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ONCOLYTIC ADENOVIRUSES FOR MALIGNANT GLIOMA THERAPY

Hong Jiang, Charles Conrad, Juan Fueyo, Candelaria Gomez-Manzano, Ta-Jen Liu

Department of Neuro-Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA

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1. ABSTRACT

Malignant gliomas are devastating diseases that localize within the central nervous system and are notoriously invasive. Despite recent advances in established treatment modalities such as postoperative radiotherapy and chemotherapy for gliomas, no definitive improvement in survival has been observed. However, progress in the understanding of the biology of these tumors allows for the development of translational research projects and new therapeutic approaches such as gene therapy. One of the most recent strategies is based on the use of targeted oncolytic adenoviruses. The progress of the oncolytic adenoviral system relies on the knowledge of the molecular biology of both the adenovirus and cancer. This review outlines the main strategies currently used to improve adenoviral infection, to restrict adenoviral replication to tumor cells, and to optimize the anticancer effect of oncolytic adenoviruses. Specifically, we discuss the concepts of conditionally replicative adenoviruses, tropism modifications used to efficiently redirect infectivity to cancer cells, and the transcription/transduction systems that limit the adenovirus to the target host cell. Mastery of the mechanisms of adenoviral infection and replication will lead to a full realization of the potential for adenoviruses as critical anticancer tools, and may result in the improvement of the prognosis of patients with brain tumors.

2. INTRODUCTION

Great challenges are inherent in finding an efficacious treatment for malignant gliomas. Since glioma cells are extremely resistant to apoptosis, chemotherapy alone has not been an entirely effective treatment. Due to the fact that gliomas generally localize within the central nervous system, local treatment strategies have traditionally been utilized. Surgical removal followed by external beam irradiation represents a standard treatment that has often led

to delayed progression and prolonged survival (1). Nevertheless, tumor recidivism followed by unstoppable progression is the rule in glioblastoma multiforme (GBM), the most malignant type of brain tumor. Despite the explosive growth of knowledge in the field of cancer biology and extraordinary improvements in the treatment of many common human cancers, the treatment of malignant gliomas has improved little during the past several decades (2). As the genetic basis of glioma formation has been delineated, numerous genetic targets have arisen as promising candidates for gene therapy. This effort has, however, been limited by the lack of an efficient system that can be used to deliver the exogenous gene to enough tumor cells *in vivo* to induce a therapeutic effect. Oncolytic adenoviruses appear to be a reliable solution for the "delivery problem" that is halting the expected promise of cancer gene therapy.

Viral therapy against cancer made its debut approximately fifty years ago after observations of occasional tumor regression in cancer patients with viral infections or in those who had been vaccinated (3). Although cancer treatment using adenoviruses induced a short-term response in most of the patients treated, it did not prevent tumor progression and resulted in toxicity (4). Currently, adenoviruses are being modified to improve anticancer potency and target specificity. The main paradigm describes an adenoviral system that uses abnormally expressed cell receptors to exclusively infect cancer cells and replicate preferentially in cells with abnormal tumor suppressor pathways or in cells with overexpressed cancer-related proteins. The ideal oncolytic adenovirus would have modifications in the genome that would be more effective than wild-type adenovirus in lysing cancer cells. Safety measures involve the control of adenoviral replication with the use of conventional drugs and the insertion of suicide genes in the genome to produce a bystander effect and to incapacitate the adenovirus in the case of a runaway infection.

3. CONDITIONALLY REPLICATIVE ADENOVIRUS (CRAd)

3.1. Targeting aberrant tumor suppressor pathways

Wild-type adenovirus induces cell death by replicating in and lysing the infected cell. This inherent cytotoxicity, together with the efficiency with which viruses can spread from one cell to another, inspired the notion that replication-competent viruses could be part of the solution for the "vector gap" delivery problem (see above) in cancer gene therapy. Interestingly, as the molecular biology of adenovirus and cancer evolved together, study of the interactions between adenovirus and critical cell proteins led to the discovery of important tumor suppressor genes. For these reasons, it seems natural to design replication-competent adenoviruses that are unable to interact with critical cell proteins and thus induce selective lysis in cancer cells. The concept of a conditionally replicative adenovirus (CRAd) refers to a genetically modified adenovirus that, given specific circumstances, is unable to dominate cellular division machinery, and ideally becomes attenuated in normal cells while retaining the ability to efficiently lyse tumor cells.

