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Downmodulation of E1A Protein Expression as a Novel Strategy to Design Cancer-Selective Adenoviruses[1]

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Abstract

Oncolytic adenoviruses are being tested as potential therapies for human malignant tumors, including gliomas. Here we report for the first time that a mutation in the *E1A* gene results in low levels of E1A protein, conditioning the replication of mutant adenoviruses specifically to cancer cells. In this study, we compared the oncolytic potencies of three mutant adenoviruses encompassing deletions within the CR1 (Delta-39), CR2 (Delta-24) regions, or both regions (Delta-24/39) of the E1A protein. Delta-39 and Delta-24 induced a cytopathic effect with similar efficiency in glioma cells and a comparable capacity for replication. Importantly, the activity of Delta-39 was significantly attenuated compared to Delta-24 in proliferating normal human astrocytes. Direct analyses of the activation of E2F-1 promoter demonstrated the inability of Delta-39 to induce S-phase–related transcriptional activity in normal cells. Interestingly, E1A protein levels in cells infected with Delta-39 were remarkably downmodulated. Furthermore, protein stability studies revealed enhanced degradation of CR1 mutant E1A proteins, and inhibition of the proteasome activity resulted in the striking rescue of E1A levels. We conclude that the level of E1A protein is a critical determinant of oncolytic phenotype and we propose a completely novel strategy for the design and construction of conditionally replicative adenoviruses.

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Introduction

Oncolytic adenoviruses, engineered to selectively replicate in and lyse cancer cells, hold great potential as efficacious therapeutic agents for primary brain tumors [1–4]. One of the strategies commonly used to design oncolytic adenoviruses is to genetically modify E1A protein binding of key cellular proteins. Comparing E1A protein sequences of various adenovirus serotypes identified three evolutionary conserved regions (CR), pointing to their critical roles in E1A activity [5]. Thus, the CR1 (42–72 aa) and CR2 (115–140 aa) regions in type 5 human adenovirus (Ad5) include E1A binding sites for p300/CBP, Rb, and Rb-related proteins [5,6]. One critical consequence of their ensuing interactions is disrupting pRb binding to E2F. This dysregulation stimulates the transcription of E2F-responsive genes and induces quiescent cells to enter the S-phase [7]. Interactions between E1A and cell cycle modulators would be critical in infected quiescent normal cells in which the G1 checkpoint is tightly regulated by a network of proteins with overlapping functions. The effect of such viral–cellular protein interactions would, however, not be particularly critical in cancer cells where G1/S transition controls are already partially inactivated. This is the underlying rationale that is driving the assessment of E1A mutant adenoviruses as preferentially or exclusively acquiring lytic properties in cancer cells [1,2].

In this work, we compared the antiglioma efficacy and replication selectivity of a CR1 mutant adenovirus (Delta-39) with the CR2 mutant adenovirus, Delta-24 [3]. Importantly, we uncover that cells infected with Delta-39 expressed low levels of E1A protein due to protein instability and enhanced proteosomal-mediated degradation. However, although the low level of E1A did not substantially impair the ability of Delta-39 to replicate in cancer cells, the activity of Delta-39 was significantly attenuated in proliferating normal cells. Delta-39 is thus an excellent potential candidate for further development as an antiglioma therapeutic tool. This is the first report showing that the level of E1A protein is a critical determinant of the selectivity of oncolytic adenoviruses and proposes a completely novel strategy for the design and construction of conditionally replicative adenovirus.

Abbreviations: CR, conserved regions; CPE, cytopathic effect; MOI, multiplicity of infection; PFU, plaque-forming units

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Materials and Methods

Cell Lines

U-87 MG, D-54 MG, U-251 MG, and 293 cells were cultured as previously described [3]. Normal human astrocytes (NHA) were obtained from Clonetics/BioWhittaker (Wakersville, MD) and cultured using the manufacturer's specifications.

Adenoviruses

Delta-24 characteristics were reported previously [2]. The 39-nucleotide deletion, corresponding to 48 to 60 aa in the CR1 region of the E1A protein, in pXC1 (Microbix Biosystems, Inc., Ontario, Canada) or pXC1-D24 [3]) was accomplished by replacing the *Bam*HI/*Xba*I fragment, encompassing the 701 to 739 nt region of the Ad5 genome, with two polymerase chain reaction (PCR) fragments flanking this deleted region, resulting in pXC1-D39 (harboring a 701–739 nt deletion in the adenoviral genome) and pXC1-D24/39 (harboring deletions of 701–739 nt and 923–946 nt in the Ad5 genome). XC1-D39 or pXC1-D24/39 was cotransfected with pBHG10 (Microbix Biosystems, Inc.) into 293 cells to generate the Delta-39 and Delta-24/39 adenoviruses, respectively, and then amplified in 293 cells and purified by CsCl gradient centrifugation [8]. To confirm the deletions, PCR amplification of a region of the Ad5 genome, which encompasses the 39- and 24-bp deletions in the E1A region, was followed by *Xho*II or *Bst*XI enzymatic digestion. Controls include wild-type adenovirus (Adwt) [9], ultraviolet-inactivated adenovirus, and mock infections [3].

