Adenovirus-Based Strategies Overcome Temozolomide Resistance by Silencing the O\textsuperscript{6}-Methylguanine-DNA Methyltransferase Promoter

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Abstract
Currently, the most efficacious treatment for malignant gliomas is temozolomide; however, gliomas expressing the DNA repair enzyme O\textsuperscript{6}-methylguanine-DNA methyltransferase (MGMT) are resistant to this drug. Strong clinical evidence shows that gliomas with methylation and subsequent silencing of the MGMT promoter are sensitive to temozolomide. Based on the fact that adenoviral proteins directly target and inactivate key DNA repair genes, we hypothesized that the oncolytic adenovirus Δ24-RGD could be successfully combined with temozolomide to overcome the reported MGMT-mediated resistance. Our studies showed that the combination of Δ24-RGD and temozolomide induces a profound therapeutic synergy in glioma cells. We observed that Δ24-RGD treatment overrides the temozolomide-mediated G\textsubscript{2}-M arrest. Furthermore, Δ24-RGD infection was followed by down-modulation of the RNA levels of MGMT. Chromatin immunoprecipitation assays showed that Δ24-RGD prevented the recruitment of p300 to the MGMT promoter. Importantly, using mutant adenoviruses and wild-type and dominant-negative forms of the p300 protein, we showed that Δ24-RGD interaction with p300 was required to induce silencing of the MGMT gene. Of further clinical relevance, the combination of Δ24-RGD and temozolomide significantly improved the survival of glioma-bearing mice. Collectively, our data provide a strong mechanistic rationale for the combination of oncolytic adenoviruses and temozolomide, and should propel the clinical testing of this therapy approach in patients with malignant gliomas.

Introduction
At the moment, temozolomide is the mainstay of treatment for glioblastomas (1). However, a phase 3 evaluation of temozolomide combined with radiotherapy for newly diagnosed gliomas showed that the expression of the DNA repair enzyme O(6)-methylguanine DNA methyltransferase (MGMT; ref. 2) rendered these tumors resistant to temozolomide (3). Δ24-RGD is an oncolytic adenovirus that harbors a 24-bp deletion in the E1A region responsible for binding Rb protein (4) and presents enhanced infectivity due to the addition of an RGD-4C motif in the fiber HI loop (5). Δ24-RGD prevents the recruitment of p300 to the MGMT promoter. Importantly, using mutant adenoviruses and wild-type and dominant-negative forms of the p300 protein, we showed that Δ24-RGD interaction with p300 was required to induce silencing of the MGMT gene. Of further clinical relevance, the combination of Δ24-RGD and temozolomide significantly improved the survival of glioma-bearing mice. Collectively, our data provide a strong mechanistic rationale for the combination of oncolytic adenoviruses and temozolomide, and should propel the clinical testing of this therapy approach in patients with malignant gliomas.

Materials and Methods

Animal studies. U87 MG human glioma cells (5 \times 10\textsuperscript{5}) were engrafted into the caudate nucleus of athymic mice using a guide-screw system as previously described (8). Mice received a single intratumoral injection (day 3 postimplantation) of Δ24-RGD (10\textsuperscript{5} plaque-forming units/animal). Temozolomide was administered at 7.5 mg/kg during 5 days.

Immunohistochemical analysis. The paraffin-embedded sections of the mice brains were immunostained for antibodies specific for adenoviral E1A (Santa Cruz Biotechnology) and hexon (Chemicon International, Inc.) proteins following conventional procedures.

Cell lines and culture conditions. The glioma cell lines U87 MG and T98G were obtained from the American Type Culture Collection. Both cell lines were maintained in DMEM/F12 (1:1, vol/vol) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO\textsubscript{2} at 37°C.

