Efficient Oncolysis by a Replicating Adenovirus (Ad) in Vivo Is Critically Dependent on Tumor Expression of Primary Ad Receptors

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Abstract

Replicating adenoviruses (Ads) are designed to replicate in and destroy cancer cells, generating viral progeny that spread within the tumor. To address the importance of the primary cellular receptor for Ads, the coxsackievirus and Ad receptor (CAR), in permitting intratumoral spread of a replicating Ad, we have used a pair of tumor cell lines differing only in the expression of a primary receptor for Ad5. This novel system thus allowed the first direct evaluation of the relationship between the efficacy of a replicating Ad and the primary receptor levels of the host cell without the confounding influence of other variable cellular factors. We demonstrate that the absence of the primary cellular receptor on the tumor cells restricts the oncolytic potency of a replicating Ad both in vitro and in vivo. Based on these findings, it is apparent that the potential therapeutic advantages afforded by viral replication would be negated by poor intratumoral spread of the viral progeny due to the failure to infect neighboring tumor cells. Because a number of studies have reported that primary cancer cells express only low levels of CAR, our results suggest that strategies to redirect Ads to achieve CAR-independent infection will be necessary to realize the full potential of replicating Ads in the clinical setting.

Introduction

The utility of replication-defective Ad vectors for cancer gene therapy is restricted by their inability to infect every cell within a solid tumor mass (1). The realization of this limitation has led to the development of a novel class of anticancer agents, conditionally replicating Ads. These agents are designed to selectively replicate in and destroy cancer cells, followed by the release of the viral progeny by the lysed cells (2). The relative specificity of viral replication in tumor versus normal cells will therefore play a major role in dictating the safety and efficacy of replicating Ads. To this end, strategies to restrict the replication of Ads to tumor cells have either involved placing the expression of viral genes, most commonly the E1A gene, under the control of tumor- or tissue-specific promoters, or have been based on the complete or partial deletion of viral genes required for replication in normal cells, but not in tumor cells (3, 4). However, the efficacy of replicating Ads as oncolytic agents will also be dependent on the ability of the viral progeny to achieve lateral infection and thereby spread within the tumor (3, 4).

The first step in Ad infection is the high-affinity binding of the COOH-terminal knob domain of the fiber capsid protein (5, 6) to the primary cellular receptor, CAR (7, 8). Subsequent internalization of the virion by receptor-mediated endocytosis is potentiated by the interaction of Arg-Gly-Asp (RGD) peptide sequences in the penton base with secondary host cell receptors, integrins αvβ3 and αvβ5 (9, 10). A number of studies have reported that primary cancer cells express only low levels of CAR and are therefore poorly infected by Ads (11–13). In accordance with this observation, the efficacy of Ad-mediated cancer gene therapy has been limited in preclinical and clinical studies by the resistance of the CAR-deficient tumor cells to Ad infection (14, 15). Consequently, considerable attention is being focused on strategies to modify Ad vectors to achieve efficient, CAR-independent gene transfer (16).

Based on these findings with replication-defective Ad vectors, we hypothesized that a low level of CAR expression on tumor cells would also restrict the efficacy of replicating Ads. In this regard, not only would a deficiency of CAR limit the efficiency of infection by the initial viral inoculum, but, more importantly, the potential therapeutic advantages afforded by viral replication would be negated by poor intratumoral spread of the viral progeny due to the failure to infect neighboring tumor cells.

In this study, we have investigated the hypothesis that the oncolytic potency of replicating Ads could be restricted by poor dissemination of the viral progeny due to the inability to infect tumor cells expressing low levels of CAR. To address this issue, we have used a pair of tumor cell lines that differ only in the expression of a primary receptor for Ad5. This novel system thus allowed the first direct evaluation of the relationship between the efficacy of a replicating Ad and the primary receptor levels of the host cell without the confounding influence of other variable cellular factors. We demonstrate that a deficiency of the primary Ad receptor on the tumor cells restricts the oncolytic potency of a replicating Ad, both in vitro and in vivo. This suggests that the efficacy of replicating Ads could be improved by modifications that allow CAR-independent infection of target cancer cells.