3.1.1. Abrogating E1B-55kD/p53 binding—*dl*1520

Since adenoviruses lack their own enzymes for replication, they rely on the use of the enzymatic machinery of the host cell to replicate the adenoviral genome. To conquer the host DNA replication machinery, the adenovirus expresses a series of genes immediately after infection. These genes are called "early" genes and encode proteins that bind and inactivate cell proteins that regulate cell cycle progression and apoptosis. The first adenoviral gene expressed after infection is called E1A (E stands for early). The E1A products bind to and inactivate retinoblastoma protein (Rb), propelling the cell into unscheduled DNA synthesis in order to create an environment for replication. Forced entry into S phase may result in the trigger of p53-mediated apoptosis and the cessation of adenoviral replication. To abrogate the p53-induced apoptotic response, adenoviruses encode E1B-55kD, which binds to and inactivates p53, resulting in a prolonged cell life that facilitates the propagation of viral particles. Based on this knowledge, the McCormick group constructed and tested an adenovirus unable to express the p53-antagonical E1B-55kD protein (5). The idea behind this strategy was to construct a replication-competent adenovirus unable to replicate in wild-type p53 cells but capable of acquiring a completely developed replication phenotype in mutant p53 cells. Since nearly one-half of all tumors have been shown to harbor a p53 abnormality, they may have a preconditioned environment that favors adenoviral replication. Furthermore, disabling E1B-55kD would prevent viral propagation in normal, non-cancerous cells. This suggested the hypothesis that adenoviruses would be able to selectively replicate in tumor cells. This concept was initially bolstered by the observation that the mutant virus Ad2 *dl*1520 (a double mutant unable to synthesize the E1B-55kD protein) was extremely deficient in its ability to transform rat embryo fibroblast cells (6). A similar virus, ONYX-015 (*dl*1520), which is a chimeric human group C (Ad2 and Ad5) adenovirus, was the first CRAd to demonstrate preferential replication as well as antitumor efficacy in some p53-deficient human tumor cells (5,7). However, in human malignant glioma xenografts, ONYX-015 showed cell lysis and antitumor activity independent of p53 status (8). Phase I and II clinical trials revealed that *dl*1520 was well tolerated at the highest, practical doses that could be administrated (2×10^{12} to 2×10^{13}) via intratumoral, intraperitoneal, intra-arterial, and intravenous routes (9,10). However, a limitation in the efficacy of a single-agent antitumor treatment was observed in head and neck cancers despite repeated, direct injections (a 13% unconfirmed regression rate). No objective responses were documented by patients with pancreatic, colorectal, or ovarian carcinomas (10). These results suggested that efficacy, rather than toxicity, seemed to be the primary limitation to the use of adenoviruses. Ultimately, a favorable and potential synergistic interaction with chemotherapy was demonstrated in multiple tumor types via multiple routes of administration (10).

The replication selectivity of *dl*1520 is controversial. The complexity is compounded by the fact that the loss of p53 function in many cancers occurs through mechanisms other than gene mutation. These include the overexpression of p53-binding inhibitors (such as mdm2 and human papillomavirus E6) and loss of proteins (such as $p14^{ARF}$) that indirectly modulate the function of p53 (11). For instance, it has been reported that the loss of $p14^{ARF}$ in tumor cells facilitates replication of *dl*1520 (12). Furthermore, the precise role that p53 plays in inhibiting adenoviral replication has not yet been clearly defined (9,10). Confounding clarity further is the fact that some adenoviral proteins, such as E4ORF6, exert inhibitory effects on p53 (13). In addition, E1B-55kD functions involve mechanisms other than the inactivation of p53. For instance, this adenoviral protein also regulates viral mRNA transport and downregulates host cell protein and adenoviral E1A protein synthesis (14,15). Deletion of E1B-55kD therefore results in inappropriately high E1A expression, likely leading to the induction of E1A-mediated apoptosis and prematurely truncating the adenoviral replication cycle (16). Although *dl*1520 and its functional relationship with p53 have fueled the field of oncolytic adenoviruses, *dl*1520 is still not the perfect tool for cancer gene therapy.

3.1.2. Attenuating E1A/Rb interaction—E1A mutants

The Rb protein is the prototype of tumor suppressor genes and the master regulator of the G1 checkpoint. In its hypophosphorylated state, Rb negatively controls the G1/S transition. Rb exerts its function by binding the E2F transcription factors, which are the main positive force behind the transcription of genes that are needed for the transition from G1 to S phase. Once Rb is hyperphosphorylated by upstream cyclin-dependent kinases (CDKs), it is disabled and releases E2Fs, thereby allowing the transition to S phase to occur (17). The Rb pathway is one of the most frequently inactivated in various cancers. Abnormalities of the Rb pathway have already been characterized in most high-grade gliomas (18). As mentioned before, E1A is the first viral gene that is transcribed after infection, and it mainly produces two related proteins, 243R and 289R. The 243R species differs from the 289R species only by the absence of 46 internally located amino acids (19). The main function of E1A is to induce the transcription of other early viral gene regions and to release the E2F transcription factors from Rb (20-22). The physical interaction of E1A with Rb requires the presence of two noncontiguous regions of amino acids, 30 to 60 and 122 to 129 (20). Notably, disruption by mutation of the 122-129 region is sufficient to prevent E1A binding to Rb (23).

