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REVIEW ARTICLE

Oncolytic adenoviruses: A thorny path to glioma cure

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Received 8 September 2014; accepted 24 September 2014

Available online 13 October 2014

KEYWORDS

Adenovirus;
Brain tumor;
Glioma;
Self-replicated
vector;
Stem cells

Abstract Glioblastoma Multiforme (GBM) is a rapidly progressing brain tumor. Despite the relatively low percentage of cancer patients with glioma diagnoses, recent statistics indicate that the number of glioma patients may have increased over the past decade. Current therapeutic options for glioma patients include tumor resection, chemotherapy, and concomitant radiation therapy with an average survival of approximately 16 months. The rapid progression of gliomas has spurred the development of novel treatment options, such as cancer gene therapy and oncolytic virotherapy. Preclinical testing of oncolytic adenoviruses using glioma models revealed both positive and negative sides of the virotherapy approach. Here we present a detailed overview of the glioma virotherapy field and discuss auxiliary therapeutic strategies with the potential for augmenting clinical efficacy of GBM virotherapy treatment.

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List of abbreviations: GBM, glioblastoma multiforme; Ad, Adenovirus; DNA, Deoxyribonucleic Acid; RNA, Ribonucleic acid; Wt, wild type; CSC, Cancer stem cells; GSC, Glioma stem cells; IFN, interferon; FACS, Fluorescent Assisted Cell Sorting; PCR, Polymerase chain reaction; RT-PCR, Reverse Transcriptase Polymerase Chain Reaction; mRNA, Messenger mRNA; MLP, Major Late Promoter; CMV, cytomegalovirus.

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Peer review under responsibility of Chongqing Medical University.

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<http://dx.doi.org/10.1016/j.gendis.2014.09.009>

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Glioma as a target for gene therapy

Glioblastoma Multiforme (GBM) is the most common primary brain cancer in humans. In the cancer hierarchy, patients with brain tumors represent a relatively small cohort with an estimated 500,000 total cases diagnosed, and 20,000 – deaths reported annually. The incidence of GBM has risen¹ lately in Europe and North America with 3.19 cases per 100,000 patients diagnosed yearly² in the US alone. While current GBM diagnostic techniques have improved tumor detection sensitivities, the average survival is a dismal 16–20 months, and less than 20% of GBM patients survive more than 5 years after diagnosis.³

Histologically GBM can be defined as a tumor of astrocytes, which represent 80% of normal brain tissue. Astrocytomas are characterized based on several parameters, such as tumor localization in the brain, molecular features and invasiveness. According to the WHO classification, there are 4 different stages of brain cancer progression (where stage 4 is the most advanced), based largely on cell differentiation markers. Transition of astrocytoma grade 2–grade 4 (GBM) is associated with changes in cellular signaling pathways such as TP53, EGFR, PTEN, etc. Also, it is well documented that during this transition astrocytomas incur genomic deletions (IDH1, PTEN), which activate various signaling pathways responsible for new aggressive phenotypes. Based on such genomic rearrangements Verhaak et al. grouped gliomas into mesenchymal, classical, neural, and proneural subtypes.⁴ Each glioma subtype is characterized by a specific gene expression pattern that ultimately determines the tumor behavior.

Another constituent of glioma tumors is glioma stem cells (GSCs), or cancer stem cells, which demonstrate the ability to form tumors upon intracranial injection. Cancer stem cells are capable of forming spherical structures *in vitro*, called neurospheres, which may account for both chemo- and radioresistance of glioma tumors in patients.^{5,6} Stem cell properties have been ascribed to those cells based on their capability to maintain the tumor cell population, which implicates them in tumorigenesis and tumor recurrence mechanisms.^{7,8} It remains unclear whether the differentiation of cancer stem cells into a tumor requires environmental factors to accelerate tumorigenesis. However, scientific reports in the last 10 years suggest that one of the most devastating human cancers, such as glioma, originates from neural progenitor cells with a strong proliferative capability. Moreover, infection of progenitor cells with cytomegalovirus (CMV) significantly promotes progression of glioma in mouse experimental models of the disease.⁹ Additionally, a growing body of evidence suggests that both immunotherapeutic¹⁰ and chemotherapeutic¹¹ approaches targeting CMV improve overall patient survival. This data points towards CMV as a new potential etiological factor of GBM progression, representing an ideal target for gene therapy.

Gene therapy is an alternative approach for glioma therapy

Treatment of extremely vascularized tumors, such as gliomas is very challenging. The standard of GBM patient care

includes surgical resection, radiation, and chemotherapy. Although, temozolomide, bevacizumab and carmustine provide longer survival times, neither drug prevents glioma recurrences, mainly due to the activation of a mechanism that enables immune evasion and causes drug resistance. For instance, a recent study suggests that bevacizumab treatment promotes tumor invasion via activation of MMP2,¹² while other scientific reports implicate activation of the mTOR pathway.^{13,14} This is one of the key pathways responsible for the induction of cellular autophagy, which negatively affects glioma cells and triggers an inflammatory response. The fact that glioma stem cells (GSCs) cannot be targeted and destroyed by chemotherapy and radiation implicates them in the observed resistance of gliomas to traditional therapies, which makes treatment of the disease extremely challenging. Therefore, there is an urgent need for a new therapeutic approach with an improved efficacy that would target both the tumor cell, and the stem cell components of gliomas. Such new therapeutic approaches should target GSCs, while simultaneously comprising the existing therapeutic options, such as ionizing radiation and temozolomide. Since conventional chemotherapeutic agents exhibit strong toxicity towards cancer cells, and in most cases do not spare normal cells, cancer gene therapy seems promising with regard to its higher potential specificity and efficacy. Cancer gene therapy, therefore, is a unique approach capable of utilizing a multifunctional platform for tumor targeting, imaging, and gene delivery. This approach is based on the design of vectors capable of delivering any payload to the tumor cells using various injection routes. Viral vectors exhibit great advantages over non-viral means of gene delivery owing to their natural capability of highly efficient cell attachment and entry (perfected in the course of viral evolution) as a crucial part of gene delivery mechanism, and provide the highest level of transgene expression as part of the viral replication cycle, resulting from high amplification of transgene expression (for replication-competent vectors).

