Advances in Brief

The Presence of the Adenovirus E3 Region Improves the Oncolytic Potency of Conditionally Replicative Adenoviruses

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Abstract

Purpose: The initial development of conditionally replicative adenoviruses (CRAds) for cancer treatment has aimed at achieving selective replication in and killing of malignant cells. Other aspects such as the potentiation of the cytolytic capacity have also been investigated but still require new endeavors. As an extension of our prior work, we analyzed the effect of the E3 region, which includes the adenovirus death protein, in the context of CRAd oncolytic potency.

Experimental Design: We constructed E3-positive (E3+) and E3-negative (E3−) variants of the previously characterized CRAd, Ad5-AD24, and its infectivity enhanced version, Ad5-AD24RGD, and compared their oncolytic effect in human cancer cell lines infected with 0.01 viral particle/cell and in s.c. xenografts of A549 human lung cancer cells injected intratumorally with a single dose of 10^7 adenoviral particles in immunodeficient mice.

Results: The in vitro experiments showed that the E3+ viruses kill tumor cells 1.6–20 times more effectively in different cell lines. As well, the in vivo study demonstrated that the administration of E3+ CRAds resulted in a more potent oncolytic effect compared with the same dose of their E3− counterparts 35 days after virus administration. Moreover, a time course study of virus replication within the tumor xenografts established a correlation between higher in situ propagation of E3+ CRAds and tumor growth inhibition compared with E3− viruses.

Conclusions: These results indicate that the presence of E3 can enhance the antitumor potency of CRAds over and above the levels conferred by the enhancement of infectivity via Arg-Gly-Asp (RGD).

Introduction

In the last 5 years, virotherapy of cancer has made remarkable advancements and has been rapidly tested in several clinical trials. The variety of viruses designed to replicate selectively in malignant tissue is widening, and Ad1 (1–4), herpes simplex virus (5), influenza virus (6), Newcastle disease virus (7, 8), poliovirus (9), reovirus (10, 11), vaccinia virus (12, 13), and vesicular stomatitis virus (14), among others, are being developed for this purpose. In the context of CRAds, two strategies have been used to achieve tumor or tissue selectivity (15). One is the generation of partial deletions of E1 genes that are necessary for a virus to replicate in normal cells but are dispensable in cancer cells. Ads deleted of the p53-binding protein E1B-55K or the retinoblastoma (pRB) binding site of protein E1A are examples of this group (1–4). The other is the introduction of tissue- or tumor-specific promoters to control transcription units in adenoviral genes essential for replication, namely, E1, E2, and/or E4 (2, 16–21). There are more than 10 clinical trials in Phases I to III (reviewed in Refs. 22 and 23) that demonstrated tolerability and safety of CRAd administration; however, the antitumor efficacy of the CRAds used as a single agent was found to be modest (22, 24, 25).

Efforts to improve oncolytic potency of CRAds include the infectivity enhancement of Ad by the incorporation of motifs like the integrin-binding RGD into the HI loop of the fiber knob (26) or the heparan sulfate-binding polylysine at the COOH-terminal of the fiber protein (27). Further strategies are the combination with chemotherapeutic agents and/or radiotherapy (28–31) or the generation of “armed” CRAds with pro-drug-activating genes (32, 33). Nevertheless, these strategies have to cope with the possibility that the action of the drugs themselves may operate at cross-purposes with the viral replication (34). Moreover, the strategy of “arming” the replicative virus with other genes has to be carefully evaluated against a possible detrimental effect of deleting viral genes to create space for the new transgenes (35, 36). Few expression cassettes can be incorporated into the adenoviral genome without deletions (35), and frequently these are introduced in the E3 region, especially if conservation of the replication capacity is the concern (37).

Therefore, it is important to quantify the decrease in oncolytic...
potency that results from E3 deletions. The Ad E3 region encodes, among other proteins, a M_{r} 11,600 protein known as ADP (38). Low-level expression of ADP starts at early phases of the viral life cycle; however, this protein exerts its function at very late stages, provoking the cytolysis of the infected cell, the release of viral progeny, and the spread of the virus to the surrounding cells (39–41). In a previous study, we demonstrated the superiority of the oncolytic effect of an infectivity-enhanced CRAd, Ad5-Δ24RGD, compared with the unmodified Ad5-Δ24 in vitro and in vivo (26). This mutant contains an RGD motif in the fiber knob that binds to α_v integrins and allows cell entry via a pathway independent from the CAR, the primary Ad receptor. At the initial stages of the development of Δ24 Ads, the role of the proteins encoded by the E3 region in the context of CRAd efficacy was not clear. Along with the realization of the importance of the E3 region, we performed subsequent studies. The results from these experiments indicate that the presence of E3 augments the oncolytic potency of both Ad5-Δ24 and Ad5-Δ24RGD in vitro and in vivo. These observations are explained by the occurrence of amplification loops consisting of earlier host cell disruption by E3-containing CRAds, lateral dispersion of the progeny, and viral replication that leads to further cytolysis. Therefore, we reason that the conservation of E3 will certainly induce a significant increase in the antitumor efficacy of CRAds.