Materials and Methods

Viruses. Ad300wt, a wild-type human Ad serotype 5, was obtained from the American Type Culture Collection (Manassas, VA). AdGFP, an E1-E3-deleted replication-deficient Ad5 vector that expresses GFP under the control of the cytomegalovirus promoter, has been described previously (17). The wild-type Ad and the vector were propagated in the permissive 293 cell line and purified by two rounds of cesium chloride density centrifugation. To determine the viral particle concentration, the virus was diluted in 10 mm Tris (pH 8.0), 1 mm EDTA, and 0.1% SDS and incubated at 56°C for 10 min, and the absorbance at 260 nm was measured. Under these conditions, an absorbance of 1 corresponds to 1.1 × 1012 particles/ml (18).

Cell Lines. Human U118 MG glioma cells were obtained from the American Type Culture Collection. U118 MG-AR cells (previously designated...
U118 MG-Ad5KsFv.rec cells), which express an artificial primary receptor for Ad5, have been described previously (19). The cells were propagated at 37°C in a 5% CO₂ atmosphere in DMEM/Ham’s F-12 supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. U118 MG-AR cells were maintained in medium supplemented with 1 μg/ml BrdUrd (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Attached and detached cells were harvested 4, 6, and 8 days after infection, and encapsidated DNA was purified after precipitating unencapsidated DNA with spermere (20). The viral DNA was digested with XhoI and resolved on a 1% agarose gel. The incorporation of BrdUrd into the DNA as a result of viral replication was determined by a Western blot using a mouse anti-BrdUrd primary antibody (Dako Corp., Carpinteria, CA) followed by a HRP-conjugated rabbit antiamouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and detection with a Western blot chemiluminescence reagent (New England Nuclear Life Science Products, Boston, MA).

**Ad DNA Replication.** U118 MG and U118 MG-AR cells cultured in 6-well plates were infected with Ad300wt or AdGFP at an MOI of 0.1 viral particle/cell. The culture medium was supplemented with 1 μg/ml BrdUrd (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Attached and detached cells were harvested 4, 6, and 8 days after infection, and encapsidated DNA was purified after precipitating unencapsidated DNA with spermere (20). The viral DNA was digested with XhoI and resolved on a 1% agarose gel. The incorporation of BrdUrd into the DNA as a result of viral replication was determined by a Western blot using a mouse anti-BrdUrd primary antibody (Dako Corp., Carpinteria, CA) followed by a HRP-conjugated rabbit antiamouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and detection with a Western blot chemiluminescence reagent (New England Nuclear Life Science Products, Boston, MA).

**Ad Yield Assay.** U118 MG and U118 MG-AR cells cultured in 6-well plates were infected with Ad300wt or AdGFP at an MOI of 0.1 viral particle/cell. Eight days after infection, the cells and media were harvested, and the viral titer was determined by plaque assay on 293 cells.

**CPE Assay.** U118 MG and U118 MG-AR cells cultured in 24-well plates were infected with Ad300wt or AdGFP at an MOI of 0.1 viral particle/cell. Eight days after infection, the cells were fixed and stained with crystal violet.

**In Vitro Cytotoxicity Assay.** U118 MG and U118 MG-AR cells cultured in 24-well plates were infected with Ad300wt or AdGFP at an MOI of 1 viral particle/cell. Eight days after infection, a commercial cell proliferation assay (Promega, Madison, WI) was used to measure cell survival according to the manufacturer’s instructions.

**Animal Experiments.** U118 MG and U118 MG-AR tumor xenografts were established by s.c. injection of 5 x 10⁶ cells into the flank of 8–10-week-old female athymic nude mice (nu/nu; National Cancer Center, Fredrick, MD). On reaching 60–100 mm³, the tumor nodules were injected with PBS or with a single dose of 10⁶ particles of AdGFP or 10⁶ particles of Ad300wt. Eight days after injection, the cells and media were harvested, and the viral titer was determined by plaque assay on 293 cells.