Adenoviral vectors: Exclusive and not exclusive for glioma therapy

In the late 1950's Levy and Rowe discovered a new agent capable of passing through bacteria retention filters and infecting mammalian cells.^{15,16} It took more than 40 years after discovery of adenovirus (Ad) to accumulate knowledge about adenoviral biology critical for the development and advancement of the Ad-based vector technology for tumor targeting. Today, human Ad-based vectors have been recognized as a major tool for gene therapy with more than 100 various adenoviral vectors developed for glioma targeting. The attractiveness of adenoviruses, especially the most studied human serotypes 2 and 5, for glioma targeting applications is largely based on the knowledge that some parts of the viral genome (implicated in modulation of the host immune and inflammatory responses), can be omitted without affecting viral replication, assembly, and can be replaced with a gene of interest for therapeutic purposes. Furthermore, currently available capsid-modified Ad vectors can recognize a large variety of cell surface molecules as primary and secondary

receptors allowing efficient infection of both quiescent and rapidly proliferating tumor cells independently from the expression of the Ad native primary coxsackie-adenovirus receptor (CAR). The ability to use complementing and non-complementing cells of mammalian origin allowing human Ad propagation to high titers in culture, represents an important biotechnological advantage of using these vectors for gene and cancer gene therapy (Fig. 1). Given that many self-amplifying, or "replication-competent" Ad vectors with cancer-selective replication properties, also known as Conditionally Replicative Adenoviruses vectors (CRAds), exhibit strong oncolytic anti-glioma effects, those vectors are the primary focus of our review.

Adenovirus generation ("rescue") systems

Various strategies have been proposed to design replication-competent Ad vectors. To generate or "rescue" a replication-competent vector, a two-phase approach is commonly used. In the first phase, all necessary Ad genetic modifications/alterations are made in the viral genome. This includes initial genetic manipulations within one or more genomic regions (typically *E1*, *E3*, *E4*, hexon, or Fiber genes) in the context of a small "shuttle" plasmid using conventional DNA cloning technologies, followed by sequential transfer of the resulting modifications to a large size intermediate ("backbone construct") and/or a full-size genome ("rescue vector") by homologous recombination (HR) in mammalian cells or bacteria (*E. coli* strain BJ5183). Those modifications typically involve mutations in Ad capsid

(structural) proteins, replacement or incorporation of promoter elements (constitutive or tumor-specific), along with the transgene(s) of interest. In the second phase, a linearized form of recombinant full-size genomic DNA is transfected into mammalian (helper HEK293) cells, where the Ad genome termini, formed upon restriction digestion and release of the vector's plasmid (bacterial) portion, create a replication fork to initiate DNA replication (doubling), followed by intracellular production of viral mRNAs, proteins, and the assembly of viral particles. Most recently, Stanton et al. proposed to utilize a high throughput AdZ rescue system that allows a direct, single-step insertion of PCR products or synthesized sequences into the Ad genome and obviates the need in vector linearization prior to transfection into packaging cells.¹⁷

Glioma-associated alterations in signaling pathways offer molecular strategies for engineering anti-glioma CRAds

The rapidly growing body of knowledge on signaling pathways activated in glioma cells offers an important insight into potential molecular strategies for increasing antitumor efficacy of CRAd vectors. Genetic analysis of clinical samples demonstrates aberrations in the PTEN, p16INK4A, EGFR, and P53 signaling pathways. About 80% of glioblastoma specimens presented in The Cancer Genome Atlas (TCGA) possess aberrations in CDKN2A and Rb pathways. The latter regulate astrocytoma survival and tumor cell proliferation.^{18,19} Furthermore, deletions of the PTEN gene

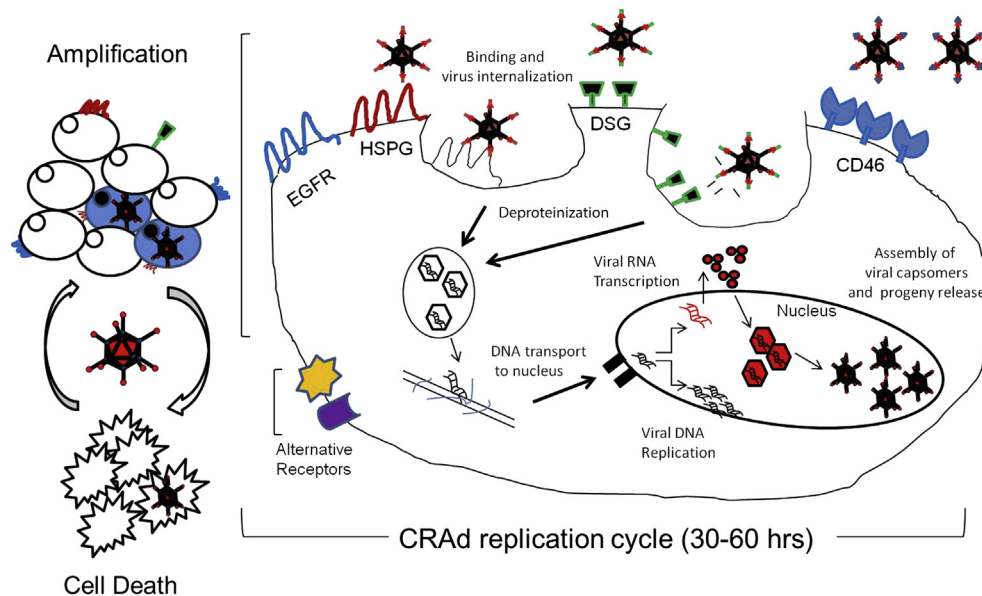


Figure 1 CRAd replication cycle resulting in target cell oncolysis. A schematic illustrating the basic mechanism of CRAd-mediated cell killing starting with binding of a CRAd particle to a tumor-specific cell surface receptor(s). This is followed by viral internalization via the endosome pathway and subsequent capsid disintegration and trafficking of the released genomic DNA (still complexed with core proteins) to the nucleus, where the recombinant genomic DNA is transcribed to produce mRNAs coding for viral proteins. Following mRNA transport into the cytoplasm and its translation into virus-specific proteins, adenoviral progeny particles are assembled from capsomers in the nucleus, following nuclear import of the Ad structural proteins. Ad progeny is then released from the infected cells via a replication-dependent (onco)lytic mechanism.