Materials and Methods

Cell Lines. A549 human lung adenocarcinoma, Hs 766T human pancreatic cancer, and SKOV-3 human ovarian adenocarcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA; ATCC numbers CCL-185, HTB-77, and HTB-134, respectively). All of them are defective in p16 disrupting the pRB/p16 pathway of cell cycle control (42–44). Human embryonic kidney 293 cells were obtained from Microbix Biosystems Inc. (Toronto, Canada). Cells were cultured in DMEM supplemented with 5% heat-inactivated FBS in the absence of antibiotics and maintained at 37°C in a 5% CO_2 atmosphere.

Virus Construction. All of the CRAds used in this study contain a 24-bp deletion (Δ24), from Ad5 bp 923 to 946 (both included), corresponding to the E1A region necessary for pRB protein binding (45). Details of the tumor-specific replication of the Δ24 mutant virus are presented elsewhere (3). Viruses were grown in A549 lung cancer cells and purified by cesium chloride banding, and the physical titer was calculated by absorbance measurement at a wavelength of 260 nm (A_{260 nm}; 1 A_{260} unit = 1.1 x 10^{12} vp/ml; Ref. 46). The vp:CPE unit ratio (vp:CPE units) of the Δ24 mutants ranged from 9 to 42. AdGFP is a complete E1-deleted virus that contains a GFP expression cassette driven by the CMV promoter (47).

Ad5-Δ24/FGF (E3−). The plasmid pE3-GFPzeo carries the expression cassette containing the CMV promoter and the GFP-Zeocin fusion gene (1,771 bp) from pTracer-SV40 (In Vitrogen, Carlsbad, CA) flanked by Ad5 sequences (left, bp 27,040–28,045; right, bp 30,863–31,948; Ref. 48). An A/III/SpH fragment from this plasmid was inserted by homologous recombination into the E3 region of pKS4050 that contains the viral genome except the E1 and E3 genes generating pKS4050-GFPzeo. The plasmid pXC1-Δ24 has been described previously (3). In brief, this plasmid was constructed by site-directed mutagenesis of pXC1 (Microbix Biosystems Inc.) to loop out bp 923–946 from the E1A transcription unit. pKS4050-GFPzeo and pXC1-Δ24 were cotransfected into 293 cells using the calcium phosphate method to generate the virus.

Ad5-Δ24E3 (E3+). Two fragments from pXC1-Δ24 (BsrGI/XbaI and XbaI/MfeI) containing E1A with the 24-bp deletion and the E1B transcription unit were ligated with the BsrGI/MfeI fragment of pShuttle (Stratagene, La Jolla, CA) to generate pShuttle-Δ24. Plasmid p24E3+ was generated by homologous recombination between a pShuttle-Δ24 linearized by PmeI/EcoRI digestion and pTG3602 linearized by PacI digestion (49). The resulting adenoviral genome was transfected into 293 cells to rescue the virus.

Ad5-Δ24GFP/RGD (E3−). The A/III/SpH fragment from pE3-GFPzeo containing the CMV promoter-driven GFP-Zeocin gene flanked by the left and right arms of E3 was introduced into pVK526 (26) by homologous recombination, and the resulting adenoviral genome linearized with PacI was transfected into 293 cells to rescue the virus.

Ad5-Δ24RGD (E3+). This virus was constructed by homologous recombination of the E1 fragment that was isolated from the plasmid pXC1-Δ24 and the ClaI-digested plasmid pVK503 containing the RGD fiber (50) to generate pVK526. To rescue the virus, the resulting Ad genome was excised from the plasmid with PacI and transfected in 293 cells (26).

Validation of Virus Constructs. The Δ24 deletion in all of the Ad mutants was analyzed by PCR with primers E1a-1 (5′-ATTACCGAGAATAATGCCGC-3′) and E1a-2 (5′-CCATTTAACACGCGCATGCA-3′) followed by BsrXI digestion. This deletion results in the loss of a BsrXI cleavage site yielding only one band of 1023 bp upon BsrXI digestion, as opposed to the two bands (725 and 322 bp) obtained from the wild-type E1A. The presence/absence of the RGD motif in all of the viruses was confirmed by PCR of the modified fiber region with the primers FiberUp (5′-CAACCGTGTGATTCATATC-3′) and FiberDown (5′-GTGGTGAAGGATGTGCGAAT-3′). The expected sizes of the PCR products are 247 bp for the viruses with wild-type fiber and 274 bp for the RGD-containing mutants (26). Further verification was performed by sequence analysis (CEQ2000 Dye Terminator Cycle Sequencing kit; Beckman-Coulter, Fullerton, CA) with primers E1a-1 and Fiber-up and E3-Adp (5′-CAGCGAACCCACCTACAACAGA-3′).

