Examination of the Therapeutic Potential of Delta-24-RGD in Brain Tumor Stem Cells: Role of Autophagic Cell Death

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The eradication of brain tumor stem cells is essential for long-term brain tumor remission after treatment. In this study, we examined the therapeutic potential of an oncolytic adenovirus, Delta-24-RGD, targeted to the abnormal p16INK4/Rb pathway in brain tumor stem cells. Four brain tumor stem cell lines from surgical glioblastoma specimens expressed high levels of adenoviral receptors and allowed for efficient viral infection, replication, and oncolysis in an Rb-dependent manner. Delta-24-RGD induced autophagic cell death, as indicated by accumulation of Atg5 and LC3-II protein and autophagic vacuoles. Treatment of xenografts derived from brain tumor stem cells with Delta-24-RGD statistically significantly improved the survival of glioma-bearing mice (means: 38.5 versus 66.3 days, difference = 27.8 days, 95% confidence interval = 19.5 to 35.9 days, *P*<.001). Analyses of treated tumors showed that Atg5 expression colocalized with viral fiber protein and delineated a wave front of autophagic cells that circumscribed areas of virally induced necrosis. Our results show for the first time that brain tumor stem cells are susceptible to adenovirus-mediated cell death via autophagy in vitro and in vivo.

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The brain tumor stem cell hypothesis proposes the existence of multipotent glioma cells of origin that are characterized by the expression of stem cell markers and by the capacity for self-renewal, multilineage differentiation, and reestablishment of tumors after transplantation (1–5). An implication of the brain tumor stem cell model is that brain tumor stem cells are resistant to radiation and chemotherapy and may therefore be responsible for tumor recurrence (6,7). Because adenoviral proteins can completely overcome the molecular machinery of the infected cell, we hypothesized that Delta-24-RGD, an oncolytic adenovirus with enhanced tropism to glioma cells and selective replication in cancer cells with an abnormal Rb pathway (8,9), may act as a potent therapeutic agent to target brain tumor stem cells and prevent them from developing resistance to other forms of therapy. However, adenoviral receptor expression, infectibility, the susceptibility to adenoviral replication, as well as the characteristics of adenovirus-mediated cell death have not previously been examined in cancer stem cells.

In this study, we isolated neurosphere-forming cells from four fresh surgical specimens of glioblastoma multiforme (3) (Fig. 1, A). These cells exhibited the in vitro stem cell characteristics of extensive self-renewal (more than five passages in culture) and the ability to differentiate to neurons and astrocytes (Supplementary Fig. 1, available online). Flow cytometric analyses showed that 20%–80% of the cells expressed the neural stem cell protein CD133, which was recently identified as a potential brain tumor stem cell marker in brain cancer (1) and in other solid tumors (10,11) (Fig. 1, A). When clonally derived, these cells initiated new tumors when transplanted into the basal ganglia of immunodeficient mice (Supplementary Fig. 2, available online). Because Delta-24-RGD is targeted to Rb-deficient cells (8,9), we examined the levels of Rb and p16INK4a proteins, whose expression is mutually exclusive in glioblastoma multiforme (12,13), in brain tumor stem cells. As reported for glioblastoma multiforme, immunoblotting analyses showed that the expression of either Rb or p16 protein was absent in these cell lines (Fig. 1, B). Next, using flow cytometric analysis, we demonstrated that the cell lines expressed high levels (>50% positive cells) of coxsackie–adenovirus receptor, the main adenoviral receptor required for virus attachment (14), and/or Arg-Gly-Asp (RGD)–recognizing integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ for virus internalization (15) (Fig. 1, C). Consequently, the four glioma stem cell lines were susceptible to adenoviral infection (Supplementary Fig. 3, A, available online). Accordingly, treatment of the cell lines with Delta-24-RGD resulted in a drastic reduction in cell viability, and at 6 days after viral infection, the dose inducing 50% cell death (ID50) was less than 2 pfu/cell in the majority of the cell lines, as assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Fig. 1, D). As expected, pretreatment with Rb protein resulted in rescue of the viability of Delta-24-RGD–infected cells that was due

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to the restriction of an efficient replication phenotype (Fig. 1, E and F; Supplementary Fig. 3, B, available online).