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grafts and is currently awaiting clinical trial evaluation. Because each of the new pharmacologic and oncolytic viral therapies tested in gliomas shows no toxicity but generates only partial responses, glioblastoma treatment currently requires multimodal therapy. Interestingly, adenoviral proteins directly target key DNA repair genes in order to replicate efficiently (6, 7). We hypothesized that the combination of the oncolytic adenovirus Δ24-RGD and temozolomide would effectively overcome MGMT-mediated chemoresistance.

Cell viability assay. U87 MG or T98G cells were plated at a density of 5 \times 10\textsuperscript{3} cells per well in 96-well plates, and the next day, cells were infected with Δ24-RGD or UV-inactivated (UVi) at multiplicities of infection (MOI) of 0.1, 1, and 5. In addition, cells were treated with temozolomide at a concentration ranging from 0.01 to 1.00 μmol/L. Cell viability was assessed 7 days later using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich), as previously described (10). Dose-response curves were analyzed using CalcuSyn Software (Biosoft). CalcuSyn fits the dose-response curves to Chou-Talalay lines (11). IC\textsubscript{50} is the median-effect dose (the dose causing 50% of cells to be affected, i.e., 50% survival). After fitting the combined dose-response curve from a single representative experiment to a Chou-Talalay line, Chou-Talalay combination indices (CI) were calculated (12). A mean CI was calculated from data points with the fraction of cells affected at > 0.5 (13).

Immunoblotting. U87 MG and T98G cells were plated and 24 h later infected with Δ24-RGD (10 MOI for U87 MG cells and 25 MOI for T98G cells) alone or in combination with temozolomide (100 μmol/L for U87 MG cells and 300 μmol/L for T98G cells). Cells were harvested 36 h after the infection, and proteins were extracted and separated in a 10% SDS-PAGE, and the membranes were probed with antibodies for E1A (Santa Cruz Biotechnology) and fiber (NeoMarkers).

Viral replication assay. The tissue culture infection dose replication assay (TCID\textsubscript{50}) method was used to determine the final viral titration, as previously described (5). Final titers were determined as plaque-forming units, using the validation method developed by Quantum Biotechnology.
Cell cycle analysis. Cell cycle phase distribution was analyzed by measuring DNA content. Cell samples were collected at different time points after infection of U87 MG or T98G cells with Δ24-RGD (10 MOI) and/or treatment with temozolomide (100 μmol/L).

Alkaline comet assay. Cells were plated and the next day treated with Δ24-RGD (10 and 25 MOI for U87 MG and T98G cells, respectively) and/or temozolomide (100 or 300 μmol/L for U87 MG and T98G cells, respectively). Cells were harvested 48 h after treatment, and DNA damage was quantified using the alkaline comet assay kit (Trevigen, Inc.). Data are shown as the percentage of comet tails found per treatment (n = 500 cells per treatment).

Reverse transcriptase-PCR and RT quantitative-PCR. U87 MG and T98G cells were treated with temozolomide (100 or 300 μmol/L for U87 MG and T98G cells, respectively), Δ24-RGD (10 and 25 MOI for U87 MG and T98G cells, respectively), D24R (50 MOI), p300 (50 MOI), or AdTL2 (50 MOI) alone or in combination as indicated. Cells were collected 48 h after infection, and RNA was extracted using RNeasy (Qiagen) following the manufacturer’s directions and subjected to reverse transcription-PCR (RT-PCR) and RT-quantitative (RT-QPCR). MGMT mRNA was amplified using the same primers for both types of PCR and are published elsewhere (14). In both cases, input RNA was normalized with the amplification of glyceraldehyde-3-phosphate dehydrogenase RNA. Reaction products for the RT-PCR were resolved on 2% agarose gel. QPCR analysis was performed on a Chromo 4 sequence detection system (Bio-Rad). To determine relative gene expression, the comparative threshold cycle method was used (15).

Chromatin immunoprecipitation assay. Cells were treated with the indicated single or combined treatments. The chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit (Upstate) according to the manufacturer’s instructions. Cell lysates were collected 24 and 48 h after infection and immunoprecipitated with p300 antibody (Santa Cruz Biotechnology). MGMT promoter primers have been described elsewhere (16).