Virus Infection. Cells were infected at indicated concentrations (vp/cell) in DMEM supplemented with 2.5% FBS. After an absorption time of 2 h, cells were washed once with serum-free DMEM and maintained in DMEM supplemented with 2.5% or 5% FBS at 37°C in a 5% CO_2 atmosphere. Physical titers (vp) were used because the viruses contained different capsids.

Virus Spread Assay. A549 cells cultured in 2-well chamber slides (Nalge Nunc International Corp., Naperville, IL) were infected with 0.1 vp/cell Ad5-Δ24GFP or Ad5-Δ24E3 as described. On days 2, 3, 4, and 5 after infection, the slides were fixed with 3% formaldehyde, blocked, incubated with goat anti-hexon polyclonal antibody (25 μg/ml; Chemicon Inc., Temecula, CA) followed by Texas red-labeled donkey anti-goat.
IgG (10 μg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and counterstained with Hoechst 33342 (20 μg/ml; Molecular Probes, Eugene, OR). The slides were analyzed by fluorescence microscopy (Olympus 1X70; Olympus America, Inc., Melville, NY), and photographs were taken using the MagnaFire system (Optronics, Goleta, CA).

**Plaque Development Assay.** A549 cells cultured in 6-well plates were infected with 0.1 vp/cell Ad5-Δ24GFP or Ad5-Δ24E3. After a 2-h adsorption time, they were washed once with serum-free DMEM and a 6-ml agar overlay consisting of a 1:1 mix of autoclaved 1.33% Bacto-agar (Difco, Detroit, MI), and 2× DMEM supplemented with 5% FBS was added. Formation of plaques was observed during a period of 12 days, and individual plaques were photographed using an inverted microscope (Olympus 1X70) and the MagnaFire imaging system.

**Oncolysis Assay.** A549, Hs 766T, and SKOV-3 cells were cultured at 80% confluence in 12-well plates in triplicate, infected with Ads at a dose of 0.01 vp/cell as described, and cultured until CPE was evident in 90% of the cells in any of the treatment groups. Cell viability was assessed using the methods described next.

**In Vitro Cytotoxicity Assay (XTT).** Cell survival was determined using a colorimetric assay based on XTT (Sigma, St. Louis, MO) that quantifies the number of living cells based on mitochondrial activity. The media were carefully aspirated from the wells, and 300 μl/well DMEM without phenol red supplemented with 2.5% FBS and 20% XTT was added. After an incubation time of 2 h, the absorbance of the media from each well was measured at a wavelength of 450 nm. The percentage of viable cells/well was calculated considering the uninfected well was 100% viable.

**Crystal Violet Staining.** After the XTT assay was performed, the cells were fixed with 10% buffered-formaldehyde for 10 min and stained with 2% crystal violet solution.

**Spectrophotometric Titration of Ad.** To analyze multiple samples originating from both in vitro (cell pellets and supernatants) and in vivo (tumor extracts) experiments, we used a modified version of the titration method proposed by O’Carroll et al. (51). Briefly, 293 cells were seeded in 96-well plates (4 × 10^4 cells/well) in DMEM containing 0.5% FBS. After an overnight attachment period, cells were infected with serial dilutions of each sample. Four days after the infection of 293 cells, media and detached cells were removed, and cell loss (CPE) was determined by measuring changes in total protein concentrations in each well. This concentration is obtained by adding 200 μl of BCA protein assay reagent (Pierce, Rockford, IL) to each well, shaking for 2 h at room temperature, and measuring the absorbance in a microtiter plate reader at a wavelength of 595 nm. The readings were expressed as a percentage of the respective uninoculated controls, and graphs were plotted against the virus dilutions using the Origin scientific graphing and analysis software (OriginLab, Northampton, MA). The Ad titer (CPE units/ml) were calculated as the dilution that corresponds to CPE_{50} multiplied by the number of cells killed (20,000), and divided by the inoculum (0.1 ml).

**Dynamics of Production and Release of Ad Progeny.** A549 cells seeded in 6-well plates were infected with 0.1 vp/cell Ad5-Δ24GFP or Ad5-Δ24E3 as described previously and maintained in DMEM with 2.5% FBS. Supernatants and cells were harvested separately on days 3, 6, and 9 and kept frozen until analysis. Cell pellets were resuspended in 1 ml of DMEM with 2.5% FBS and subjected to three freeze/thaw cycles before titration as described above.