**Results**

To evaluate the hypothesis that the oncolytic potency of replicating Ads correlates with their ability to achieve intratumoral spread based on lateral infection of tumor cells by the viral progeny, we used a pair of cell lines that differ only in the expression of a primary receptor for Ad5. Human U118 MG glioma cells are refractory to Ad5 infection due to a paucity of CAR, although they express the αv integrins necessary for virus internalization (12). We have previously generated a derivative cell line, designated U118 MG-AR, which is sensitive to Ad5 infection due to the expression on the cell surface of an artificial primary Ad5 receptor, in which the extracellular domain is derived from a single-chain antibody with specificity for the Ad5 knob (19). In preliminary experiments, we confirmed that a replication-defective Ad5 vector expressing GFP, AdGFP, was able to infect U118 MG-AR cells, although it failed to infect the parental U118 MG cells, which lack a primary receptor for Ad (data not shown).

Monolayers of U118 MG and U118 MG-AR cells maintained in medium supplemented with BrdUrd were infected at an MOI of 0.1 viral particle/cell with a wild-type, replicating Ad5, Ad300wt, or with a replication-defective Ad5 vector, AdGFP, as a control. Encapsidated Ad DNA was isolated from equivalent numbers of cells at various times after infection, digested with XhoI, and subjected to Southern blot analysis using an anti-BrdUrd antibody. As shown in Fig. 1, Ad DNA could not be detected 6 or 8 days after infection of U118 MG or U118 MG-AR cells with the replication-defective Ad5 vector AdGFP, although a very small amount of newly synthesized DNA was present at 4 days. In contrast, newly synthesized Ad DNA could be purified from cells infected with the wild-type Ad, with a significantly greater amount of encapsidated DNA present at all time points in U118 MG-AR cells compared with U118 MG-AR cells. Thus, more Ad DNA was synthesized in the cells that express a primary Ad receptor and can therefore be infected by the replicating Ad.

We next sought to investigate whether the increased synthesis of viral DNA by the replicating Ad in cells expressing a primary cellular receptor for Ad5 would lead to an increase in the production of infectious viral progeny. To this end, monolayers of U118 MG and U118 MG-AR cells were infected with Ad300wt or AdGFP at an MOI of 0.1 viral particle/cell. Eight days after infection, the cells and media were harvested, and the viral titer was determined by plaque assay on 293 cells. As expected, no infectious virus was produced in cells infected with AdGFP, a replication-deficient vector. Infection of primary receptor-negative U118 MG cells with Ad300wt resulted in...
4.0 × 10^7 plaque-forming units/ml, whereas the viral yield in U118 MG-AR cells was more than 57 times greater (2.3 × 10^9 plaque-forming units/ml). Thus, more Ad virions were produced in the cells that express a primary Ad receptor and can therefore be infected by the replicating Ad, allowing the viral progeny to spread efficiently throughout the monolayer. This finding was confirmed by a quantitative assay in which viable cells were counted (Fig. 2B). Hence, the absence of the primary Ad receptor on the U118 MG cancer cells significantly reduced the oncolytic potency of the replicating Ad in vitro.

We next wished to determine whether the absence of the primary Ad receptor from cells in a solid tumor mass would restrict the ability of a replicating Ad to achieve lateral infection and spread within the tumor. Athymic nude mice bearing s.c. U118 MG or U118 MG-AR xenografts on the flank were injected intratumorally with a single dose of 10^9 particles of Ad300wt or with 10^8 particles of AdGFP or PBS alone as controls (five mice/group). Tumor growth kinetics are shown in Fig. 3. We observed that U118 MG tumors treated with PBS grew at a faster rate than U118 MG-AR tumors treated with PBS (P = 0.0001). Treatment of U118 MG tumors with the replication-defective AdGFP vector did not cause a significant reduction in volume compared to treatment with PBS (P = 0.085; Fig. 3A). Similarly, there was no significant difference in the size of U118 MG-AR tumors treated with either PBS or AdGFP (P = 0.314; Fig. 3B). Hence, intratumoral injection of the replication-defective vector