are observed in ~50% GBM specimens, while 30% of clinical samples exhibit EGFR amplification, and about 11% of samples reveal mutations in P53 and IDH1 genes.²⁰

Ad capability for selective replication in gliomas is determined by genetic information encoded by the self-amplifying Ad genome. The first anti-glioma CRAds were designed using deletion of Immediate Early (*E1*) viral genes such as *E1B-55K*, which blocked vector replication in normal, but not in cancer cells. The glioma-specific oncolytic vector, referred to as ONYX105 or dl1520, was designed to replicate in the p53 deficient tumor cells with functional defects in p53 tumor suppressor signaling²¹ and induce non-apoptotic cell death during viral infection.²² However, over the past several decades, multiple scientific reports have evidenced that dl1520 can also replicate in normal (non-cancer) cells, suggesting involvement of a p53-independent replication mechanism.^{23,24}

The delta24 CRAd vector (also known as dl922-947, or ONYX-838) was constructed by introducing a 24 bp deletion in the *E1A* gene. In the course of Ad infection, the *E1A*-encoded protein binds to the cellular tumor suppressor protein retinoblastoma (pRb) to displace transcription factor E2F from the intracellular E2F/Rb complex, thereby controlling the intracellular pool of free E2F. The release of E2F results in entry of the infected cell into the S-phase, a prerequisite for Ad DNA replication. The delta24 Ad mutant can replicate in actively dividing cells that have an aberrant G1-S checkpoint.²⁵ While normal cells do not support replication of the delta24 CRAd, the virus is effective against U251 and U87 glioma cells at doses of 10 infectious units per cell (iu) *in vitro*, and 100 iu per cell *in vivo*.²⁶ Recently, Gomez-Manzano et al. reported a new vector *E1A-E1B* (CB1), which combines both delta24, and *E1B-55K* deletions. Although the CB1 vector demonstrates a more robust replication, resulting in greater cytotoxicity *in vitro* than delta24, intracranial injection of the double mutant vector into mice results in the same animal survival rates ($p = 0.28$, Mean percent survival is 59 vs. 51 days) as those found for delta24 CRAd.²⁷

Clinical use of dl1520, delta24, or the double mutant CB1 as individual vectors (monotherapy) for gene therapy applications demonstrated limitations for each of those agents. For instance, Georger et al demonstrated that 5 consecutive intratumoral injections of human xenografts with dl1520 are not sufficient to prevent tumor progression in mice. This observation suggests that additional modifications are required to create a more specific and efficacious CRAd agent. Therefore, combinations of various strategies based on utilization of molecular features of glioma tumors are needed to design a potent anti-glioma therapeutic CRAd.

Improving Ad targeting and internalization

It is unclear if incorporation of capsid modifications into recombinant Ad genomes that could potentially affect therapeutic potency of the vector is always justified, i.e. whether those modifications are really necessary to achieve successful gene targeting. For example, to treat prostate cancer Freytag and collaborators used a capsid-unmodified oncolytic adenovirus for successful delivery of cytokines

and two suicide genes.²⁸ On the contrary, given that glioma cells express low levels²⁹ of primary Ad5 receptor (Coxsackie-and-adenovirus receptor, CAR), payload delivery to the tumor cells via capsid-unmodified viral particles might be inefficient, and could induce normal cell toxicity due to CAR expression on healthy cells (Fig. 2). This evidence exposes one of the major limitations of Ad vectors, i.e. the intrinsically low efficiency of tumor cell transduction.

To increase Ad vector specificity several strategies have been developed. One of them involves Ad serotype chimerism. Currently, over 100 types/serotypes of the *Adenoviridae* family have been characterized. Those comprise 5 genera, capable of infecting humans and a large number of animal species. Human Ad species belong to the *Mastadenovirus* genus comprising 57 characterized serotypes (Ad1–Ad57) and 7 distinct species/groups (A-G) based on the variety of serotype-specific and group-specific characteristics. An important group-specific characteristic is the ability to recognize common (group-specific) receptor(s) located on the surface of target cells, such as a glioma cells. Adenovirus type 5 of group C has been a predominant vector used for gene therapy applications.

Adenoviral particles transduce target cells by a mechanism involving a direct initial interaction between the fiber protein of the Ad capsid, and the primary Ad receptors on the surface of tumor cells. It has been suggested that Desmoglein 2 (DSG2) and CD46 molecules represent such native primary receptors of the Ad group B2 serotypes (Ad11, 14, 34 and 35 and others), or group B1 serotypes (Ad3, 7, 16, 21, 50 and others), respectively. Therefore, replacing the Ad5 fiber knob (C-terminal) domain, or the knob-shaft region of the wild type Ad5 with those of other serotypes allows potential retargeting of adenoviral particles from CAR (Ad5 native receptor) to alternate (other serotype-specific) receptors such as DSG2, CD46 etc. In line with this, Nandi et al.,³⁰ Wohfahrt et al.³¹ and Li et al.³² independently demonstrated that pseudotyping Ad5 particles with fibers from serotypes 3, 35 or 11 significantly

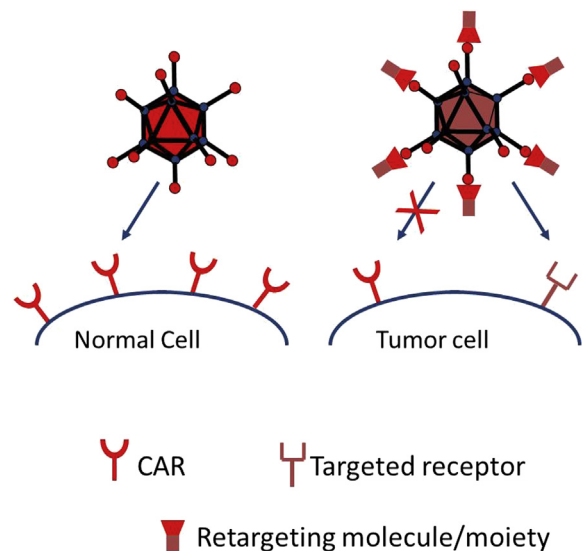


Figure 2 Retargeting of adenoviral particles to an alternate receptor improves targeting specificity of replication-competent adenoviral vectors.

improved transduction of glioma cells compared to the wild type (WT) Ad5 both *in vitro*, and *in vivo*.