**s.c. Tumor Xenograft Model.** Female 5–7-week-old athymic nu/nu mice (Frederick Cancer Research, Frederick, MD) were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Eight million A549 cells were xenografted s.c. into the right flank of the mice under anesthesia. When the nodules reached a volume of 70–110 mm^3, a single Ad dose (10^7 vp in 30 μl of PBS) or the same volume of PBS was administered intratumorally (n = 5). This administration scheme was based on previous studies by us that demonstrated that a single low Ad dose suffices to produce tumor cell destruction across the entire tumor mass (26). Tumor size was monitored twice a week, and fractional volume was calculated by the formula: (length × width × depth/2) (52). The mice were euthanized 35 days after the treatment to prevent excessive tumor growth in the control group. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

**Ad Hexon Immunodetection.** The presence of Ad particles in the tumor xenografts was assessed by immunofluorescence detection of the hexon protein. A549 tumors were dissected aseptically, embedded in tissue-freezing media (Triangle Biomedical Sciences, Durham, NC), flash-frozen in liquid nitrogen, and stored at −80°C until sectioning. Tumor sections were fixed with 3% formaldehyde, blocked, incubated with goat anti-hexon polyclonal antibody (25 μg/ml; Chemicon Inc.) followed by Alexa Fluor 488-labeled donkey antigoat IgG (10 μg/ml; Molecular Probes), and counterstained with Hoechst 33342 (20 μg/ml; Molecular Probes). The slides were analyzed with an inverted fluorescence microscope (Olympus 1X70), and photographs were taken using the MagnaFire system.

**Virus Replication in Tumor Xenografts.** The in vivo replication of the viruses was assessed using the s.c. tumor xenograft model described in a previous paragraph. Similarly, the nodules were injected with a single dose of 10^7 vp of conditionally replicative viruses, a nonreplicative AdGFP, or PBS. On days 3, 6, and 9 after injection, whole tumors were harvested (n = 3), weighed, frozen, and stored at −80°C until analysis. To make the extracts, the tumors were thawed, minced using sterile scissors, and digested with Liberase Blendzyme 3 (0.1 mg/ml; Roche Molecular Biochemicals, Indianapolis, IN) with constant agitation for 1.5 h at 37°C. The resulting cell suspensions were freeze/thawed three times and spun, and the supernatants were filtered through a low protein binding 0.2-μm-pore membrane (Pall Gelman Laboratory, Ann Arbor, MI). The concentration of virus progeny present in the extracts was titered by the spectrophotometric method.

**Statistical Analysis.** Determinations of significant differences among groups were assessed by calculating the value of Student’s t using the Origin data analysis and graphing software (OriginLab).
Fig. 1  Schematic representation of the CRAds. The white boxes indicate the 24-bp deletion in the CR2 region of the E1A gene, the gray-shaded boxes represent the GFP gene substituting the E3 gene, and the black boxes symbolize the 27-bp sequence encoding the RGD motif in the fiber knob region.

**Results**

**Virus Structure.** Fig. 1 shows the structure of the mutant Ads used in this study. All of the viruses contain a 24-bp deletion corresponding to the amino acid sequence L122 TCHEAGF129 of the E1A protein known to be necessary for pRB protein binding (45). Ad5-Δ24/GFP and Ad5-Δ24GFP/RGD E3 regions (Ad5 bp 28,045–30,863) were substituted with a CMV promoter-driven GFP-Zeoicin expression cassette, whereas Ad5-Δ24E3 and Ad5-Δ24RGD contain the wild-type E3 region (bp 27,858–30,828). The presence or absence of a GFP cassette in the deleted E3 region did not change the cytolytic capacity of Ad5-Δ24E3 (formerly named Ad5-Δ24; Ref. 26) versus Ad5-Δ24/GFP (data not shown), and we chose to use the viruses containing GFP as E3− viruses considering the possibility of direct observation of the virus presence. Additionally, Ad5-Δ24GFP/RGD and Ad5-Δ24RGD contain 27 bp in the HI loop of the fiber knob that code for the amino acid sequence CDCRGRDFC (50).