Statistical analysis. For the in vitro experiments, statistical analyses were performed using a two-tailed Student’s t test. Data are expressed as mean ± SD. The in vivo cytopathic effect of Δ24-RGD 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. The P values for pair-wise comparisons were calculated using the Bonferroni method.

Results and Discussion

We assessed the antiglioma efficacy of Δ24-RGD plus temozolomide in vivo using an orthotopic model of human glioma...
Figure 2. Characterization of the anticancer effect of the combination of temozolomide and Δ24-RGD in gliomas. A, examination of the cytopathic effect of temozolomide alone or in combination with Δ24-RGD. Cells were seeded at a density of 5 × 10^3 cells per well in 96-well plates. The next day, the cells were infected with D24R at a MOI of 0.1, 1, or 5 or UViD24R at a MOI of 5. Cells were treated with temozolomide at a concentration ranging from 0 to 2 × 10^{-3} μmol/L. Cell viability was assessed 7 d later. Points, mean of three independent experiments represented as cell viability relative to cells treated with UViD24R and temozolomide 0 μmol/L (equal to 100%); bars, SD. B, median-effect doses (IC_{50}) of temozolomide alone or in combination with Δ24-RGD. Cells were seeded at a density of 5 × 10^3 cells per well in 96-well plates and the next day were infected with Δ24-RGD at MOI of 0.1, 1, or 5 or with UVi-Δ24-RGD at 5 MOI. Where indicated, cells were treated with temozolomide at a concentration ranging from 0 to 2 × 10^{-3} μmol/L. Cell viability was assessed 7 d later using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. IC_{50} is the median-effect dose (the dose causing 50% of cells to be affected, i.e., 50% survival). C, interaction between temozolomide and Δ24-RGD in glioma cell lines. The range of CI corresponds with the different doses tested. Interaction was measured by combination index values. Combination index values >1.3 indicated antagonism, values between 1.1 and 1.3 indicated moderate antagonism, values between 0.9 and 1.1 indicated additivity, values between 0.8 and 0.9 indicated slight synergy, values between 0.6 and 0.8 indicated moderate synergy, values between 0.4 and 0.6 indicated strong synergy, and values <0.4 indicated strong synergy. Each combination was studied in three independent experiments, the differences of which were not statistically significant. Results of single experiments are shown. D24R, Δ24-RGD; UViD24R, UV-inactivated Δ24-RGD; TMZ, temozolomide; ND, nondetermined. D, analysis of the replication phenotype of Δ24-RGD in combination with temozolomide. Left, expression of early and late adenoviral genes. U87 MG and T98G cells were plated and 24 h later infected with Δ24-RGD (10 MOI for U87 MG cells and 25 MOI for T98G cells) alone or in combination with temozolomide (100 μmol/L for U87 MG cells and 300 μmol/L for T98G cells). Cells were harvested 36 h after the infection. Actin is shown as a loading control (representative immunoblot). Right, quantification of the replication phenotype of Δ24-RGD in combination with temozolomide. Glioma cell lines were plated and treated with Δ24-RGD (1 MOI for U87 MG cells and 5 MOI for T98G cells) alone or in combination with temozolomide (100 μmol/L for U87 MG cells and 300 μmol/L for T98G cells). Three days after infection, cell lysates were used to infect 293QBI cells. Viral titers were determined by the tissue culture infection dose-50 method, as described previously.
xenograft in immunocompromised mice. The median survival duration for animals treated with PBS in two independent studies was 30 and 35 days, respectively. Treatment with temozolomide alone extended the survival duration to a median 38 and 33.6 days, respectively. A single dose of Δ24-RGD administered in combination with temozolomide significantly prolonged the survival of these mice in comparison to animals treated with Δ24-RGD alone (P < 0.05, log-rank test for both experiments). Moreover, 50% of Δ24-RGD–treated mice were alive 50 days after tumor implantation. Importantly, 90% of the animals treated with the Δ24-RGD/temozolomide combination were alive at the end of both experiments (Fig. 1A and B). Next, we asked whether viral replication was impaired by the drug effect. To this end, three additional animals from each group were sacrificed 25 days after the treatment started. Animals treated with Δ24-RGD and temozolomide showed hexon expression levels indicating that viral replication capability was not compromised in the presence of temozolomide (Fig. 1C).