The pathways involved in adenovirus-mediated cell death remain unclear. Here, we showed that Delta-24-RGD induced the formation of acidic vesicular organelles in the four cell lines ($P<.001$) (Fig. 2, A; Supplementary Fig. 4, A, available online). The Delta-24-RGD–induced acidic vesicular organelles were further confirmed by the dramatic change in the ratio of the cytosolic LC3-I to membrane-bound LC3-II (Supplementary Fig. 4, B, available online), a modification that is essential for the formation of autophagosomes (16), in the treated cells. These data were consistent with a previous report showing adenovirus-induced autophagy in two established glioma cell lines (17). The observation of increased levels of biochemical markers and cellular acidic vesicular organelles was strongly supported by the direct demonstration of cytoplasmic autophagic vacuoles in cells treated with Delta-24-RGD using electron microscopy (Fig. 2, B). To examine the activation of proautophagic signaling by Delta-24-RGD infection, we first examined the protein levels of beclin1, a type III PI3 kinase–interacting protein that participates in the induction of autophagy (18). Western blot analyses showed no differences in the levels of beclin1 in treated versus untreated cells (Supplementary Fig. 4, B, available online). We next analyzed the protein levels of Atg5, a key molecule in the conversion of LC3-I to -II and therefore required for autophagosome formation and autophagic cell death (19), during the Delta-24-RGD replication cycle. We observed a remarkable induction of endogenous Atg5 expression that was noticeable by 48 hours after treatment and was increased by six- to eightfold 72 hours after the treatment, the latest time point examined (Fig. 2, C; Supplementary Fig. 4, B, available online). The timing of Atg5 expression suggested that activation might link Delta-24-RGD–mediated cell lysis to autophagic cell death.

We next examined the anti-tumor efficacy of Delta-24-RGD in intracranial xenografts that were derived from MDNSC11 cells in athymic mice. The mean survival time of control-treated MDNSC11-glioma–bearing mice was 38.5 days (95% confidence interval [CI] = 35.6 to 41.4 days), in contrast to a mean survival time of 66.3 days (95% CI = 55.2 to 77.3 days, the largest observed analysis time is censored, the mean is underestimated) in mice treated with Delta-24-RGD (difference = 27.8 days, 95% CI = 19.5 to 35.9 days; $P<.001$) (Fig. 2, D) with two of eight (25%) mice remaining alive without noticeable neurologic deficits until they were killed at day 92 (Fig. 2, D). Microscopic examination of the brain tissues of control mice with MDNSC11 xenografts revealed highly infiltrative tumors that recapitulated the histopathology of glioblastomas including hypercellularity, hypervascularity, and necrotic areas surrounded by cells in a pseudopalisading distribution (Supplementary Fig. 2, available online). Immunohistochemical staining of the tumor from the Delta-24-RGD–treated group revealed the expression of E1A and hexon indicating efficient adenoviral infection and replication in vivo (Supplementary Fig. 5, available online). Importantly, immunofluorescence analyses identified high levels of expression of the proautophagic protein Atg5 (Fig. 2, E). Atg5 colocalized with fiber protein and displayed a pattern of expression that clearly defined a tumor zone immediately adjacent to the necrosis area (Fig. 2, E). No other area in the tumor or any area of untreated tumors was positive for Atg5 (data not shown). Therefore, Atg5 appears to be useful to identify the adenoviral wave front of spread and may be used as a cellular indicator of viral activity and a surrogate marker of the anti-glioma effect.

In summary, our results show for the first time, to our knowledge, that brain tumor stem cells are susceptible to adenovirus-mediated cell death via autophaghy in vitro and in vivo. Our data are important because brain tumor stem cells are the driving force sustaining tumor growth (1,3), and therefore developing therapies to target the brain tumor stem cells should be a more effective strategy than conventional treatments. We reported previously that Delta-24-RGD displays efficacious anti-glioma activity in the models based on established malignant glioma cell lines (9). Together, our previous study and this study indicate that Delta-24-RGD efficiently eliminates both brain tumor stem cells and tumor mass cell populations in gliomas. An alternative theory to the cancer stem cell model hypothesizes that mutant dedifferentiated astrocytes are responsible for the emergence and phenotype of high-grade gliomas (20). Nevertheless, despite the origin of these tumor-initiating cells, the resulting phenotype of the cancer cell is probably similar (20), and the universal disruption of the Rb pathway renders them susceptible to Delta-24-RGD (Fig. 1, B; 8,9,20). Furthermore, the in vivo assessment of adenovirus-induced autophagy may be a useful way to monitor oncolytic adenovirus efficacy in future clinical trials.

CONTEXT AND CAVEATS

Prior knowledge

To achieve long-term remission after treatment for brain cancer, it is necessary to eradicate brain tumor stem cells that are resistant to radiation and chemotherapy.