**The Presence of E3 Enhances the Spread of CRAds.**

The ability of the E3− and E3+ CRAds to spread through a cell monolayer was analyzed using an assay based on the immunodetection of Ad hexon protein. A549 cells cultured in chamber slides were infected with 0.1 vp/cell Ad5-Δ24/GFP or Ad5-Δ24E3, and the number of cells positive for Ad presence was examined on days 2–5 after infection. When administered at low dose, the virus must go through several cycles of replication, cell lysis, and secondary infection to spread through the cell monolayer. We detected the presence of Ad in the cytoplasm of a similar number of cells in both groups 2 days after infection (Fig. 2, a and e). Afterward, the E3+ CRAd spread quickly. The number of virus-carrying cells increased considerably on day 3 when the virus could still be observed in the cytoplasm, but cell disruption, virus release, and spread took place on day 4, and the monolayer was completely destroyed on day 5 (Figs. 2f–h and n–p). In contrast, the number of E3− CRAd-bearing cells showed only a slight increment starting on day 4, and the majority of the virus was still located inside the cells after 5 days (Fig. 2d). When an overlay of agar is added on top of similarly infected cells, the diffusion of Ad progeny to the media is prevented, thus allowing the observation of plaque formation by local spread and CPE of the virus. In this experiment, the growth of E3− CRAd plaques was slower compared with E3+ CRAds resulting in smaller plaques after 12 days of incubation (data not shown). These results indicate that the presence of the E3 region increases the cell spreading ability of Δ24 mutants.

**E3+ CRAds Gain Oncolytic Potency in Vitro.**

The cell killing capacity of the different mutants was compared in three human cancer cell lines defective in the pRB/p16 pathway. For this purpose, we infected the cells with low doses (0.01 vp/cell) of each virus to allow multiple cycles of viral replication over the following days. Cell viability was assessed by XTT assay (Fig. 3A), and the live cells attached to the wells were stained with crystal violet on days 9 (A549), 10 (Hs 766T), and 17 (SKOV-3) after infection (Fig. 3B). Fig. 3A shows the percentage of cells killed by the different CRAd mutants in the respective experiments shown in Fig. 3B. In all three cell lines, the viruses containing the E3 gene exerted a more potent cell killing effect than the viruses lacking E3 (statistical differences ranging from at P < 0.02 to P < 0.0000004). The gain in oncolytic potency obtained by the E3+ viruses ranged from 1.6-fold in Hs 766T cells treated with Ad5-Δ24/GFP versus Ad5-Δ24E3 to 20-fold in SKOV-3 cells treated with Ad5-Δ24GFP/RGD versus Ad5-Δ24RGD. The enhancement in cell killing effect was more evident in the cell lines A549 and SKOV-3 that were more resistant to E3− CRAds. With regard to the RGD mutation, no differences were detected between the groups treated with E3− viruses in any cell type. In contrast, in the E3+ virus-treated groups, Ad5-Δ24RGD demonstrated higher oncolytic potency compared with Ad5-Δ24E3 (A549, P < 0.01; Hs 766T, P < 0.02; SKOV-3, P < 0.02).

**The Presence of E3 in a CRAd Augments the Release of Ad Progeny.**

To study the effect of the E3 region on the release of CRAd progeny and its correlation with oncolysis, we infected A549 cells with Ad5-Δ24/GFP or Ad5-Δ24E3 to compare two titers of infectious virus: one liberated to the media and the other remaining inside the cells. Media and cells were
harvested separately on days 3, 6, and 9 after infection and titrated using a spectrophotometric assay (Ref. 51; Fig. 4). Titers are expressed as total CPE units either in the supernatant (entire volume of media in the well) or in the cell pellet (totality of cells in the well). In the E3/Ad5-H900224/GFP-infected group, the titer in the cell fraction increased 5-fold on day 6 compared with day 3, indicating that virus replication took place. However, viral progeny was released to the supernatant only after 9 days (3.1 × 10^6 total CPE units; Fig. 4A). At the end of the experiment, the media:cell virus titer ratio was 4.9:1. In contrast, in the group treated with the E3/Ad5-H900224E3, the release of virus progeny to the media started as early as day 3 (2.9 × 10^6 total CPE units), increasing constantly throughout the incubation period until the titer reached 1 × 10^9 CPE units on day 9. The titer of the cell fraction did not show a big increase, presumably because the new viruses were rapidly released from the cell (Fig. 4B). The final media:cell titer ratio was 37.7:1. These findings indicated that the E3-containing CRAd released its progeny 7.7 times more efficiently than the one lacking E3. The results closely paralleled the in vitro oncolysis experiment performed in A549 cells (Fig. 3A, top panel) with the 7-fold increase in oncolytic potency of the Ad5-Δ24E3 virus compared with its E3-counterpart.