The lack of the CAR receptor on glioma cell surface is the reason for the poor gene transfer in those cells by recombinant Ad vectors with an unmodified fiber. Therefore, it would be valuable to improve Ad target cell transduction for therapeutic uses. Retargeting of particles to alternate receptors abundant on the surface of glioma cells may circumvent their intrinsically low CAR expression. One group of such surface molecules characteristically expressed on glioma cells is represented by integrins. It has been shown that insertion of RGD-4C ligand (cyclic peptide) into the fiber protein of the adenoviral capsid allows interaction of virions with cellular α_v integrins, enhancing glioma transduction.³³ Moreover, combining the integrin targeting of Ad vectors with their transcriptional targeting by placing the *E1* genes under transcriptional control of tumor-specific (survivin³⁴ or telomerase³⁵) promoters (TSPs), or mutations (delta24) in *E1* genes that abrogate their binding to Rb or p53 can further improve specificity and efficacy of glioma targeting.^{36–42}

Another type of genetic modification that redirects Ad particles to alternate receptors is incorporation of a polylysine motif at the C-terminus of the fiber protein. While this modification does not ablate CAR-mediated binding and internalization of viral particles, it improves Ad infectivity through a positively-charged heparan sulfate proteoglycan (HSPG) molecules abundant on the surface of cancer cells.³³ Zheng et al. tried to determine which of the HSPG receptors is needed for transduction using pK7-modified Ad vectors. Treatment of glioma cells with pK7-modified Ad vector in the presence of neutralizing antibodies against syndecan 1 and perlecan decreased efficiency of the cells transduction by 30–50%, implicating those molecules in attachment to the Ad5pK7 virus. The first Ad vector targeted to HSPGs through incorporation of 7 lysine amino acids (heptalysine) into the C-terminal domain of the wild type Ad5 fiber was designed by Wickham et al.⁴³ The attachment of Ad particles to the target cells can be significantly augmented given that the viral capsids retain capability of binding to CAR, typically expressed by rapidly proliferating cells, as well as to cellular integrins through an RGD motif in the penton base. However, these receptors are also expressed on muscle cells, macrophages, and endothelial cells, making them a less desirable transduction targets.⁴³ Although most cancer cell lines are highly permissive for Ad vector transduction, its efficiency in patient-derived primary tumor cells is rather poor. To further improve gene transfer of Ad5 vectors enhanced by RGD-4C or pK7 fiber modifications, the viral tropism could be expanded to additional set of receptors, not used by group C species. This can be achieved by using a small peptide/ligand (RGD-4C) modification in the context of Ad3 fiber pseudotyping.⁴⁴ Although studies using the replication deficient ($\Delta E1$) Ad5/3-RGD vector showed a great promise for gliomas,⁴⁴ the benefit of this combined fiber modification for GBM oncolytic virotherapy is yet to be determined.

Expression of EGFR localized to the cell surface is upregulated in 40–60% of gliomas.⁴⁵ Transductional targeting of the EGFR receptor was first proposed by Grill et al., in 2001.⁴⁶ Ad5 particles can be re-directed to this receptor by a bi-specific single chain antibody (scFv)

expressed from either *E1* or *E4* regions of the Ad5 genome that would bridge the fiber knob and the EGFR receptor on the target cell surface. However, retention of the CAR-binding site within the genetically-unmodified (WT) Ad5 fiber knob could interfere with the cancer specificity of transduction due to the potential ability of the scFv-complexed Ad5 to simultaneously recognize CAR receptor on the surface of non-cancer cells. Although, incorporation of 425-S11 single chain antibody into the fiber knob domain improves transduction of CAR-negative tumors by 2- to 11-fold,⁴⁶ further ablation of the native CAR tropism is required. For instance, redirection of adenoviral particle to EGFR with simultaneous ablation of CAR and α_v integrin binding ability provides selective gene transfer to glioma cells.⁴⁷ These promising results led investigators to design an 435-S11 scFv-modified CRAd⁴⁸ to target and destroy CAR-deficient tumors. The mutant version of EGFR (EGFRvIII) is present on the majority of glioma cells as well as breast and ovarian cancer cells⁴⁹ and regulates pro-survival pathways, which makes it a promising candidate for cancer gene therapy.⁵⁰

In conclusion, the data published by Nandi et al.³⁰ suggest that retargeting Ad particles by genetic pseudotyping of fiber can greatly improve CRAd cytotoxicity for tumors. However, in many cases tumor-specificity of such Ad vectors remains poor since the alternate receptors such as CD133 (expressed on the surface of glioma stem cells and neural stem cells) and CD46 they are often retargeted to, are also found on the surface of non-cancer cells. Therefore, further capsid modifications are needed to optimize CRAds for cancer gene therapy.

Limiting adenovirus transcription to glioma cells

Genetic incorporation of tumor-associated gene expression control elements into the Ad genome for regulating its early (*E1*) gene expression (transcriptional or post-transcriptional), improves CRAd vector specificity by restricting viral replication to tumor cells, and thereby preventing unfavorable vector toxicity in normal cells. To achieve this specificity, incorporation of transcription factor recognition motifs and/or microRNA binding elements upstream of the Ad5 *E1* genes has been proposed. The rationale behind this approach was to provide conditional (selective) expression of the E1A protein, crucial for triggering Ad early gene transcription and genomic DNA replication. A prototypical tumor-specific promoter (TSP) is known from the literature to selectively regulate/activate Ad early gene expression in tumor cells, yet remain inactive in healthy tissues, such as liver, involved in efficient uptake of most Ad5 entering the circulation. The “tumor on/liver off” expression ratio is a commonly accepted tumor-specificity characteristic of a TSP.