Study design

The therapeutic potential of oncolytic adenovirus Delta-24-RGD targeted to brain tumor cells was tested in vitro using cell lines with stem cell properties that were derived from gliobastoma multiforme tumors and in vivo using a mouse xenograft tumor model.

Contribution

The four cell lines were efficiently infected with an oncolytic adenovirus Delta-24-RGD, which induced autophagic cell death. Mice carrying xenograft tumors that were derived from one of the cell lines survived longer after treatment with Delta-24-RGD than with an inactivated form of the virus.

Implications

Adenovirus-mediated autophagic cell death can be induced in brain tumor cells with properties of stem cells.

Limitations

It is unclear how similar the cell lines developed in this study are to the brain tumor stem cells that exist in human brain cancer or whether the oncolytic adenovirus developed in this study would be efficacious and safe in humans.

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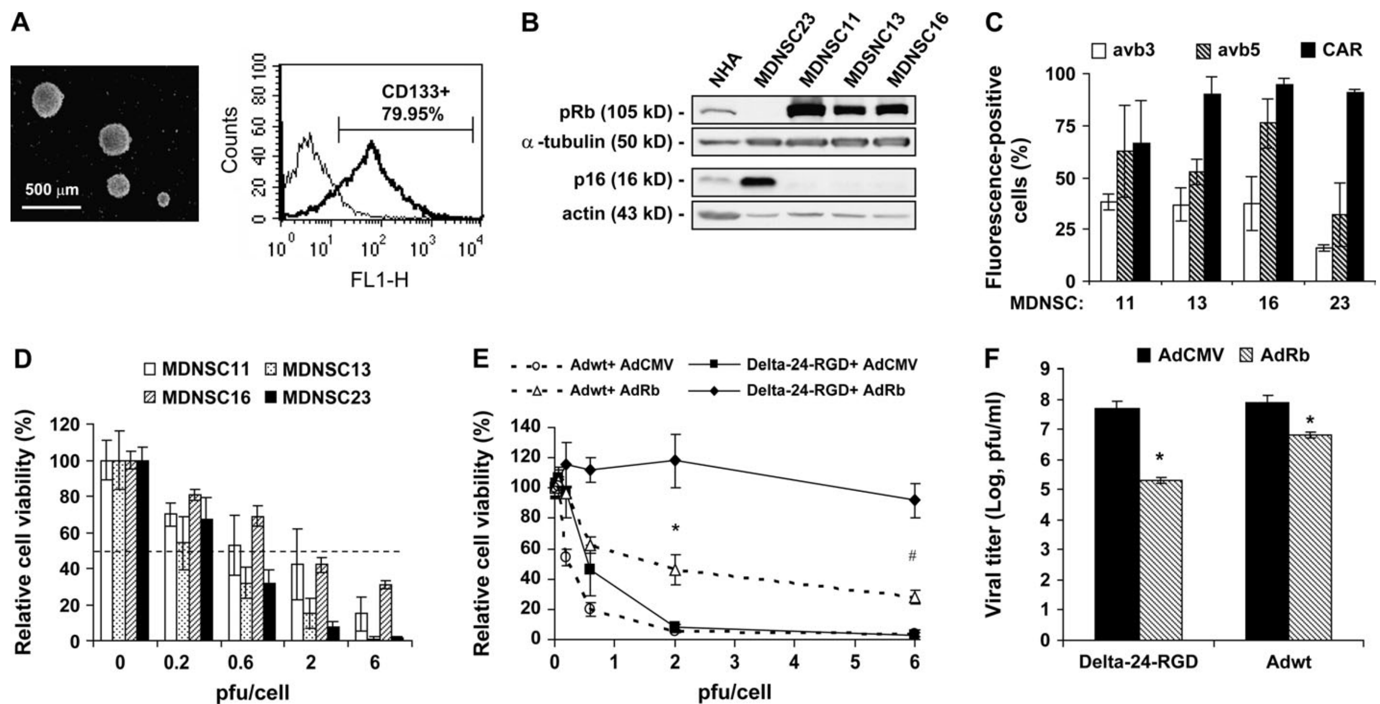