**E3+ CRAds Show More Potent Oncolytic Effect than E3− CRAds in Vivo.** To analyze the in vivo lytic potency of our viruses, A549 human lung cancer cells xenografted into nude mice were treated with a single intratumoral injection of 10^7 vp of CRAds or PBS. As observed in Fig. 5A, tumor growth was partially (Ad5-Δ24E3) or totally (Ad5-Δ24RGD) arrested during the 35 days following the administration of E3+ CRAds. In contrast, the size of the tumors treated with either E3− CRAd, nonreplicative virus AdGFP, or vehicle progressively increased in the same period [fractional tumor volumes: Ad5-Δ24E3 (3.22 ± 0.77) versus Ad5-Δ24/GFP (7.95 ± 3.54);
Ad5-Δ24GFP (1.87 ± 0.53) versus Ad5-Δ24GFP/RGD (7.83 ± 2.81)]. Also, the combination of E3 and the infectivity enhancement conferred by the RGD motif resulted in an additional increment in the oncolytic potency. The statistical significance of the tumor volume measurements compared with the vehicle-treated group was as follows: P < 0.05 for Ad5-Δ24E3 and P < 0.01 for Ad5-Δ24RGD. No significant difference was found for Ad5-Δ24/GFP and Ad5-Δ24GFP/RGD (Fig. 5A).
Also, the comparison between RGD versus non-RGD mutants of either E3− or E3+ CRAds did not show statistical differences. At the end of the experiment, we studied the presence of CRAd progeny in the tumor tissue by immunostaining of the Ad hexon (Fig. 5, B–D). All nodules were sampled and analyzed except for two E3− Ad5-Δ24E3-treated tumors, two E3+ Ad5-Δ24RGD-treated tumors, and one E3− Ad5-Δ24GFP/RGD-treated tumor due to either the small volume or the pulpy consistency of the nodule (necrotic material). Whereas vehicle-treated nodules showed no green fluorescence (Fig. 5B), E3+ viruses were readily detected in the tumor nodules with a locally concentrated pattern (Fig. 5C). Similarly, 30% of the tumors treated with E3− viruses presented a scattered faint fluorescent signal (Fig. 5D). However, the rest of the E3− CRAd-treated tumors failed to show any signal. Altogether, the in vivo data indicate that CRAds replicate in the tumor tissue and that the inclusion of the E3 region enhances the oncolytic capacity of CRAds.

Titrations of Virus Progeny Recovered from Tumor Xenografts. To correlate the in situ replication of the CRAds with the lytic effect inflicted on the tumors, we quantified the amount of infectious virus present in the tumors in a time course experiment. The extracts used for titration were obtained from the nodules by enzymatic dissociation. In a preliminary test performed to rule out possible alteration of structural and/or functional characteristics of the Ad due to protease exposure, we treated nonreplicative AdGFP with an enzyme solution at the same concentration that was used for tissue dissociation. Next, A549 cells were transduced with protease-treated or PBS-treated AdGFP virus, and the transduction efficiency was shown to be the same for both groups (data not shown). Titration of extracts from CRAd-treated tumors ranged from 3 × 10^8 CPE
units/g tissue (Ad5-Δ24/GFP) to 2 × 10^{11} CPE units/g tissue (Ad5-Δ24RGD; Fig. 6A). At all time points, the mean virus titer of the nodules treated with E3+ CRAds tended to be higher compared with that of their E3− counterparts, although statistical significance was reached only on day 9 (Ad5-Δ24/GFP versus Ad5-Δ24E3, \( P = 0.057 \); Ad5-Δ24GFP/RGD versus Ad5-Δ24RGD, \( P = 0.036 \)). Of note, Ad5-Δ24RGD-treated tumors show the highest mean amount of virus progeny, although Ad5-Δ24E3 attains a similar value at the end of the experiment. As expected, Ad was not present in any of the extracts derived from tumors injected with nonreplcative AdGFP. Tumor volumes were also measured at the moment of harvest (Fig. 6B), and there are no statistically significant differences among the treatment groups because the time points chosen for this experiment are included in the early phase of an in vivo study (Fig. 5A; see “Discussion”). However, Ad5-Δ24RGD-treated tumors clearly tend to have smaller volumes. Altogether, these data establish a correlation between virus replication and the oncolytic effect of the different CRAds in vivo.

**Discussion**

In this report, we analyzed the effect of the Ad E3 region on the oncolytic capacity of previously described CRAds, Ad5-Δ24 (3) and Ad5-Δ24RGD (26). ADP, one of the seven E3 proteins, mediates cell lysis and release of the progeny, leading to spread of Ad from cell to cell. Here, we compared the dispersion of E3− or E3+ CRAds throughout A549 cell monolayers after infection with 0.1 vp/cell by means of Ad hexon immunostaining. Our results indicate that the presence of the E3 region enhances the spreading ability of Ad5-Δ24E3, which started as early as 3 days after infection, peaked on day 4, and destroyed the cells in 5 days (Fig. 2, a−h). In contrast, Ad5-Δ24/GFP showed only a slight increase at the end of the experiment, indicative of its slower spread (Fig. 2d).