Recently, Guvenc et al., showed that tumor cells highly resistant to therapy exhibit high expression level of the survivin gene.⁵¹ The latter codes for an anti-apoptotic protein that governs spindle formation and chromatin separation during tumor cell mitosis.^{51–53} The high level of survivin protein correlates with high level of its mRNA, suggesting activity of the survivin promoter and stability of

its mRNA in those cells. A ~200 bp fragment of the survivin promoter (short version) was found to be sufficient to confer Ad replication specificity to glioma cells.^{34,54} Moreover, since the survivin promoter contains radiation-inducible elements, ionizing radiation can sensitize exposed glioma cells to infection by survivin-*E1* bearing CRAd vectors.⁵⁵

Midkine (MK) is a heparin-binding growth factor encoded by the *MDK* gene. Induced during ontogenesis and inflammation, syndecan 1 is the midkine receptor regulating cell proliferation, angiogenesis, fibrinolysis, and mRNA expression in several cancers including glioblastoma and neuroblastoma. Since MK is implicated in cancer cell proliferation, it has become a target for gene therapy. However, there are also effects of MK on genesis of normal cells such as fibroblasts, myoblasts, and renal cells,⁵⁶ and therefore cancer specificity of CRAd vectors with MK promoter-controlled replication is questionable. Nevertheless, an oncolytic vector with a ~600 bp MK promoter element, driving expression of adenoviral *E1A*, has been demonstrated to eradicate MK-positive glioma cells *in vitro* and *in vivo*.⁵⁷

The Promoter of the telomerase related gene (*TERT*) is also active in glioma cells. According to scientific literature, more than 85% of cancers express telomerase, which is required for cell proliferation. A 455 base pair (bp) promoter of the human gene, coding for the telomerase catalytic subunit, was successfully used for construction of OBP-301 CRAd (also known as Telomelysin) transductionally-retargeted to integrins on glioma cell lines U87, U373, U251, and patient-derived MDC-01.³⁵ However, not all glioma cells exhibit high telomerase activity. For instance, according to studies by Jafri et al., only 26.1% of high-grade glioma specimens exhibit high telomerase activity.^{58,59} Under certain conditions, normal cells, such as fibroblasts, possess very low levels of telomerase activity, which, however, could be induced by drugs, such as HDAC.^{60,61} This data points to some limitations for targeting primary tumors in clinical settings.

Another study conducted by Hoffmann et al evaluated potential glioma-specific promoters for oncolytic virotherapy including those of VEGF, GFAP, FGF, Ki-67, Nestin, and Midkine gene alone, or in combination with an SV40 promoter/enhancer.⁶² According to this data, elevated activity of promoters was manifested by expression of the Luciferase reporter. Based on this assay conducted in several patient-derived and established glioma cell lines (D54, U251, and U87), the top 7 promoters included: MK, hTERT, VEGFlong, VEGFshort, Ki67, GFAP, and E2F/SV40. Furthermore, *in vitro* promoter activity testing showed that the long version of the human GFAP promoter restricts replication of the CRAd5/35 vector to glioma cells with both high and low level of proliferation capability. Those *in vivo* data corroborate the ones *in vitro* and suggest that GFAP promoter-controlled CRAd prolongs survival of mice harboring fast growing U251 xenografts. A study performed by Horst et al.⁶³ showed that rapidly dividing glioma cells can be targeted by a CRAd vector with GFAP promoter-controlled replication. Since GBM cells are sensitive to ionizing radiation and temozolomide treatment, virotherapy with the GFAP promoter-controlled CRAd could be used to augment current therapeutic modalities.

Tumor selective transcription: is CMV promoter the best reference?

Several studies have compared efficiency of various promoters against that of CMV. A study by Zheng et al.⁶⁴ demonstrated that replacement of the *E1A* gene promoter with the CMV major late promoter (MLP) failed to provide cancer specificity to Ad replication. Furthermore, utilization of the CMV promoter to control CRAd replication (*E1* transcription) resulted in lower replication efficiency as compared to the native (*E1*) promoter-bearing CRAd in OE33 and OsACL cancer cell lines. In contrast, equal levels of CRAd replication resulted from the CMV-driven *E1* expression and the one controlled by the native promoter in A549 (lung adenocarcinoma cells), or WI-38 non-tumor cells. These observations suggest that the CMV promoter does not confer tumor-specificity to CRAd replication. Similar results were also obtained in other studies.⁶⁵ To date, experimental evidence suggests that most tumors,^{66,67} including brain tumors,^{68–70} contain cytomegalovirus proteins, DNA and RNA transcripts, particularly the ones of the early and immediate early CMV genes. Besides its robust activity in tumors, the CMV MLP promoter is also active in normal cells, which reduces specificity of CRAd vectors utilizing this promoter to control expression of the *E1* genes.

Recent reports have indicated that glioma resistance to conventional treatment modalities may be determined by expression of therapy-resistant proteins, such as the Y-box protein YB-1, a cellular transcription factor implicated in GBM cell survival.^{71,72} Treatment of cells with UV radiation and chemotherapy translocates YB-1 from the cytoplasm to the nucleus, indicating its possible role in DNA repair.^{73,74} Since expression of multidrug-resistance genes correlates with YB-1 activity, it is logical to expect a YB-1-dependent CRAd to selectively replicate in chemo-resistant glioma cells. In line with this expectation a YB-1-dependent CRAd dl520 ($\Delta E1A-13S$) demonstrated oncolytic activity in glioma cells, resistant to Irinotecan and Trichostatin A.⁷⁵ Most recently, a dl520-derivate Ad-Delo3-RGD, carrying an additional *E1B* gene deletion and the integrin binding motif in the fiber protein, also demonstrated a selective replication in chemo-resistant glioma stem cells.⁷⁶

The other genetic alteration in gliomas that impacts cell division is p16INK4a, leading to phosphorylation of Rb and activation of E2F1 transcriptional factor.⁷⁷ To restrict expression of the adenoviral *E1A* protein to target (glioma) cells deficient in the Rb pathway, authors placed *E1A* transcription under control of endogenous E2F1 transcription factor by cloning an E2F1 response element in place of the *E1* promoter region.⁴² As a result, replication of such CRAd (ICOVIR5) in normal cells with low level of free E2F1 (trapped in the form of Rb/E2F1 inactive complex) was suppressed. On the contrary, an excessive amount of E2F1 in glioma activated ICOVIR5 vector's replication in target cells. Thus, replication activity of this CRAd directly correlates with the E2F1 expression in the virus-infected cells.⁷⁸ However, since high level of E2F1 expression is a feature characteristic for any rapidly dividing cells,⁷⁹ the E2F1-controlled CRAd vector cannot discriminate between rapidly dividing normal cells and malignant cells.

