*A*). There were no treatment-related deaths or observable toxicity (activity or weight change) in either the BCNU monotherapy or combined BCNU plus HYPR-Ad#1 groups. HYPR-Ad#1 and BCNU monotherapies similarly reduced LN229 tumor growth 3-fold compared with PBS-treated tumors. Combined HYPR-Ad#1 and BCNU additionally reduced LN229 tumor growth with a 10-fold size reduction compared with PBS-treated tumors, including 2 of 8 tumors showing complete regressions. These findings were confirmed in an independent model, U251MG-T2 cells, which contain a hypoxia-activated HIF pathway (18). In this study, an increased dose of BCNU (LD_{50}, 3×30 mg/kg, every 4 days; refs. 27, 28) was used to try to obtain stronger antitumor efficacy leading to tumor eradication (Fig. 6*B*). As expected, 50% of the animals in the BCNU group exhibited a decrease in weight (>25% loss) and activity after the third dose and had to be terminated due to Institutional Animal Care and Use Committee regulations. The remaining mice in the BCNU group exhibited either no ill effects or a transient decrease in weight and/or activity. Animals within the combination BCNU and HYPR-Ad#1 group exhibited a similar degree of death and toxicity as the BCNU group. Animals that died before the completion of the study were excluded from the final analysis. HYPR-Ad#1 monotherapy began to have a measurable antitumor effect at 60 days when it clearly reduced the exponential initiation of tumor growth. At termination (day 97), HYPR-Ad#1-treated tumors had a 2.6-fold reduced size compared with PBS-treated tumors. In contrast, BCNU treatment was highly efficacious at reducing initial tumor growth. This effect was short-lived, and precisely at the time PBS-treated tumors started exponential growth, BCNU-treated tumors regrew aggressively and rapidly surpassed the growth of HYPR-Ad#1-treated tumors. Combined treatment with HYPR-Ad#1 and BCNU resulted in a dramatic suppression of U251MG-T2 tumor growth for 100 days after treatment. These animals were kept alive for another 70 days, and no tumor regrowth was observed (data not shown), suggesting complete tumor eradication. These results demonstrate that combining HYPR-Ad#1 treatment with conventional chemotherapies that are efficacious against normoxic tumor cells leads to improved antitumor efficacy.

DISCUSSION

Hypoxia plays a critical role in driving tumor growth (29). The presence of hypoxic tumor cells hinders tumor eradication, because they produce survival factors such as glycolytic enzymes and proangiogenic factors, are resistant to conventional

radio- and chemotherapies, are resistant to proapoptotic stimuli due to defective apoptotic pathways, and are predisposed to cell invasion and metastasis (1–4, 10). HIF, the major transcriptional mediator of biological responses to hypoxia, is overexpressed in numerous cancer types and their metastases in response to intratumoral hypoxia and tumor-specific genetic alterations (11). New therapeutic strategies that could antagonize the protumoral effects of HIF and/or specifically target hypoxic/HIF-expressing cells are urgently needed. To address this need we designed HYPR-Ad#1, a conditionally replicative adenovirus that can specifically kill hypoxic/HIF-active tumor cells. This virus was engineered by placing the Ad *E1A* viral replication gene under the regulation of an exogenous hypoxia/HIF-responsive promoter. We tested the therapeutic potential of HYPR-Ad#1 in a model that is very representative of highly aggressive human tumors (malignant gliomas), which contain extensive areas of hypoxia (30) and HIF-positive tumor cells (6, 10).

We demonstrate that a HIF-dependent replicative adenovirus (HYPR-Ad#1) specifically replicates under hypoxia in both normal and tumor cells in culture and *in vivo* in tumor xenografts. Thus, the cytolytic effect of this virus is due to its restricted replication in hypoxic cells. In culture, HYPR-Ad#1 replication generally correlated with the hypoxia-dependent expression of E1A in these cells. Most importantly, virus localization within intratumoral-treated xenografts was in a patchy and uneven distribution throughout the tumor mass that closely overlapped with detectable hypoxic regions labeled with pimonidazole. This localization and pattern of distribution was maintained 30 days after treatment demonstrating the tight regulation of active viral replication to hypoxic microregions of the tumor. There were areas of hypoxia in the tumor mass in which no viral replication was evidenced. It is possible that our viral injection dose and/or schedule was not sufficient to disseminate the virus to all of the hypoxic regions within the tumor mass. Alternatively, there is evidence that murine tumor-supporting structures, such as connective tissue cells and noncellular matrix components, are a barrier to human adenovirus spread within tumor xenografts (31). Some of these obstacles might be overcome with convection-enhanced delivery.