Our group has tested an oncolytic adenovirus that is unable to bind the Rb protein. To disrupt the Rb binding function of E1A proteins, a deletion was made in its conserved region 2 (CR2). The mutant adenovirus has a deletion of amino acids 122 to 129 (comprising a 24 bp region, and for that reason we named the adenovirus Delta-24) (23). The Delta-24 construct demonstrates cytolytic activity against glioma cells *in vitro* and *in vivo*. The presence of functionally active Rb protein in quiescent normal cells is able to halt the cytopathic effect of the mutant Delta-24 adenovirus (23). Characteristics of Delta-24 include the incapability to induce S phase in normal cells and cancer cells with a restored Rb function (23). However, Delta-24 induces accumulation of glioma cells in S phase that precedes a cytopathic effect *in vitro* and *in vivo* (23). Other groups have also tested E1A mutant adenoviruses. One of these groups has demonstrated that a similar E1A mutant adenovirus (*dl*922-947) displays a greater potency than *dl*1520 *in vitro* and *in vivo* (24).

One of the reasons why Delta-24 is more efficient in inducing an anticancer effect than dl1520 may be that the E1B deletion in dl1520 interferes with several critical functions. In addition to binding p53, E1B proteins are also required for the accumulation of late viral mRNA and affect cellular mRNA translation and transport. Lack of these functions may diminish the ability of dl1520 to replicate efficiently.

3.2. Tumor and/or tissue specific adenovirus---limiting viral spread

Another approach to improve the specificity of oncolytic viruses is to limit their replication by transcriptionally targeting viral genes that are essential for replication using tumor- and/or tissue-specific promoters. This is usually accomplished by inserting the promoter upstream of the E1A gene, restricting the replication and propagation of the virus within the tumor and/or a certain tissue. Controlling the dissemination of the virus in this manner can greatly improve the safety of oncolytic therapy.

Various gene regulators have been utilized to construct tumor-specific replication-restricted adenoviral vectors. One regulator is the minimal enhancer/promoter derived from the 5' flank of the human PSA gene (prostate-specific enhancer). This promoter controls the expression of PSA, which is overexpressed in prostate cancer. The prostate-attenuated replication-competent adenovirus has a selective cytotoxicity for PSA positive prostate cancer cells and demonstrates antitumor activity in the animal model system (25). Data from phase I of the clinical trial for the oncolytic adenovirus CV706 show that it can be safely administrated to patients via intraprostatic delivery, even at high doses. The trial also suggests that CV706 possesses enough clinical activity, as reflected by changes in serum PSA, to warrant additional clinical and laboratory investigation (26). The alpha-feto-protein gene promoter was selected as a regulator because the alpha-feto-protein gene is highly expressed in 70% to 80% of patients with hepatocellular carcinoma (HCC) but not in normal adults. The tumor-specific replication-restricted adenoviral (TSRRA) vector for HCC demonstrated both specificity and efficacy *in vitro* and *in vivo* (27). Finally, the DF3/MUC1 promoter/enhancer is aberrantly overexpressed in human breast and other types of carcinomas. A recent report indicates that the DF3/MUC1 promoter confers competence for selective replication of the adenovirus in MUC1-positive breast tumor cells, and that the antitumor activity of this vector is potentiated by integration of the TNF cDNA (28).