Suppression of endogenous gene expression on post-transcriptional level involving microRNA (miRNA), found in gliomas, can also be utilized in gene therapy approaches. MicroRNAs are small non-coding RNAs complementary to target cellular mRNAs. MicroRNA binding to target mRNA leads to specific repression of regulatory genes either on transcriptional or translational levels. It is well documented that gliomas exhibit diverse microRNA expression patterns or "signatures". Given that miRNAs can regulate cell proliferation, invasion and angiogenesis, incorporation of miRNA coding sequences into CRAds could aid to CRAd's cytotoxic activity. It was recently shown that glioma cells express high levels of microRNA 124, -128, -146B and 218,⁸⁰ and therefore incorporation of microRNA recognizing elements (MREs) into the Ad genome could inhibit CRAd replication in tumor tissues. It remains to be investigated whether the microRNA-mediated CRAd targeting approach will be efficient in human tissues with regard to patient's age, and the course of treatment as those might affect miRNA expression.

Analysis of scientific literature suggests that cancer specificity of CRAd agents can be achieved by proper selection of transcription regulatory elements and their genetic incorporation in CRAd genomes to control viral *E1* transcription. However, despite strong antitumor effects, incorporation of a given TSP may not prevent *E1A* transcription leakage from the inverted terminal repeat (ITR) sequences in the Ad genomic DNA.⁸¹ For instance, previous studies have shown that multiple enhancer elements and cryptic promoter elements exist within the Ad ITRs, which can contribute to undesired "leaky" *E1* transcription in healthy cells.^{64,82,83}

Strategies to improve anti-glioma efficacy of CRAds

As it was mentioned above, glioma cell populations responsible for tumor recurrence exhibit strong resistance to conventional treatment modalities. Therefore, it is critical to find a new and more effective therapeutic approach devoid of cytotoxicity caused by the conventional anti-glioma treatments. Besides, a growing body of evidence suggests that intratumoral injection of CRAds activates antiviral immune response. To circumvent the immune response problem various Ad shielding methods, such as coating with polyethylene glycol (PEG) or using stem cells as Ad delivery vehicle, have been developed.

a) Strategies to improve CRAd-mediated toxicity: RT and chemotherapeutic drugs.

It has been shown that chemotherapeutic agents activate cellular pathways that contribute to CRAd-mediated toxicity. We^{54,84,85} and others^{86–91} have shown that CRAds induce cell death via two main mechanisms: apoptosis, and autophagy. Despite activation of pro-apoptotic genes, such as BAX2, BIM, and BIK, CRAd infection does not trigger caspase-dependent apoptosis. Several reports suggest that CRAds induce autophagy, which involves the formation of double-membrane phagosomes. Moreover, recent evidence suggests that the *E4* region of the Ad genome is required to

induce autophagy upon the viral infection. Augmentation of CRAd-induced autophagy is one strategy to boost CRAd toxicity. Ionizing radiation,^{36,40,55,89} Rad001^{35,39} and clinically approved temozolomide (TMZ)^{35,38,76,92} have been utilized to promote CRAd-mediated autophagy. However, it is, still unclear whether this effect can be directly attributed to CRAd, or is merely a result of CRAd-induced cell defense mechanism that requires more inhibition to improve CRAd cytotoxicity.

b) Sensitization of cells to the CRAd-mediated toxicity using TRAIL and CD gene expression

To successfully design and develop effective treatments for GBM, combinational approaches to targeting several molecular pathways may be necessary. Despite our advancing knowledge of the genetic alterations involved in this disease, identification of new therapeutic combinations may prove advantageous. To augment CRAd-mediated toxicity, expression of pro-apoptotic molecules, such as TRAIL (TNF-related apoptosis-inducing ligand), represents a new anti-glioma approach. TRAIL represents an extracellular carboxy-terminal portion of the type II transmembrane protein that sensitizes tumor cells to apoptosis via binding to the DR4 (DR5) receptor. This, in turn, activates caspase 8/10 or the intrinsic cytochrome C release pathway, which subsequently activates SMAC/DIABLO to translocate pro-apoptotic BIK, BID and BAX.^{93–96} The common activation of BAX and suppression of anti-apoptotic BCL-2 apparently provides a mechanistic link for the additive effect between CRAd and TRAIL expression.^{97–99} Wolhardt et al.³¹ first proposed targeting glioma cells with the CRAd-TERT-5/35 vector encoding TRAIL as a therapeutic transgene, while expressing proteins of the *E1* region under control of the human telomerase promoter. In addition, the capsid of this vector was retargeted to an alternate receptor by replacing the wild type fiber knob domain with that of serotype 35. Later, this approach was elaborated by Li et al.,³² who used a delta24-5/11 backbone, and by Tsamis et al.,¹⁰⁰ who used a delta24 backbone with the WT fiber. In all cases, expression of TRAIL in the context of an oncolytic vector resulted in strong anti-cancer effect compared to the unarmed CRAd.