Immunoprecipitation and Immunoblotting

Cells infected with Adwt, Delta-24 at 20 MOI, or Delta-24/39 at 50 MOI, or mock-infected were collected 16 hours after infection and suspended in ice-cold PBS plus 0.5% NP40 and a protease inhibitor cocktail (Sigma, St. Louis, MO), and then lysed by sonication. For each sample, 2 μg of anti-E1A antibody and 20 μl of Protein A Agarose beads (Oncogene, Cambridge, MA) were added to 400 μg of cell lysate proteins. After 4 hours of immunoprecipitation at 4°C, the beads were pelleted and washed. The precipitated proteins or cell lysate proteins were dissolved in 1× SDS loading buffer, separated by SDS-PAGE, and probed with antibodies against adenovirus 2 E1A (13 S-5), p300 (C-20), Rb (C-15)-G, or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). For Western blot experiments, cells were infected with 50 MOI of each adenovirus; however, different viral dosages were used for infection to achieve similar E1A expression levels in cell lysates for immunoprecipitation.

Cell Viability and Viral Replication Assays

Crystal violet, MTT, and tissue culture infection dose$_{50}$ ($TCID_{50}$) assays were conducted as described previously [3,10]. Final viral titers were determined as plaque-forming units (pfu/ml) according to the validated method developed by Quantum Biotechnology (Carlsbad, CA).

Luciferase Assay

A total of 3×10^4/well cells was seeded in 24-well plates and grown for 24 hours. Each well was cotransfected with 250 ng of the E2F-1 reporter plasmid [11] and 1 ng of pRL-CMV as control for transfection efficiency (Promega Life Science, Madison, MI) with FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). The cells were infected 2 hours later with 5 MOI of the adenoviruses. Twenty-four hours after transfection, the cells were lysed and luciferase activity was assayed with a luminometer.

E1A Protein Stability Studies

A total of 6×10^4 cells/well was seeded in 24-well plates and infected 24 hours later with adenoviruses at 50 MOI. Sixteen hours later, 100 μM anisomycin (Sigma) was added to the culture to inhibit protein synthesis. Cell lysates were collected in 1× SDS loading buffer (100 μl/well). An equal volume of cell lysates was used for immunoblotting analysis of E1A protein expression.

Proteasomal-Mediated Degradation of E1A

Cells were infected with adenoviruses for 16 hours at 50 MOI and then either mock-treated or treated with 10 μM lactacystin (Calbiochem, La Jolla, CA) for 24 hours. The cell lysates were subjected to immunoblotting analysis.

Results

Construction and Characterization of Delta-39 and Delta-24/39 Adenoviruses

The Delta-24 adenovirus harbors a 24-nucleotide deletion (from 923 to 946 nt in the Ad5 genome) corresponding to 122 to 129 aa in the CR2 region of E1A protein, which is responsible for pRb binding [3]. The Delta-39 adenovirus encompasses a deletion of a 48 to 60 aa region (from 701 to 739 nt in the Ad5 genome) in the CR1 region of the E1A protein, which is responsible for p300 binding [12]. The Delta-24/39 adenovirus was constructed by generating a CR1 mutation similar to the one in Delta-39, on a Delta-24 backbone [3] (Figure 1*A*). Adenoviral genome deletions were verified through PCR amplification followed by restriction enzyme digestion (Figure 1*B*), and then confirmed by sequencing (data not shown). As expected, immunoprecipitation analyses of mutant E1A complexes showed that CR1 mutant E1A proteins do not bind p300, and that CR2 mutant proteins do not physically interact with pRb (Figure 1*C*).

Delta-39 and Delta-24 Induced Comparable Oncolytic Effects in Glioma Cells

We first studied the *in vitro* antiglioma activity of the Delta-24, Delta-39, and Delta-24/39 adenoviruses in three glioma cell lines: U-251 MG, D-54 MG, and U-87 MG. Crystal violet assays showed that all three E1A mutant adenoviruses displayed antiglioma activity and that there was no marked difference between the oncolytic effects of Delta-24 and Delta-39 (Figure 2*A*). The antiglioma activity of Delta-24/39 was inferior to the other two mutant adenoviruses (Figure 2*A*). A quantitative assessment of cell viability by MTT demonstrated a consistent adenovirus-mediated