Nestin is similarly overexpressed by gliomas (29,30). The gene regulator, nestin's second intron, drives glioma-specific gene expression when inserted before the 5' upstream region of the gene (30). Another glioma-related gene regulating element that can be used for conditional transcription is the enhancer region of the glial fibrillary acidic protein (GFAP). It contributes to high level expression of the reporter gene in cells of glial origin whereas there is no detectable expression in nonglial cells (31). Since glioma cells are of glial origin, the target gene would theoretically be expressed preferentially in gliomas, thereby resulting in low toxicity for neurons. In addition, to take the advantage of the hypoxic microenvironment in tumor tissue but not in normal tissue, a DNA cassette composed of a hypoxia-inducible promoter upstream of the Ad type 5 E1 gene region was introduced into the deleted E1 region of a replication-deficient Ad vector. The resulting replication-competent virus shows specific oncolytic activity in tumor cells under hypoxic conditions and reduces human glioblastoma growth in nu/nu mice (32). Moreover, because malignant gliomas have disrupted pRb function, there is an excess of "free" E2F in the cells. E2F-responsive promoters, such as the E2F-1 promoter, should thus be more active in glioma cells relative to normal cells. Based on this prediction, adenoviral vectors that contain transgenes driven by the E2F-1 promoter were constructed. These vectors showed tumor-selective gene expression in gliomas *in vivo* (33). Recently, a replication-competent adenovirus was constructed by inserting the human E2F-1 promoter element upstream of E1A gene. It resulted in extensive cell death in a panel of tumor cells but not in non-proliferating normal cells *in vitro* (34). Therefore, these regulators could be applied to regulate E1A expression in the construction of oncolytic adenoviruses that specifically replicate in glioma cells.

Tissue specific promoters can limit viral replication within a certain tissue type but cannot guarantee exclusive viral replication within cancer cells. Nevertheless, if combined with other targeting systems, these strategies should improve the replication selectivity of the virus.

4. MODIFYING THE TROPISM OF THE ADENOVIRUS---TARGETING THE GLIOMA CELL SURFACE

The entry of adenovirus into cells is initiated by its fiber protein binding with the adenoviral receptor CAR (coxsackie and adenovirus receptor) (35,36). Binding is further strengthened by the interaction between the tripeptide amino acid sequence RGD (located at the adenoviral penton base) with $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins (37). The bound adenovirus is then transported to clathrin-coated pits by the process of random diffusion. In fewer than five minutes after initial adenoviral binding to the cell surface, the virus makes its way to cellular endosomes through endocytosis (38). CAR is expressed by most cell types, including epithelial, neural, fibroblast, and muscle cells (39).

The broad tropism of adenoviruses may constitute a disadvantage when they are intended as exclusive anti-glioma tools. Wild-type adenovirus infects normal and tumor cells indiscriminately. Modification of the natural tropism of the adenovirus is required to generate cancer-targeted oncolytic viruses. The most direct strategy for inducing selective infection of adenovirus into tumor cells is to abolish the adenoviral interaction with its natural receptor and then redirect the viral affinity to a tumor-specific receptor. Such selective infectivity should improve therapeutic efficacy and decrease the potential for viral-associated toxicity. For these reasons, extensive efforts have been made to modify the tropism of adenoviruses. Retargeting has been achieved by the use of bispecific antibodies or oligopeptides with dual binding specificity for fiber protein and receptors on the cell surface (40,41). Adenoviral retargeting has also involved genetic engineering of the adenovirus genome. One strategy consists of introducing heterologous cell targeting peptides into the knob domain. This technology requires meticulous consideration of the structural limitations of the three-dimensional configuration of the fiber. The fiber is synthesized as a monomer which undergoes trimerization prior to its attachment to the penton base (42). Thus, it is critical that modifications of the fiber knob do not impair trimer formation. In addition, the final configuration of the engineered fiber should make the incorporated ligand accessible to target cell receptor recognition and binding. A different retargeting technology focuses on modification of the penton base, which mediates the internalization of the adenovirus (43). In this case, the targeted peptide is used as a substitute for the RGD motif in the penton base.

The role of targeting is essential for systemic delivery of adenoviral vectors or oncolytic adenoviruses. In this setting, the identification of ideal ligands alone is not sufficient for accomplishing clinically meaningful targeting. Problems associated with systemic adenoviral delivery must first be addressed, including the systemic immune response against adenoviruses. Another issue is the stability of adenovirus/ bifunctional crosslinker complexes in the bloodstream. Ongoing clinical trials using systemic adenoviral administration will hopefully yield detailed information about adenoviral pharmacokinetics after single or repeated systemic administration. This knowledge could contribute to the development of a more advanced design for redirecting anticancer-specific adenoviruses.

In glioma cell lines, the expression of CAR varies significantly (44,45). As a result, the sensitivity of glioma cell lines to adenoviral transduction is not homogenous (44,45). Retargeting the adenovirus to glioma cells should improve the efficacy of viral entry into cancer cells and decrease the capability of the targeted construct to infect normal cells. Described below are several potential targets for future targeting of oncolytic adenoviruses designed to predominantly infect glioma cells.