![Fig. 2. A, CPE assay. Monolayers of U118 MG and U118 MG-AR cells were infected with AdGFP or Ad300wt at the indicated MOI. Eight days after infection, the CPE was monitored by staining the viable cells with crystal violet. The replicating Ad did not kill the parental U118 MG cells but caused extensive CPE in the U118 MG-AR cells. Representative results are shown. B, cell viability assay. Monolayers of U118 MG and U118 MG-AR cells were infected with AdGFP or Ad300wt at a MOI of 1 viral particle/cell. Eight days after infection, a cell proliferation assay was performed to count viable cells. The replicating Ad did not kill the parental U118 MG cells but caused a significant reduction in survival of the U118 MG-AR cells. Results are the mean of triplicate experiments.](image)

![Fig. 3. Growth kinetics of s.c. U118 MG (A) and U118 MG-AR (B) tumors in athymic nude mice. Tumors were injected with a single dose of 10^9 particles AdGFP (green squares), 10^8 particles of Ad300wt (blue triangles), or with PBS (red circles). Data points represent the mean ± SE of the tumor size in each group at the indicated time points (n = 5). The oncolytic potency of the replicating Ad was significantly greater in the U118 MG-AR tumors than in the U118 MG tumors.](image)
CAR DEFICIENCY RESTRICTS POTENCY OF REPLICATING AD

In this study, we have investigated the hypothesis that the efficacy of replicating Ads could be restricted by poor dissemination of the viral progeny due to the inability to infect tumor cells expressing low levels of the primary Ad receptor. To address this issue, we have used a pair of tumor cell lines that differ only in the expression of a primary receptor for Ad5. We demonstrated that the oncolytic potency of a replicating Ad was significantly greater in the receptor-positive cell line, both in monolayers of cells in vitro and in solid tumors in vivo. Moreover, the greater efficacy of the replicating Ad in the receptor-positive tumors was due to increased spread of the virus. Therefore, we have shown that it is necessary for a replicating Ad to achieve efficient lateral infection of the tumor cells to realize its full potential as an anticancer agent. This novel model system thus allowed the first direct evaluation of the relationship between the efficacy of a replicating Ad and the primary receptor levels of the host cell without the confounding influence of other variable cellular factors.

A number of studies have reported that primary cancer cells from human patients express only low levels of the primary Ad receptor, CAR, and are therefore poorly infected by Ads (11–13). Based on our results, this suggests that the efficacy of replicating viruses dependent on CAR-mediated infection pathways will be restricted in the clinical setting. In accordance with this, Phase I and II clinical trials in which patients with recurrent squamous cell carcinoma of the head and neck received direct intratumoral injection of a replicating Ad, ONYX-015, resulted in clinical benefit in less than 15% of cases (22, 23). Only when combined with standard chemotherapy did this oncolytic Ad cause an objective response (at least a 50% reduction in tumor size) in 19 of 30 cases, with 8 complete responses (24).

The CAR deficiency of primary human cancer cells suggests that the efficacy of replicating Ads could be improved by modifying the viruses to allow efficient infection via a CAR-independent pathway. In this regard, Shinoura et al. (25) have reported that the potency of...
a replicating Ad in glioma cell lines in vitro and in vivo could be improved by the addition of a stretch of 20 lysine residues to the COOH-terminal of the fiber protein, allowing the virus to bind to cellular heparan sulfate receptors. Similarly, Suzuki et al. (26) have shown that the efficacy of a replicating Ad can be enhanced by incorporating an RGD peptide motif into the fiber protein, permitting the virus to bind to αv integrins. Each of these strategies to enhance the infectivity, and hence the potency, of replicating Ads resulted in expanded viral tropism; the viruses retained the ability to recognize the native primary Ad receptor, CAR, which is expressed by normal cells. Therefore, modifications to the fiber protein that both introduce a tumor cell-specific targeting motif and ablate recognition of CAR would simultaneously improve both the efficacy and safety of replicating viruses by permitting efficient, CAR-independent infection of tumor cells while preventing infection of normal cells (16). This would complement other strategies to restrict the replication of Ads to tumor cells, either by placing the expression of viral genes, most commonly the E1A gene, under the control of tumor- or tissue-specific promoters or by the complete or partial deletion of viral genes required for replication in normal cells, but not in tumor cells.

References

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