Most importantly, we show that HYPR-Ad#1 monotherapy displays antitumor activity including the prevention of tumor formation and growth inhibition of established tumors using three genetically distinct malignant tumor models. These preclinical studies demonstrate that the virus antitumor activity is evidenced by a slower growth rate that results in a reduction of tumor volume by up to 5-fold. In some animals, viral treatment resulted in tumor growth stabilization or regression. The ability of HYPR-Ad#1 to reduce the growth of tumors that are histologically, biologically, and genetically distinct and possess either a hypoxia-activated or constitutively active HIF pathway indicates that all of the cancer types that contain hypoxic regions and/or transcriptionally active HIF are potential therapeutic targets, regardless of their tissue origin or genetic alterations. We anticipate that HYPR-Ad#1 will be particularly effective against central nervous system hemangioblastomas and clear cell carcinomas of the kidney, which contain a constitutively active HIF due to loss of the pVHL tumor suppressor protein function. pVHL directly binds to the α-subunit of HIF under normoxia and targets it for ubiquitin-mediated proteosomal degradation (11, 12). Whereas our studies clearly demonstrate the antitumor activity of HYPR-Ad monotherapy, this effect was not sufficient to eradicate the tumors and is consistent with its ability to specifically kill the hypoxic/HIF-active fraction of tumors.

This problem could be overcome by combining HYPR-Ad#1–mediated oncolysis of hypoxic tumor cells with an antitumor treatment that is effective against normoxic cells leading to a greater antitumor effect. Combination therapy of HYPR-Ad#1 with BCNU resulted in dramatic antitumor effects using two independent glioma models with 50% of tumors showing complete long-term regression. Similarly, combination therapy was also necessary to increase the therapeutic index of two independent hypoxia-targeted antitumor strategies using small molecules such as tirapazamine (32) and the genetically altered anaerobic bacteria *Clostridium novyi-NT* (COBALT; ref. 33). In addition, enhanced synergistic antitumor effects have been evidenced when oncolytic adenoviruses are combined with chemo- or radiotherapies (13). This is the first study demonstrating an enhanced antitumor effect when an oncolytic adenovirus is combined with BCNU chemotherapy. For the treatment of other tumor types with HYPR-Ad#1, it will be important to expand these studies to other chemotherapeutic agents and evaluate combining HYPR-Ad#1 with radiotherapy. Another strategy that we are actively pursuing to augment the virus antitumor effects is to arm it with an adjuvant therapeutic gene. HYPR-Ad design is modular with a bidirectional hypoxia/HIF-responsive promoter (Fig. 2*A*) that can be used to conditionally coregulate the expression of *E1A* and a therapeutic gene up to 2.8 Kb in length. The targeted delivery and expression of therapeutic genes in the tumor microenvironment is important, because the antitumor effects of conditionally replicating adenoviruses as single agents in clinical trials have been modest (13).

The data presented in this article establish the hypoxia/HIF-dependent replication and antitumor activity of HYPR-Ad#1. These findings now justify the initiation of preclinical safety and toxicity studies. It will be important to determine whether increased HIF transcriptional activity can occur under certain physiologic conditions, which would lead to local HYPR-Ad#1 replication at unwanted sites and subsequent cell death in normal tissues (34). This information will help in the design of clinical trials with regards to optimal risk to benefit ratio for patient subgroups and whether local or systemic delivery will be most beneficial. Currently available animal models are limited with regards to preclinical evaluation of human adenovirus safety and toxicity, because these viruses do not replicate in most murine cells. Despite this limitation, the antitumor testing of four oncolytic adenoviruses (ONYX-015, CN706, and CV787, Ad5-CD/TK*rep*) has moved from the laboratory to clinical trials (13). To date, treatment of cancer patients with oncolytic adenoviruses has demonstrated overall safety with minor toxicity of grade 1–2 flu symptoms and modest antitumor activity.

In conclusion, we have developed and shown the efficacy of a novel approach that specifically targets the hypoxic microenvironment of tumors. We designed an adenovirus-based oncolytic therapy using a tumor-restrictive hypoxia/HIF promoter to drive viral *E1A* gene expression and subsequent viral replication resulting in the targeted death of

hypoxic tumor cells. Our preclinical studies demonstrate that such a virus selectively replicates in hypoxic/HIF active cells thereby inducing cytolysis and has antitumor activity resulting in tumor growth reduction. These findings are a significant new development in the targeted treatment of hypoxic/HIF-active tumor cells and demonstrate that targeting the hypoxic fraction of a tumor with an oncolytic adenovirus leads to a measurable therapeutic effect by itself. In addition, this virus represents a new paradigm in which tumor-selective targeting does not rely on an intrinsic tumor cell property but rather targets the unique microenvironment that develops in tumors. This is a clear advantage, because viral replication will dynamically adapt to a changing milieu and will be less prone to cell intrinsic resistance mechanisms. This virus is suitable for the treatment of a variety of solid neoplasms independently of genetic composition or tissue origin. Moreover, its combination with chemotherapy results in enhanced cytotoxicity and augmented antitumor activity. These studies support the future clinical development and use of HYPR-Ads as a novel antitumor therapy against the hypoxic/HIF-active fraction of tumors and their incorporation into a multimodal cancer therapy regimen. This therapeutic strategy can be extended to targeting other physiologic parameters characteristic of the tumor microenvironment (pH, redox, or metabolic status).

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