Fig. 1. Delta-24-RGD–mediated cytotoxicity in cell lines with properties of brain tumor stem cells. **A**) Neurosphere cultures were established from acute cell dissociation of four human glioblastoma multiforme surgical specimens (2,3) and maintained in Dulbecco's modified Eagle medium/F12 medium supplemented with B27 (Invitrogen, Carlsbad, CA), epidermal growth factor, and basic fibroblast growth factor (20 ng/mL each, Sigma–Aldrich, St Louis, MO) according to the procedures described elsewhere (2,3). The study was approved by the Institutional Review Board at University of Texas M. D. Anderson Cancer Center. Written informed consent was obtained from every patient. Shown are results from one representative of four experiments. **Left**, representative image of formation of typical neurospheres in MDNSC16 cultures, phase-bright microphotograph. **Right**, CD133 expression in MDNSC16 cells. The cells were dissociated by exposure to accutase solution (Sigma–Aldrich) and pipetting and stained with monoclonal antibody specific for CD133 (1:10 dilution; Miltenyi Biotec, Auburn, CA) and then with fluorescein isothiocyanate (FITC)–labeled secondary antibody (anti-mouse IgG, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) according to the instructions provided by the manufacturer. After staining, the cells were analyzed by flow cytometry for cell surface expression of CD133 with FacsCalibur (Becton Dickinson, San Jose, CA). **B**) Expression of Rb protein (pRb) and p16INK4a (p16) protein in the cell lines. Normal human astrocytes (NHAs) were purchased from Cambrex Bio Science Walkersville, Inc (Walkersville, MD) and maintained in astrocyte growth medium prepared from an AGM-Astroctye Medium BulletKit (Cambrex Bio Science Walkersville, Inc) as instructed by the manufacturer. Western blots were performed as described previously (9). Briefly, equal amount of proteins from whole-cell lysates were separated by 8% (pRb) or 15% (p16) sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes, and the membranes were probed with rabbit polyclonal anti-pRb (1:200 dilution), goat polyclonal anti-p16INK4a (1:100 dilution), goat polyclonal anti-actin (1:1000 dilution) (Santa Cruz Biotechnology), and mouse monoclonal anti–α-tubulin (1:4000 dilution; Sigma–Aldrich). The protein–antibody complexes were visualized using the enhanced chemiluminescence western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). α-tubulin and actin were used as loading controls. One representative experiment of two is shown. **C**) Flow cytometric analysis of αvβ3, αvβ5 integrins and coxsackie–adenovirus receptor (CAR) expression in the cell lines. The analysis was performed as described previously (9). Dissociated cells (5×10^5) were stained with mouse monoclonal anti-αvβ3 (1:200 dilution; Chemicon International, Inc, Temecula, CA), anti-αvβ5 (1:400 dilution; Chemicon International, Inc), or anti-CAR (1:2000 dilution; Upstate Biotechnology, Lake Placid, NY) and then with FITC-labeled secondary antibody goat anti-mouse IgG (1:100 dilution, Santa Cruz Biotechnology). The stained cells were analyzed with flow cytometry for cell surface expression of viral receptors using a FacsCalibur flow cytometer (Becton Dickinson). Three independent experiments were performed. Data are shown as the mean (and 95% confidence intervals [CIs]) percentage of cells expressing the receptors. **D**) Cytotoxicity induced by Delta-24-RGD in the cell lines. The cells were dissociated and plated in 96-well plates at 2×10^4 cells per well and were immediately infected with Delta-24-RGD at the indicated doses. Six days after infection, cells were dissociated with accutase solution (400–600 U/mL, Sigma–Aldrich) and by pipetting before cell viability was assessed using the MTS assay according to the manufacturer's instructions (Promega Life Science, Madison, MI). The experiments were performed once in triplicate. Data are shown as mean (and 95% CIs) percentage of viable cells relative to that of mock-treated cells (equal to 100%). **E**) Effect of pRb restoration on the Delta-24-RGD–mediated cytotoxicity in MDNSC23 cells. The cells were infected with replication-deficient adenovirus expressing Rb protein, AdRb, or the control E1A-deleted virus AdCMV at 150 pfu/cell, as previously reported (9). Forty-eight hours later, cells were plated (2×10^4 per well) in 96-well plates and infected with Delta-24-RGD or wild-type adenovirus (Adwt) at the indicated doses. Six days later, cell viability was determined using the MTS assay as described in (**D**). The experiments were performed once in triplicate. Data are shown as the mean (and 95% CIs) percentage of viable cells relative to that of mock-treated cells (equal to 100%). At viral dosages of 2 and 6 pfu/cell, the Rb-mediated rescue of the cytotoxicity induced by Delta-24-RGD treatment is statistically significantly greater than the Rb-mediated rescue in cells treated with Adwt (*P = .01, #P = .03, two-tailed Student's *t*-test). **F**) Effect of pRb restoration on the replication of Delta-24-RGD in MDNSC23. Cells were infected with AdRb or control virus AdCMV at 150 pfu/cell, as described in (**E**). Forty-eight hours later, the cells were infected with Delta-24-RGD or Adwt at 1 pfu/cell. The titers of the viral progenies were determined 72 hours after viral infection using the tissue culture infection dose$_{50}$ method, as described previously (9). Shown are the titers of viral progenies from 1×10^5 cells in 1 mL infected with the indicated viruses. The experiments were performed once in triplicate. Means (and 95% CIs) are shown. The Rb-mediated restriction on Delta-24-RGD replication is statistically significantly greater than that on Adwt in MDNSC23 cells (*P = .009, two-tailed Student's *t*-test).