These in vivo data strongly suggest that the antitumoral effect exhibited by the tandem adenovirus/chemotherapy modality was more than simple additivity. To confirm the potential synergy between Δ24-RGD and temozolomide, we first quantified the anticancer effect of the combination of these agents in U87 MG and T98G glioma cells. We showed that the median-effect doses (IC50 = dose causing 50% of survival) of Δ24-RGD were 0.57 and 4.93 MOI in U87 MG and T98G cells, respectively, and that the IC50 of temozolomide was 204 and 1,585 μmol/L in U87 MG and T98G cells, respectively. Importantly, we observed a decrease of between three and five orders of magnitude in the IC50 of the drug in the combination treatment in both cell lines (Fig. 2A and B). The analysis of the combination index values showed that the association between temozolomide and Δ24-RGD globally resulted in a profound synergistic effect in both glioma cell lines (Fig. 2C).

Because temozolomide did not increase adenoviral replication (Fig. 2D), we next asked whether Δ24-RGD overcomes chemoresistance to temozolomide. Because cell cycle arrest in G2-M phase renders cells more resistant to temozolomide (17), we analyzed the cell cycle profile of U87 MG and T98G cells treated with temozolomide alone and in combination with Δ24-RGD (Fig. 3A). We observed that temozolomide treatment induced G2-M arrest but that the addition of Δ24-RGD was sufficient to override this arrest and resulted in over-representation of the S phase populations of U87 MG and T98G cells (60.8 ± 5.7%, P < 0.05 and 53 ± 95%; CI, 7.2, P < 0.005, respectively). Because the G2-M arrest as well as the cytotoxic mechanism of temozolomide are related to the induction of DNA damage (18), we next used the alkaline comet assay to determine whether the Δ24-RGD infection results in the impairment of DNA repair. We showed that T98G was less susceptible than U87 MG to temozolomide-induced DNA damage (24.1 ± 3.9% and 60.2 ± 4.2%, respectively); however, the percentage of T98G and U87 MG cells with comet tails increased significantly in cells treated with Δ24-RGD and temozolomide...
(80.1 ± 3%, P < 0.0001 in T98G, and 98.2 ± 6%, P < 0.0001 in U87 MG; Fig. 3B). These results suggest that the adenovirus-mediated abrogation of cell cycle arrest might hinder the extra allocation of time provided by the temozolomide-induced arrest that allows the DNA repair machinery to reverse the cytotoxic effect of the drug (17), thus eventually speeding the process of cell death.

It has been proven that the expression of MGMT in cancer cells creates a resistant phenotype by blunting the therapeutic effect of temozolomide in patients with glioblastoma (3). Therefore, we evaluated the role of MGMT expression in the resistance of U87 MG and T98G cells to temozolomide. We showed that the two cell lines expressed different levels of MGMT (Fig. 4A), with U87 MG cells expressing low basal levels of MGMT (due to partial methylation of the promoter) and T98G cells expressing high basal levels of MGMT (promoter is wild-type). Importantly, these differences were consistent with both sensitivity to temozolomide (Fig. 2A) and with the extent of DNA damage induced by temozolomide (Fig. 3B).