4.1. Epidermal Growth Factor Receptor (EGFR)

Increased and/or aberrant EGFR activity in human cancer is well documented and is closely related to

high-grade gliomas (46-49). As many as 90% of high-grade astrocytic gliomas express EGFR, which is associated with EGFR gene amplification in 40% to 50% of GBMs (46,50,51). EGFR protein overexpression without gene amplification has been reported in 12% to 38% of GBMs (51-53). To take advantage of the fact that EGFR expression is higher in glioma cells than in postmitotic glia and neurons, bispecific antibody conjugates against the fiber knob and EGFR have been used to ablate adenoviral binding to fiber receptors and to retarget binding to EGFR (40,54). A further improved system is an adenoviral construct that expresses a secretory adapter comprised of a recombinant soluble form of truncated CAR (sCAR) fused to human EGF (55). These approaches could be used to retarget adenovirus to EGFR-specific cells independent of fiber-fiber receptor interactions (40,55,56).

The variant III mutant of EGFR (EGFR vIII), which comprises the in-frame deletion of exons 2-7 from the extracellular domain, is the most frequently detected genomic variant. Between 50% to 67% of GBMs that exhibit EGFR gene amplification express EGFR vIII (approximately 30% of all GBMs) (51,57-60). Targeting the EGFR vIII receptor is a promising strategy due to its exclusive expression on the surface of cancer cells. In addition, it has been demonstrated that EGFR vIII allows both the binding and internalization of peptides and antibodies (51).

4.2. Fibroblast Growth Factor Receptor (FGFR)

Fibroblast growth factors (FGFs) and the FGF signaling pathway appear to play significant roles in tumor development and progression (61). FGF is produced in more than 90% of human glioma and meningioma tissues (62). A significant correlation has been found between the expression levels of basic FGF (bFGF and bFGF2) and FGFR1 in human glioma tissues. The increased expression of FGFR1 in tumor cells correlates directly with the numbers of endothelial cells in glioma tissue (62). A successful solution for accomplishing viral retargeting using FGF2 was recently described (63). Similar to the EGFR approach, FGF2 is chemically conjugated to a neutralizing anti-adenoviral antibody to ablate normal viral tropism and confer FGF2 receptor specificity. FGF2-retargeted vectors transduced cells at higher levels than non-retargeted vectors. Furthermore, when adenoviral vectors encoding therapeutic transgenes were administered to tumor-bearing animals, the clinical benefit of enhanced transduction was demonstrated by significantly improved survival rates in groups treated with retargeted FGF2 compared with non-retargeted vectors (64). These observations indicate that FGFR may constitute an excellent glioma cell surface target.

4.3. Urokinase-type Plasminogen Activator (uPAR)

Urokinase-type plasminogen activator (uPA) and its receptor, uPAR, play key roles in glioma invasion. The uPA protein is a serine protease that catalyzes the conversion of inactive plasminogen into plasmin, which degrades extracellular matrix proteins and activates metalloproteases and growth factors (65,66). uPA binds its own cell-surface receptor (uPAR), thereby increasing proteolytic activity at the cell surface (67). The binding of uPA to uPAR not only augments the proteolytic activity of uPA but also favors the focal and directional proteolysis of extracellular matrix molecules (68). In early human glioma studies, uPAR expression was found to be greater in high-grade than in low-grade gliomas (69,70). The binding between uPA and uPAR is thought to play a major role in the invasion of glioblastoma cells into normal brain by concentrating proteolytic activity at the leading edge of the tumor (69,71). The expression of uPAR by human glioblastoma cells may significantly contribute to their invasive capacity (72). Additionally, a low-grade neuroglioma cell line transfected with full-length uPAR cDNA was more invasive than the parental cells (73). Downregulating the level of uPAR using an antisense strategy inhibited glioma invasion *in vitro* and tumor formation and growth *in vivo* (74-76).

Because uPAR is overexpressed on the malignant glioma cell surface, an uPA-retargeted adenovirus would specifically and potently transduce adenovirus into gliomas. A recent report provided support for this hypothesis. A 7-residue peptide derived from uPA (u7-peptide) was coupled to an adenovirus with bifunctional polyethylene glycol (PEG). This retargeted virus transferred the gene more efficiently into human airway epithelial cells which express uPAR (77). Therefore, the uPA/uPAR system could be an excellent target for producing an efficacious therapy for gliomas.