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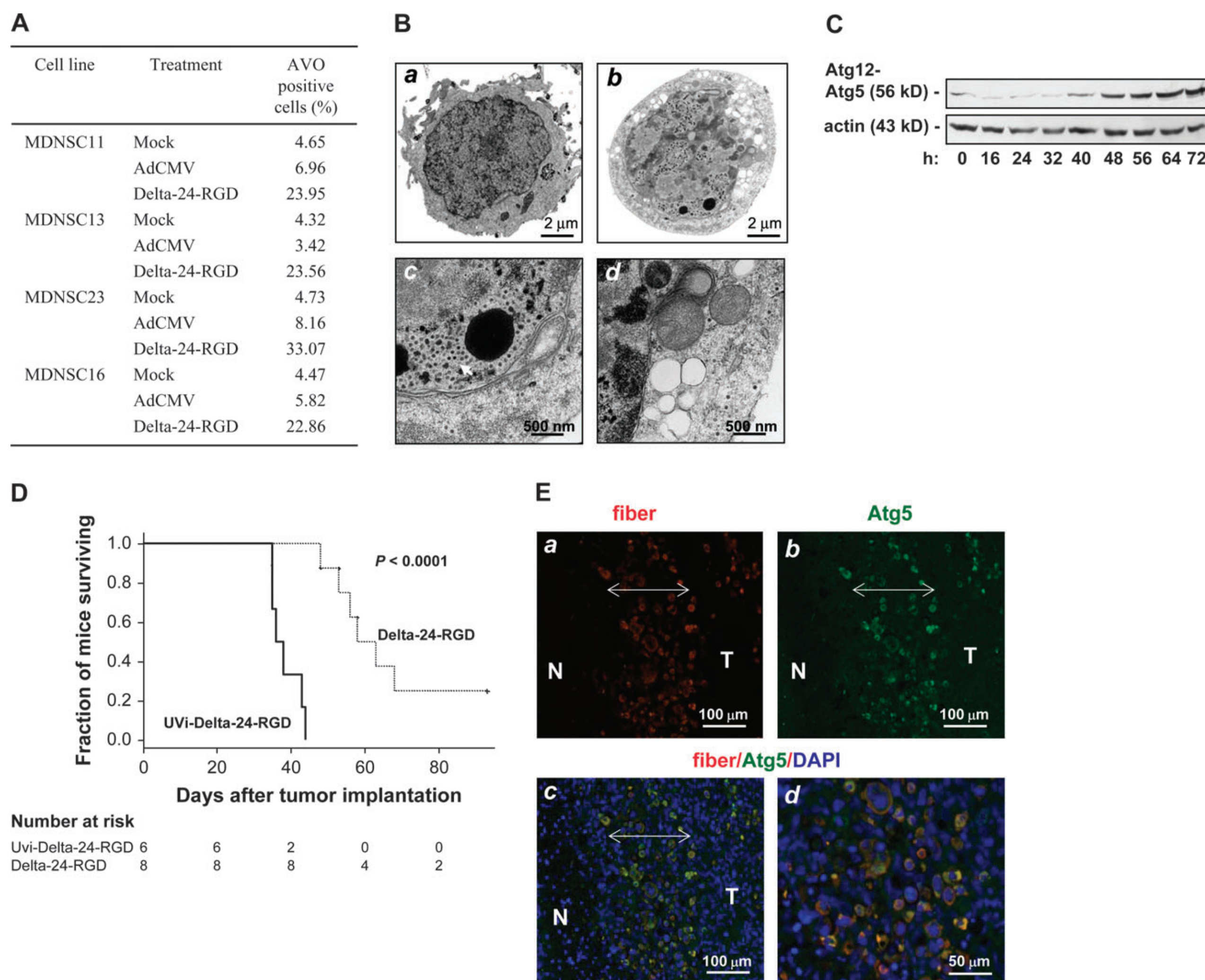

