Efficient Oncolyis 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 813

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3 The abbreviations used are: Ad, adenovirus; BrdUrd, bromodeoxyuridine; CAR, coxsackievirus and adenovirus receptor; CPE, cytopathic effect; GFP, green fluorescent protein; HRP, horseradish peroxidase; MOI, multiplicity of infection.
CAR DEFICIENCY RESTRICTS POTENCY OF REPLICATING AD

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 400 μg/ml G418. FCS was purchased from Life Technologies, Inc. (Grand Island, NY), and media and supplements were from Mediatech (Herndon, VA).

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 μCi/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 Southern blot using a mouse anti-BrdUrd primary antibody (Dako Corp., Carpinteria, CA) followed by a HRP-conjugated rabbit antimouse 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 24-well plates were infected with Ad300wt or AdGFP at MOIs of 0.1 and 1 viral particle/cell. Eight days after infection, the cells and media were harvested, and the viral titer was determined by a 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 commercial cell proliferation assay (Promega, Madison, WI) was used to measure cell survival according to the manufacturer’s instructions.

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 × 10⁶ cells into the flank of 8–10-week-old female athymic nude mice (nu/nu; National Cancer Center, Frederick, MD). On reaching 60–100 mm², the tumor nodules were injected with 50 μl of PBS or with a single dose of 10⁶ particles of AdGFP or 10⁶ particles of Ad300wt in 50 μl of PBS (five mice/group). Bidimensional tumor measurements were taken twice a week with calipers, and the tumor volume was calculated using the simplified formula for a rotational ellipsoid: 0.5 × length × width² (21). Animals were followed for 38 days, until the tumor burden in some of the control groups became excessive, and the mice were sacrificed. Experiments were performed in accordance with federal and institutional guidelines for animal care.

Statistical Methods. Descriptive statistics (mean and SD) on tumor volume (mm³) were calculated per day for each treatment group. The percentage change in volume was calculated for U118 MG tumors infected with Ad300wt compared with the control groups treated with AdGFP or PBS. A similar procedure was used to calculate the percentage change in the volume of U118 MG-AR tumors infected with Ad300wt compared to the two controls, AdGFP and PBS. The mean percentage change in tumor volumes was compared between U118 MG and U118 MG-AR cells infected with Ad300wt using one-way ANOVA and tests of repeated measures using SAS software (version 6.12; SAS Institute, Inc. Cary, NC). The mean tumor volumes were compared between U118 MG tumors treated with PBS and U118 MG tumors treated with AdGFP using the ANOVA (t test). Likewise, the mean tumor volumes were compared between U118 MG-AR tumors treated with PBS and U118 MG-AR tumors treated with AdGFP. Similar tests were performed to compare mean tumor volumes between controls and Ad300wt-treated tumors. P < 0.05 was considered statistically significant in all of the analyses.

Immunohistochemistry. The presence of Ad5 capsid proteins in U118 MG and U118 MG-AR tumor sections was determined by immunohistochemical analysis using polyclonal rabbit anti-Ad5 antisera (Cocalico, Reamstown, PA) as the primary antibody with an HRP-conjugated goat antirabbit secondary antibody (Jackson ImmunoResearch Laboratories). Diaminobenzidine (Sigma, St. Louis, MO) was used as the chromogenic substrate.

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 α₅ 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 Southwestern 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 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 a 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

![Fig. 1. Southwestern blot analysis of Ad DNA synthesis. Monolayers of U118 MG and U118 MG-AR cells maintained in medium supplemented with BrdUrd were infected with AdGFP or Ad300wt at an MOI of 0.1 viral particle/cell. Encapsidated Ad DNA was isolated from equivalent numbers of cells at the indicated times after infection, digested with XhoI, and subjected to Southwestern blot analysis using an anti-BrdUrd antibody. A significantly greater amount of DNA was synthesized by the replicating Ad in U118 MG-AR cells compared to U118 MG cells. Representative results are shown.](image-url)
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.

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
did not affect tumor growth kinetics. Before statistical analysis of the effects of the replicating virus, it was necessary to adjust for the differential growth rates of the U118 MG and U118 MG-AR tumors in the control treatment groups. Ad300wt caused a significant reduction in the size of U118 MG-AR tumors compared to U118 MG tumors ($P = 0.0007$ when adjusted for treatment with AdGFP). Whereas U118 MG tumors treated with Ad300wt grew at rates comparable to the control groups ($P = 0.1119$ versus AdGFP), U118 MG-AR tumors injected with Ad300wt actually decreased in size ($P = 0.0001$ versus AdGFP), with complete tumor regression being observed in three of the five mice. Thus, the oncolytic potency of the replicating Ad was significantly greater in the solid tumors expressing a primary Ad receptor.

To confirm that the reduction in size of the U118 MG-AR tumors was due to intratumoral spread of Ad300wt, tumor sections were analyzed for Ad capsid proteins. Immunohistochemical staining with rabbit anti-Ad5 antiserum indicated that Ad300wt replicated and was disseminated throughout the U118 MG-AR tumors (Fig. 4). No staining was seen in U118 MG tumors injected with Ad300wt or in either U118 MG or U118 MG-AR tumors treated with AdGFP, the replication-defective Ad5 vector. Thus, the enhanced oncolytic potency of the replicating Ad in the solid tumors expressing a primary Ad receptor was due to increased intratumoral spread of the virus.

**Discussion**

A major limitation of cancer gene therapy strategies is the inability of replication-defective Ad vectors to disseminate throughout a solid tumor. A number of groups are seeking to address this problem by using Ads that replicate in cancer cells, thereby lysing the cells and releasing viral progeny that spread to neighboring cells. The potency of this novel class of anticancer agents will therefore depend on the efficiency of dissemination of the virus throughout the tumor.

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 $\alpha_v$ 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.

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