The higher capacity of E3+ CRAds to spread resulted in a superior oncolytic efficacy compared with E3− CRAds in vitro. Cytotoxicity assays indicated that the increment in oncolytic efficiency ranges from 1.6× to 20×, depending on the cell line (Fig. 3, A and B). Also, although modest, Ad5-Δ24RGD demonstrated a statistically significant oncolytic advantage over the non-RGD virus in all of the cell lines. In low CAR-expressing cell lines, such as Hs 766T (53) and SKOV-3 (54), the presence of \( \alpha_v \) integrins led to infectivity enhancement via the interaction with RGD modified fiber increasing the oncolytic effect (\( P < 0.02 \) for both cell lines). Also in A549 cells, which express moderate levels of CAR, the notably high expression of \( \alpha_v \beta_3 \) integrin (Refs. 55 and 56; data not shown) contributed to enhance infectivity and oncolysis (Ad5-Δ24E3 versus Ad5-Δ24RGD, \( P < 0.01 \)).

Recently, viral burst has been proposed as a more relevant measurement of oncolytic potency of CRAds compared with live-dead assays (57). Therefore, we conducted a burst titration experiment comparing Ad5-Δ24/GFP and Ad5-Δ24E3 in vitro. The resulting progeny release patterns revealed remarkable differences dependent on the E3 status when virus titers of the media versus those of the cell fraction were compared in A549 cells infected with the same dose of either virus. Of note, the results of the early time points correlate to the observations of the virus spread assay. The E3− Ad5-Δ24/GFP was not able to disrupt the host cell and escape until day 9, at which time the majority of the progeny is released into the media, leaving a small amount of virus in the remaining cells (Fig. 4A). In contrast, virus was detected in the E3+ Ad5-Δ24E3-infected cells and supernatants as early as day 3, indicating the onset of viral replication, early cell lysis, and progeny release. The virus titer of the cell fraction increased 62-fold from day 3 to day 6. Afterward, this number diminished due to the low number of live cells remaining that can support further viral replication after 9 days (Fig. 4B). The titer in the media showed a constant increment up to 1 × 10^{10} total CPE units on day 9, and the total virus yield (sum of media and cells compartments) at the end of the experiment was 274 times higher in the E3+ virus than in...
the E3− one. ADP mutants are affected only in the cell lysis capacity and release of the progeny, but the virus growth remains unchanged (40). Consistently, we do not think that this 274-fold increase reflects an advantage in the E3+ virus replication process itself, but rather that the faster virus release led to more free virus to continue further rounds of infection, replication, release, and logarithmic expansion. In conclusion, these results confirmed the existence of a strong correlation between a bigger viral progeny burst (Fig. 4B) and a greater oncolytic potency of the E3+ Ad5-Δ24E3 (Fig. 3A) in vitro.

The oncolysis study performed in vivo confirmed the in vitro findings and showed that a single dose of 10^7 vp injected intratumorally sufficed to demonstrate the superior antitumor effect of E3+ CRAds over E3− CRAds. Ad5-Δ24E3 and Ad5-Δ24RGD exerted, respectively, partial or total oncolytic effects 35 days after treatment in comparison with the nodules treated with the E3− viruses (Fig. 5A). This graph shows the strong role that E3 plays in the Ad5-Δ24 context. As mentioned, the Ad5-Δ24 used in previous reports does not contain E3 (26), corresponding to Ad5-Δ24/GFP in the present report. Accordingly, the differences between Ad5-Δ24 and Ad5-Δ24RGD reported previously (26) were more evident due to a contribution of both E3 and RGD. The present work dissects these effects and shows that both E3 and RGD contribute to enhance the oncolytic potency. The fact that we were able to detect viruses in cryosections of CRAd-treated tumors 35 days after injection suggested that virus progeny was produced. The growth curves of E3+ CRAd-treated tumors together with the immunodetection data indicated that cell lysis and lateral spread were strong and fast enough to abolish tumor growth, and although complete tumor eradication was rarely seen, some of these nodules (40%) were composed mostly of necrotic material. In contrast, the presence of E3− CRAds in the growing tumors suggested that their cell killing capacity is not potent or fast enough to oppose tumor growth (Fig. 5A). Nevertheless, it has to be considered that the interaction of each of the mutant viruses with the cells is dynamic. It is possible to have more inefficiently released virus (E3−) if this accumulates slowly during the 35 days of treatment. In contrast, the E3+ viruses that rupture cells and expand faster might face a lack of host cells after overcoming the balance between tumor cell growth and cell killing by the virus, and the number of virus might decrease together with the tumor lysis. From these results, we concluded that the presence of E3 confers the advantages of early cell disruption and progeny release that are required for an efficient oncolytic effect in vivo and that the combination with the infectivity-enhancing RGD motif results in additional enhancement of the antitumoral effect. In fact, work done in our group sustains the superiority of the RGD-containing viruses in the context of oncolysis.4