4.4. RGD motif

The first successful and encouraging effort made to genetically modify adenoviral tropism was the insertion of the RGD motif (Arg-Gly-Asp) into the HI loop, capsid, or C-terminal of the viral fiber protein (43,78-80). This strategy resulted in improved adenoviral entry into cells independent of CAR expression. The RGD-retargeted vector increased gene delivery to endothelial and smooth muscle cells expressing αv integrins (80,81). Both $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are expressed in glioma cells and in the vasculature of the tumor. Integrin expression generally correlates with the histologic grade of the tumor. For example, $\alpha v\beta 3$ expression is prominent in astrocytic tumors. $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins are markers of tumor vascularization, particularly endothelial proliferation, which suggests that they have roles in glioma-associated angiogenesis. The $\alpha v\beta 3$ integrin also functions in neoangiogenesis and cell migration at the periphery of high-grade gliomas (82). Therefore, the cell-receptor scenario present in glioma cells consists of low levels of CAR and high levels of RGD-related integrins (44,45). These observations indicate that adenoviruses retargeted to bind integrins should circumvent the lack of CAR expression on the glioma cell surface. The potential effectiveness of the RGD retargeted vector as an anticancer tool was first suggested by the enhanced gene transfer in ovarian cancer cells (83). In the first report of an oncolytic adenovirus (Delta-24) modified by the genetic introduction of an RGD sequence in the fiber of a CRAd, the authors showed that the fiber modification allowed CAR-independent infection leading to the enhancement of viral propagation and oncolytic effect *in vitro* and *in vivo* in

Table 1. Examples of glioma targets

Targets	Cellular localization	Gene function	Targeting strategy
Targeting strategy			
Aberrant p53 pathway	▪ nucleus	▪ tumor suppressor	▪ deletion in E1B
Aberrant Rb pathway	▪ nucleus	▪ tumor suppressor	▪ deletion in E1A
Transcription			
Nestin promoter	▪ nucleus	▪ glial cell marker	▪ control of viral gene transcription
GFAP promoter	▪ nucleus	▪ glial cell marker	▪ control of viral gene transcription
E2F1 promoter	▪ nucleus	▪ transcription factor	▪ control of viral gene transcription
Tropism			
EGFR	▪ cell surface	▪ growth factor receptor	▪ tropism modification
FGFR	▪ cell surface	▪ growth factor receptor	▪ tropism modification
UPAR	▪ cell surface	▪ invasion of GBM cells	▪ tropism modification
Integrins $\alpha v\beta 3$ & $\alpha v\beta 5$	▪ cell surface	▪ binding with RGD motif	▪ tropism modification

prostate and lung cancers (84). The mechanisms of the improved infectivity of the RGD-modified Delta-24 adenovirus have also been tested in sarcomas (85).

The glioma targets are summarized in Table 1. Since the expression of CAR, the natural receptor for adenovirus, varies significantly in glioma cells, modification of the natural tropism of the virus to target glioma-related molecules on the cell surface should greatly facilitate viral infectivity among tumor cells while reducing infectivity among normal cells.

5. IMPROVING CELL LYSIS---ENHANCEMENT OF VIRAL SPREAD

Efficient lysis of the Ad-infected cell is critical for viral spread to adjacent cells and it is now known that the Ad death protein (ADP) is responsible for this particular event. ADP is encoded by the E3 transcription unit and synthesized in small amounts at early stages of infection but synthesized in large amounts at very late stages of infection. ADP is required for efficient lysis of Ad-infected cells and its function, shown in studies of ADP mutants, is to mediate the release of Ad progeny from infected cells. Of interest is that ADP has no sequence homology with other known death-promoting proteins (86).

One method used to increase cell lysis is to construct replication-competent E1A-mutant adenoviral vectors that overexpress ADP (87,88). Incremental ADP expression has been achieved by removing all the ORFs in the E3 region with the exception of the one for ADP. This strategy increases the probability that a major late pre-mRNA could become spliced such that ADP-specific mRNA is synthesized in abundance (87). These vectors lyse cells and spread from cell to cell more rapidly than either the control virus or wild-type adenovirus in cultured tumor cells (87,88). In addition, they are three to four-fold more efficient at inhibiting tumor growth in mice compared with the control virus (87).

In addition to ADP, another adenoviral protein has been shown to induce cell death. The adenoviral E4orf4 protein is a multifunctional viral regulator that induces p53-independent apoptosis in transformed cells, but not in normal cells. Although the E4orf4 protein accomplishes specific functions that impel viral replication, it is highly toxic. This effect could dampen the infectious cycle, but might also facilitate the release of viral progeny. The ability of this adenoviral protein to induce cell death could be used to improve adenoviral replication given favorable circumstances or to induce cell death as a direct therapeutic effect of its overexpression in cancer cells (89).