In 2005 Conrad et al.¹⁰¹ attempted to combine an oncolytic Ad vector and pro-drug therapy to suppress gliomas. In addition to the oncolytic effect the virus elicited cytotoxicity owing to the expression of the delivered "suicide" gene (humanized form of yeast cytosine deaminase, hCD) converting 5-FC substrate to a toxic metabolite 5-FU in tumor cells.¹⁰² Given the limitation in achieving an effective therapeutic dose without hepatotoxicity in the U87 intracranial glioma mouse model, delivery of pro-drug therapy in the context of an oncolytic vector holds a great promise.

c) Modification of CRAd genome to improve CRAd-mediated cytotoxicity.

The adenoviral genome encodes immediate early and early genes transcribed before the onset of DNA replication, as well as late genes transcribed after DNA replication. The functions of the Ad5 early proteins include:

controlling cell division (*E1*), containment of host immune responses to Ad infection, preventing apoptosis (*E3*) and activation of Ad replication⁹¹ (*E2*). The *E3* genes encode 7 proteins, including adenoviral death protein (ADP), which is exclusively expressed during the late stage of infection¹⁰³ and is responsible for efficient cell lysis and progeny release.¹⁰⁴ In efforts to improve adenoviral oncolysis Yun et al. designed an anti-glioma CRAd that harbors a 55Kda-*E1B* deletion and expresses ADP under the control of the adenoviral Major Late Promoter (MLP) or CMV promoter.⁹¹ The vector expressing a CMV-driven ADP exhibited a strong cytotoxicity towards human U343 glioma cells. Although, *in vitro* data suggest that ~40% of glioma cells were sensitive to infection with ADP-overexpressing CRAd, this observation has not been confirmed *in vivo*. Taken together, all this data suggest that overexpression of ADP facilitates CRAd-mediated oncolytic effect.

d) Improvement of CRAd-mediated toxicity by indirect activation of viral replication in the presence of hypoxia.

One of the disadvantages of using adenoviral vectors in cancer gene therapy is uneven dissemination of the vector across the tumor mass.¹⁰⁵ One study showed that hypoxia may compromise blood supply to certain regions of tumor tissue, which ultimately limits intratumoral distribution of CRAds.¹⁰⁶ Given that hypoxia contributes to GBM invasion and proliferation by maintaining its CSC component,^{107–109} targeting GBM CSCs via accelerating CRAd replication under hypoxic conditions may improve anti-glioma therapy. It has been shown that hypoxia affects tumor progression through the blood, and regulates activity of target genes via binding of hypoxia induced-transcription factors to hypoxia response elements (HRE). These transcriptional regulators allow cells to survive hypoxia by activating proliferation.¹¹⁰ Therefore, CRAds designed to aggressively replicate in hypoxic environment by utilizing hypoxia-inducible factors (HIF) to control their replication may be effective in suppressing gliomas.¹⁰⁸ In line with the above Post et al demonstrated that incorporation of HRE in the *E1* region improved CRAd replication in hypoxic areas of tumors.¹¹¹

e) Strategies to modulate the anti-Ad immune response?

It is known that patients with GBM develop a strong immunosuppression resulting from chemotherapeutic drugs, ionizing radiation, accumulation of cancer stem cells, etc. This evidence has initiated the debate as to whether brain tumor patients might benefit from immune system stimulation through experimental therapies, such as gene therapy with CRAds, which have been shown to induce a proinflammatory response in glioma mouse models. Since the mechanism of antitumor effect achieved by glioma virotherapy is still unclear, it cannot be ruled out that modulation of the patient's immune response might strongly interfere with the effectiveness of the treatment. A recent publication by Liikanen et al.¹¹² reignited discussions about the role of immune system activation in CRAd-mediated tumor oncolysis. Considering that immune response to Ad vector could significantly compromise the

efficacy of CRAd-mediated tumor oncolysis by rapid clearing of the viral particles, the overall impact of immune response on the clinical outcome of glioma virotherapy treatment is still in question.

The immunological responses to CRAd infection has been investigated by several research groups. One study using immunosuppressed hamsters suggested that the host immune response neither significantly contributes to CRAd clearance, nor to the antiviral immune response.¹¹³ Moreover, steady levels of the virus were detected in immunosuppressed hamsters, similar to those found in mouse xenografts. In immunocompetent animals, the level of CRAd dropped 22 days after tumor implantation, suggesting activation of viral clearance. In this regard, of interest is a recent data from Klejin et al.¹¹⁴ demonstrating that the immune response to delta24-RGD affects therapeutic efficacy in the rodent immunocompetent glioma model. In fact, a local production of proinflammatory cytokines in response to intratumoral vector injection increased along with the number of infiltrating CD4+ and CD8+ lymphocytes and macrophages. It still needs to be determined whether the observed immune response was activated due to the host's protective response against CRAd replication, or was a result of the viral mechanism contributing to its replication.

The main role of stem cell-based vectors is to preserve and deliver CRAds to tumors in tumor-specific fashion, while avoiding activation of anti-Ad immune responses. Delivery of CRAd payloads to tumors and passing those payloads on to neighboring cancer cells within the tumor mass by means of GSCs is based on their intrinsic tumor homing properties. Glioma cells release chemokines and angiogenic factors, such as TGF- β ,¹¹⁵ PDGF β ,¹¹⁶ VEGF,¹¹⁷ which attract stem cells administered via various routes. Indeed, the bone-marrow-derived mesenchymal stem cells (MSC) have been reported to improve CRAd persistence and dissemination *in vivo*.^{118,119} Moreover, a delta24 CRAd MSC-delivered to glioma xenografts, significantly prolonged mice survival in glioma xenografts.¹²⁰ Of note, regardless of the delivery route, intravenous,¹¹⁵ or intracranial (delivery of payload from one brain hemisphere to another hemisphere bearing a tumor), MSC successfully targeted glioma xenografts. Similarly, we have shown that HB1.F3.CD NSCs, which lack HLA class I antigen, when loaded with the CRAd-S-pk7 vector, exhibit a robust anti-glioma effects *in vitro* and in a U87 intracranial mouse model.¹²¹ A side-by-side comparison between the MSC (mesenchymal stem cells)- and NSC (neural stem cells)-based CRAd delivery cell vectors showed that the NSC delivery system is more advantageous.¹²² One significant disadvantage of using NSCs for targeted CRAd delivery is their sensitivity to CRAd infection, which prevents effective migration and delivery of CRAd payload to other sites within the tumor due to CRAd leakage. To circumvent this drawback Kim et al.¹²³ utilized N-acetylcysteine (NACA) for treatment of NSCs loaded with CRAd-S-pk7 to attenuate the CRAd-induced apoptosis. The NACA-treated loaded NSCs lived longer and maintained properties necessary to deliver their payload. Although these results suggest that stem cells improve CRAd distribution *in vivo*, the use of immunocompromised animal models makes it difficult to assess the induction of an antiviral immune response.