To further study the in vivo behavior of the CRAds, we recovered infectious virus particles from the tumors without compromising their infectivity by using an enzymatic dissociation of the tissue. As the method of titration, we chose the spectrophotometric assay reported by O’Carroll et al. (51). This is based on the biological activity of the virus, unlike other techniques such as quantitative PCR that are based on the DNA (or RNA) sequences in the sample. Considering that not all of the viral DNA is packaged and not all of the encapsidated viruses are capable of infection (58, 59), we wished to measure exclusively the bioactive progeny virus. The result showed that the tumors treated with E3+ CRAds have higher mean values compared with the E3− counterparts, and Ad5-Δ24RGD is highest among all of the treatment groups (Fig. 6A). The 293 cells used for the titration assay were not affected by exposure to either the extracts obtained from PBS-treated tumors or the nonreplicative AdGFP-treated tumor extracts. This assured that the CPE, on which the titration is based, was indeed provoked by the presence of CRAd progeny and not by toxic substances derived from the tumor. The side-by-side comparison with the corresponding tumor sizes indicated that higher virus titers corresponded to smaller tumor volumes. However, several factors should be taken into account when quantitative analyses of this type are done. It is important to include the entire tumor because the Ad is not homogeneously distributed throughout the tumor mass. As suggested by Harrison et al. (60), structural barriers inside the established tumor may preclude the spread of the CRAds, and, as a result, a sample taken from the tumor might not be representative of what is happening to the nodule as a whole. Another point is that the tumor is composed not only of cells but also of fibrous and necrotic tissue, making representative sampling a problem. As a matter of fact, previous experiments have shown that Ad5-Δ24RGD-treated nodules were composed mainly of necrotic material 35 days after the injection and showed lower titers than the nodules that still contained cellular components (data not shown). Necrotic tissue does not support virus production. This is the reason why early time points were chosen for the in vivo titration of virus growth (Fig. 6). As described in Fig. 5A and also in our previous work (26), it is not possible to demonstrate statistical difference in size at this initial phase, but this time frame allows the recovery of tumors without necrosis that lead to more real comparisons of virus replication capacity.

Similar to the study performed with promoter-based CRAds by Yu et al. (41), the E3+ mutants used in the present report contain the complete E3 region. For this reason, we cannot dissect the effect of the individual genes within this region. However, we speculate that ADP is the principal molecule eliciting cell lysis. It is known that the dynamics of plaque formation and cell lysis by mutants that lack other proteins encoded by E3 is normal in vitro (40). In addition, there is proof that transgene substitutions for ADP, but not for the other E3 genes, reduce the effectiveness of the virus cell killing in vitro (37, 61, 62). The role of ADP would therefore be manifested in preclinical antitumor assays and could have an important implication in therapy. Nevertheless, other E3 genes with immune suppressive function may also have an important role for oncolysis in the presence of an immune system. As mentioned previously, the E3 region encodes six other proteins, four of which are involved in immunoevasion, namely protein 14.7K, the RIDa/β complex (formerly 10.4K/14.5K), and gp-19K (63–

65). The preservation of immunoregulatory E3 genes in a CRAd may thus be favorable because it enhances the immune evasion potential of the virus (37). On the other hand, it has been suggested that the deletion of the E3 region combined with an overexpression of the ADP gene in a CRAd may lead to a combined tumor cell killing effect elicited by the CRAd and the immune system (66, 67). The interplay of the other E3 proteins with the immune system and its influence on the oncolytic effect of CRAds requires further investigation in immune-competent models for Ad oncology.

In the present report, we have extended our studies on the enhancement of the oncolytic potency of Ad5-Δ24 and Ad5-Δ24RGD by the inclusion of the E3 region. We have demonstrated that the addition of the E3 gene enhances the antitumor effect of CRAds by promoting an earlier cell lysis, progeny release, and faster spread compared with E3−viruses. Improvements such as the incorporation of the RGD motif for infectivity enhancement and the E3 region for oncolysis enhancement will have a major impact on the clinical applications of CRAds.

Acknowledgments

We thank Cristina Balague (Research Center, Almirall Prodesfarma, Barcelona, Spain) and Dirk M. Nettelbeck (Gene Therapy Center, University of Alabama at Birmingham) for critical review of the manuscript and Dr. Albert Tousson (High Resolution Imaging Facility, University of Alabama at Birmingham) for valuable technical advice.

References


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