6. COMBINATION OF ONCOLYSIS WITH OTHER ANTITUMOR THERAPIES

6.1. Combination of oncolysis and gene therapy

To take advantage of their greater efficiency at disseminating in the tumor than replication-deficient vectors, oncolytic viruses have been used to transport genes to enhance anticancer efficacy by activating prodrugs expressing cytokines or other anticancer molecules. The enzyme/prodrug systems delivered by replicating adenoviruses include herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV), cytosine deaminase/5-fluorocytosine (CD/5-FC), and rat cytochrome P450 2B1/cyclophosphamide (CYP2B1/CPA) (90,91). It has been reported that the intrinsic oncolytic effects of E1B-55k-deleted adenoviruses could be significantly enhanced in several solid xenograft tumor models when followed by the use of a HSV-tk/GVC system, alone or in combination with the CD/5-FC protocol (90,92,93). A shortcoming of this approach is that because activated prodrugs like GCV and 5-FC interfere with DNA metabolism, they can also cause antiviral activity and can antagonize viral propagation and cell lysis (94,95). Further research is necessary to assess the replicative potential of the virus and the optimal schedule for drug administration to improve the oncolytic effect of the system used. In contrast with the HSVptk/GCV and CD/5-FU paradigms, the CYP2B1/CPA system does not significantly inhibit viral replication, and the addition of CPA was shown to enhance the oncolytic effects of the virus (90,96).

The cytokines expressed by oncolytic viruses have provided another area of investigation. In animal model experiments, tumor cells transduced with cytokine interleukin-4 (IL-4) demonstrated *in vivo* inhibition of tumor growth by stimulating local inflammatory and/or

immune responses (97,98). In contrast, interleukin-10 (IL-10) did not cause localized tumor killing or generate host immunity (99). When murine genes encoding IL-4 or IL-10 were delivered via oncolytic herpes simplex viruses (HSV) into intracranial gliomas in immunocompetent mice, IL-4 HSV significantly prolonged the survival of the tumor-bearing mice. In contrast, tumor-bearing mice that received IL-10 HSV demonstrated a median survival identical to that of the saline-treated controls (100). A similar strategy proposes to deliver the human interferon consensus gene into tumors using an oncolytic adenovirus (101). Complete regression of breast cancer demonstrates the validity of the approach (101). In addition, strategies to deliver exogenous genes through cloning in the E3 region of a replicating human adenovirus have shown superior transgene expression levels when compared to those generated from a replication-deficient virus (102-104).

6.2. Combination of oncolysis and chemotherapy

Combination therapy with agents that act through different mechanisms will hopefully make the emergence of treatment-resistant disease less likely. Ideally, the toxicities associated with the agents used would be non-overlapping, thereby creating safe combinations for treatment (105). Viral therapy is well suited for use in combination with chemotherapy, although the rationale for combining adenoviruses with chemotherapy has not been completely established. The additive synergistic mechanisms of combined therapies that have been observed in the clinical setting are also not yet entirely understood. One hypothesis is that the induction of S phase by the E1A adenoviral protein may be responsible for cell cycle-mediated chemosensitivity. In fact, adenoviruses can induce a significant number of cells to enter the S phase. An E1A-mutant adenovirus unable to bind the Rb protein also produces similar results in cancer cells (23,106). Overrepresentation of the S phase cell population could augment the effect of concurrently used chemotherapeutic agents, and also dramatically improve the anticancer effect of S phase-specific agents such as topoisomerase inhibitors.

Intratumoral adenoviral replication results in the expression of pro-apoptotic molecules, including tumor necrosis factor (107). The presence of tumor necrosis factor in the tumor milieu may improve the efficacy of pro-apoptotic drugs because of its synergistic action with some chemotherapeutic drugs to induce apoptosis (108,109). The specific ability of adenoviruses to infect and kill arrested cancer cells should improve the anticancer effect of many chemotherapeutic agents that are most effective in actively dividing cells. Also, the adenovirus could target a population of cells that are normally "resistant" to the chemotherapeutic drugs. Further investigation is needed to identify the mechanisms underlying the potential synergy between replication-competent adenoviral agents and chemotherapy.