Cell line	Treatment	AVO positive cells (%)
MDNSC11	Mock	4.65
	AdCMV	6.96
	Delta-24-RGD	23.95
MDNSC13	Mock	4.32
	AdCMV	3.42
	Delta-24-RGD	23.56
MDNSC23	Mock	4.73
	AdCMV	8.16
	Delta-24-RGD	33.07
MDNSC16	Mock	4.47
	AdCMV	5.82
	Delta-24-RGD	22.86

Fig. 2. Examination of the in vitro and in vivo Delta-24-RGD–induced autophagy. **A**) Induction of acidic vesicular organelles (AVOs) by Delta-24-RGD in cell lines with properties of brain tumor stem cells. Spheroids were dissociated and immediately infected with the indicated virus at 10 pfu/cell. Seventy-two hours later, cells were dissociated 2 hours before acridine orange (1µg/mL; Sigma–Aldrich) was added to the medium for 15 minutes. Percentage of cells presenting the formation of AVOs were quantified by flow cytometry as previously described (17). AdCMV was used as a control for the viral infection. Note that Delta-24-RGD induced a statistically significant increase of the percentage of AVO-positive cells in all cell lines tested compared with AdCMV (means: 25.8% versus 6.1%, difference = 19.7%, 95% confidence interval = 15.0% to 24.3%, $P<.001$, randomized block two-factor analysis of variance). **B**) Representative electron micrographs showing the ultrastructure of the mock-infected (**a**) and Delta-24-RGD–infected (**b**) MDNSC11 cells. Note the vacuoles in the virus-infected cells but not in the untreated cells. Close-ups of Delta-24-RGD–infected cell illustrated in **b** show the cluster of the progenies of Delta-24-RGD (**white arrow**) in the nucleus (**c**) and complex autophagic multivacuolar bodies in the cytoplasm (**d**). Representative images from 20 cells are shown. **C**) Kinetics of Atg12-Atg5 protein expression during Delta-24-RGD infection. MDNSC11 cells were collected at the indicated time points after Delta-24-RGD infection, and equal amounts of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose membranes, and the membranes were probed with rabbit polyclonal anti-Atg5 (1:2000 dilution; a kind gift from Dr N. Mizushima, National Institute for Basic Biology, Japan) and goat polyclonal anti-actin antibodies. The protein–antibody complexes were visualized as described (in Fig. 1, B). Actin was used as a loading control. Data from one representative experiment of two are shown. **D**) Kaplan–Meier curves of overall survival of Delta-24-RGD–treated (n = 8) and UV-inactivated (UVi) Delta-24-RGD–treated (n = 6) athymic mice with MDNSC11 intracranial xenografts. Cells (1.0×10^6) were grafted into the caudate nucleus of the athymic mice using a guide-screw system as previously described (9). On days 7, 9, and 11 after tumor cell implantation, mice were intratumorally injected with 10 µL of Delta-24-RGD or UVi-Delta-24-RGD (2.5×10^8 pfu). Survival in the two treatment groups was compared using the log-rank test (two-sided). All mouse studies were performed in the veterinary facilities of University of Texas M. D. Anderson Cancer Center in accordance with institutional, state, and federal laws and ethics guidelines for experimental animal care. **E**) Viral replication and autophagy in vivo. Immunofluorescence analysis of viral fiber and Atg5 proteins expression in the brain of a mouse from the study represented in (**D**) that was treated with Delta-24-RGD and died 59 days after implantation. The paraffin-embedded section of the mouse brain was double immunostained with mouse monoclonal antibodies specific for adenoviral fiber protein (4D2, 1:500 dilution; Lab Vision, Fremont, CA) (**a**) or rabbit polyclonal Atg5 (1:200 dilution) (**b**) and then with Texas Red (fiber)– or Alexa Fluor 488 (Atg5)–conjugated secondary antibodies (1:500 dilution; Molecular Probes, Inc, Eugene, OR). Fluorescence staining for fiber and Atg5 were merged and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining was used to visualize the cell nuclei (**c**). Expression of both proteins was positive (**double-headed arrow**) within the tumor (**T**) surrounding necrotic areas (**N**). Note, in a close-up of (**c**), that the viral and the cellular protein are localized in the same cells, around the cells that exhibit virally induced necrosis (**d**). These are the representative images from the brains of two mice treated with Delta-24-RGD.

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