Because the adenovirus E1A protein binds to and inactivates CBP/p300 and by doing so strongly inhibits MGMT promoter activity (16), we assessed MGMT expression after treatment with Δ-24-RGD alone and in combination. As expected, temozolomide treatment induced the expression of MGMT in U87 MG and T98G cells (Fig. 4A). However, Δ-24-RGD treatment was followed by a drastic down-modulation of MGMT transcript in both glioma cell lines treated with temozolomide (Fig. 4A), suggesting a critical role of p300 in these phenomena. Overexpression of p300 resulted in the up-regulation of MGMT levels in control-infected cells (30-fold in U87 MG cells and >10-fold in T98G cells). The p300-mediated up-regulation of MGMT was drastically counteracted by Δ-24-RGD, with the levels of MGMT returning to basal levels in cells treated with both Δ-24-RGD and the adenoviral vector expressing p300. Further experiments confirmed that the expression of a dominant-negative form of p300 down-modulated the basal levels of MGMT (Fig. 4A). Accordingly, we showed that infection of cancer cells with a mutant E1A adenovirus (Δ-39; ref. 19) unable to bind to and inactivate p300 did not modify the level of expression of MGMT (Fig. 4A). Together, these data indicated that Δ-24-RGD down-modulates MGMT by means of the E1A binding to and inactivation of p300. To directly examine the effect of the physical interaction of the E1A/p300 complex with the promoter of MGMT, we performed ChIP assays. We showed that 24 h after treatment with temozolomide as single treatment or with Δ-24-RGD, p300 became a component of the protein complex recruited to the MGMT promoter in glioma cells (Fig. 4B). However, the recruitment of p300 to the MGMT promoter was disrupted 48 h after the infection in cells treated with Δ-24-RGD plus temozolomide (Fig. 2B). Our data therefore indicate that adenoviral infection is followed by E1A-mediated removal of p300 from the MGMT transcriptional machinery leading to silencing of the MGMT promoter.

Other oncolytic systems have been successfully combined with temozolomide (14, 20). Interestingly, herpes simplex virus

Figure 4. Role of Δ-24-RGD in overcoming temozolomide resistance in gliomas. A, E1A-mediated inhibition of MGMT expression. U87 MG and T98G cells were treated with temozolomide (100 or 300 μmol/L for U87 MG and T98G cells, respectively), Δ-24-RGD (10 and 25 MOI for U87 MG and T98G cells, respectively), D39 (50 MOI), p300 (50 MOI), or AdTL2 (50 MOI) alone or in combination as indicated. Cells were collected 48 h after infection, and RNA was extracted and subjected to RT-PCR (top) and RT-QPCR (bottom). In both cases, input RNA was normalized with the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Bars, SD (●, P < 0.001 and □ = 0.02). B, examination of the occupancy of the MGMT promoter by p300. Cells were treated with the indicated single or combined treatments. Cell lysates were collected 24 and 48 h after infection and immunoprecipitated with p300 antibody (Santa Cruz Biotechnology). Ten percent of the total cell lysates obtained prior to the immunoprecipitation were subjected to PCR to determine the chromatin input. D39, adenovirus Δ-39; p300, adenovirus p300; AdTL2, adenovirus expressing a dominant-negative form of p300.
replication benefits from the increase in the DNA damage repair machinery function triggered by the temozolomide treatment; meanwhile, adenoviruses need to shut down this machinery to achieve an effective replication.

In summary, our results indicate a synergistic therapeutic role between Δ24-RGD and temozolomide. This work also underscores the dominant role of MGMT expression in modulating resistance to temozolomide, to date, the most active brain tumor chemotherapy. Our study suggests that therapeutic strategies targeting molecular regulation of MGMT enhances the temozolomide anticancer effect and thus link clinical observations with feasible avenues of therapy. This study also provides a preliminary rationale for a logical continuation of the imminent phase I clinical trial of Δ24-RGD in patients with recurrent glioblastoma by proposing testing in the clinical scenario the combination of Δ24-RGD and temozolomide in patients with malignant gliomas.

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