Preclinical murine tumor model studies demonstrated that *dl*1520 can be safely and effectively combined with cisplatin and 5-FU and that efficient viral replication can still occur (7,110). More importantly, evidence of favorable effects from combining adenoviral therapy and chemotherapy has been obtained in multiple trials. Promising clinical data have been obtained from patients with recurrent head and neck cancer treated with intratumoral *dl*1520 and intravenous cisplatin and 5-FU [105]. Of the 37 patients treated in one study, 19 responded to the therapy, a response rate that compares favorably with that from chemotherapy alone in previous trials (30% to 40%). Cooperation between the virus and drug was further demonstrated when patients were used as their own controls. Patients with more than one tumor mass had a single tumor injected with *dl*1520, but their remaining masses were not injected with adenovirus. By exposing the treated and untreated masses to chemotherapy, the effect of adding viral therapy to chemotherapy could be assessed. The *dl*1520-injected tumors were significantly more likely to respond and less likely to progress than non-injected tumors. Furthermore, some non-injected control tumors that progressed after patients were treated with chemotherapy alone were subsequently treated with *dl*1520. Two of these four injected tumors completely regressed. These data illustrate the potential value of combining adenoviral therapy with chemotherapeutic interventions (105).

In addition to studies of intratumoral injection of adenoviruses, a phase I/II trial of *dl*1520 administered by hepatic artery infusion in combination with intravenous 5-FU and leukovorin is already underway (n = 33 total). Following phase I dose escalation, 15 patients with colorectal carcinoma were treated with combination therapy after failing to respond to either *dl*1520 or chemotherapy alone. In this study, one patient had a partial response and 10 patients demonstrated stable disease [105]. On the other hand, viruses and drugs do not combine well for treating some types of tumors. Data from a phase I/II trial studying the combination of *dl*1520 and gemcitabine chemotherapy (n = 21 patients) in pancreatic cancer patients resulted in only two responses, and both of those patients had not received prior gemcitabine. The critical factor is, however, the inherent resistance of pancreatic tumor cells to viral replication (105). In summary, the phase I/II *dl*1520 trial suggested a potential synergy with cisplatin and/or 5-FU in two tumor types that support viral replication (head & neck and colorectal) but not in a tumor type that is resistant to viral replication (pancreatic) (105).

6.3. Combination of oncolysis and radiotherapy

Radiation is an effective means of treating localized tumors. However, in many cases, radiotherapy alone is not sufficient treatment. Oncolytic adenoviruses, which have the ability to replicate in cancer cells, force the cells into unscheduled DNA synthesis. Of note is that an increased S phase should result in sensitization to radiotherapy. Therefore, the combined use of oncolytic adenoviruses and radiotherapy may result in a broader therapeutic index.

As mentioned above, CV706, a prostate cell-specific adenovirus variant, is currently in clinical trials for the treatment of recurrent organ-confined prostate cancer. The same group demonstrates that CV706-mediated cytotoxicity is synergistic with radiation. Addition of radiation to CV706 resulted in a synergistic increase of

cytotoxicity in the human prostate cancer cell line LNCaP and its animal xenografts, as well as a significant increase of virus burst size, with no reduction in specificity of CV706-based cytopathogenicity *in vitro* and *in vivo* (111). In addition, it has been reported that the antitumor activity of *dl*1520 is augmented by radiotherapy (112).

7. PERSPECTIVE

It will be crucial for the future development of adenoviral therapy to achieve the maximum vector distribution and transgene expression possible within tumors. In that regard, a specific systemic immune effector response can be triggered against treated and untreated lesions and modulate the immune system to circumvent immune-mediated inactivation or destruction of the virus.

As far as translating laboratory findings to patient care, adenovirus researchers have been handicapped by the lack of an efficacious animal model. Human adenoviruses replicate poorly in non-human cells. The most commonly used models for testing drugs, including rodents and non-human primates, are not optimal for toxicity studies or analysis of replication ability. The cotton rat system may be the most suitable model for studying toxicity in an immunocompetent animal, but adenoviral replication in these animals has only been demonstrated in a few tissue types. The use of intracranial human glioma cell xenografts in immunodeficient mice is the most frequently used model for the study of gliomas. It is hoped, however, that the many advances in transgenic technology will yield a suitable model for studying gliomas and adenovirus replication.

The use of oncolytic adenoviruses can provide a solution for only some of the major problems encountered when using gene therapy for cancer, including a low rate of transduction, lack of selectivity, and ineffectiveness caused by the heterogeneity of genetic alterations found in human tumors. A better understanding of how the immune response mediates the effects of adenoviral therapy in cancer patients and developing animal models relevant to the clinical setting can help overcome the limitations to progress in the field of oncolysis. The use of replication-competent adenoviruses to treat patients with malignant gliomas is one of the most promising therapeutic options to date. As translational research in the oncolytic field is fast-paced, clinical trials will undoubtedly yield relevant information in the near future.

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Send correspondence to: Ta-Jen Liu, Ph.D., Department of Neuro-Oncology, Box 316, U. T. M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, Tel: 713-745-4588, Fax: 713-745-1183, E-mail tjliu@mdanderson.org