Safety concerns of using CRAds: Should we care?

Although the efficacy of CRAd delivery to glioma is a critical factor of the experimental therapy, safety of the treatment is of high importance too, especially since ongoing investigations emphasize the role of new etiological agents in glioma progression. CRAd safety testing has been performed *in vitro* using human culture of healthy adult astrocytes^{30,124,125} or cultured fibroblasts^{63,75,91,126} and *in vivo*, using animal models for neurotoxicity testing. As can be seen from the data summarized in Table 1, human non-malignant cells exhibit various sensitivities to CRAd infection, which is important to assess prior to *in vivo* CRAd testing in glioma animal models. A preliminary survival experiment in the form of a brain neurotoxicity test, using CRAd intracranially implanted into mice brain at lowest and highest doses, is recommended to determine the maximum tolerated dose (MTD) of the viral vector. Since mice are not permissive for human Ad replication as opposed to hamsters or cotton rats, those rodents represent better animal models for neurotoxicity testing.

The use of NSCs to deliver a CRAd payload requires safety testing as well. An important study by Aboody et al. showed that HB1.F3.CD NSCs (which lack HLA class I antigen) are non-tumorigenic after activating CD gene expression by pro-drug 5-fluorocytosine (5 FC).¹²⁷ Considering that cytomegalovirus was found to persist in NSCs,¹²⁸ it is important to assess the risk of stem cells application for patients. In case of high permissiveness, addition of 5FC or any other cytotoxic agent would eliminate the stem cells but may not kill cytomegalovirus harbored by those cells. Moreover, recent data suggest that the presence of cytomegalovirus may impact activation of pro-tumorigenic adenoviral regions, such as *E1A*.¹²⁹ Of note, it has been shown that *E1A* genes under certain conditions may elicit formation of oncogenic fusions¹³⁰ and form tumors in newborn hamsters^{131,132}. Therefore, it is highly unlikely, but still possible that CMV-mediated protein expression (IE1)

can trans-activate *E1A* to produce tumorigenic phenotype in stem cells. From a therapeutic standpoint, it remains unclear whether NSC passage can affect efficacy of CRAd delivery, as well as whether aged stem cells are capable of inducing tumor formation.

Concluding remarks and future directions

Obtaining an FDA approval for the use of CRAd vectors in human clinical studies requires significant time and efforts from investigators. Although in many cases *in vitro* data obtained from tumor clinical samples and *in vivo* data from mouse xenograft models look very promising, a single modality treatment is often less effective than a combination of other therapeutic approaches. Moreover, recent studies suggested that inflammation and immune response might affect efficacy and specificity of CRAds as anti-glioma agents. Although various immunomodulation strategies have been suggested, which involve either genetic modifications of the Ad genome to suppress anti-Ad immune response, or shield viral particles from the immune system by means of coating with molecular polymers or loading inside GSC as vector delivery vehicles, they all require additional experimental evaluation. Finally, the established role of autophagy in promoting Ad-mediated oncolysis and suppression of the host immune response to Ad will help determining the marker of cell resistance controlling antiviral response at the cellular and organismal levels. It remains unclear whether delivery of anti-angiogenic or immunomodulatory factors by Ad vectors actually improves oncolytic effect in patients with brain tumors. This data need to be analyzed in the future. Although oncolytic adenoviruses alone demonstrate a substantial anti-glioma potency *in vitro* and *in vivo*, recent studies suggest that the combination of virotherapy with chemotherapy and/or immunotherapy may provide greater therapeutic benefit. Therefore, tackling glioma progression from different directions, i.e. by utilizing a combination of immunotherapy, angiogenic therapy, oncolytic virotherapy, radiotherapy,

Table 1 Comparative toxicity of CRAd vectors.

	Cell type/sell type system	Vendor	Method to detect CRAd specificity/toxicity	Cytotoxic dose of CRAd/effect	Reference
Human astrocytes	Adult primary astrocytes	Lonza	LDH	10 vp per cell/ ~25% dead cells	30
		Lonza	Crystal violet toxicity test and CRAd replication	10 vp per cell/ ~90% toxicity	124
		Lonza	Progeny titration	10 MOI per cell/CRAd replication from 1.9×10^2 to 1×10^6	125
Human fibroblasts	Fetal lung fibroblasts MRC5	ATCC	Ad replication ratio to AdWT	0.1 MOI per cell/0.2-0.5	63
	Dermal fibroblasts Hs68	ATCC	Cytopathic effect, light microscopy	50 MOI per cell	75
	Normal skin fibroblasts BJ	ATCC	Crystal violet toxicity test	Various toxicity from 10 to 0.01 MOI per cell	91
	Normal skin fibroblast BJ; fung fibroblasts IMR90; lung fibroblasts WI38	ATCC	Crystal violet toxicity test	Various toxicity from 100 till 10 MOI	126

and chemotherapy could provide the most benefits for patient survival.

Conflicts of interest

All authors have no conflicts of interest.

Acknowledgments

Supported in part by National Cancer Institute (Bethesda, MD) grants R01 NS070289 (I.U., Charles Cobbs-PI), 5R03DE021758 (A.B.) and, generous support from Russian Fund of Fundamental Research (#No 11 411.0008700.13.082 and No 13 411.1008799.13.120 (A.Y.B.)). We thank Dr. Ramon Alemany (Gene and Viral Therapy Group, IDIBELL-Catalan Institute of Oncology (ICO), L'Hospitalet de Llobregat, Barcelona, Spain) for valuable advices.

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