Syrian Hamster as a Permissive Immunocompetent Animal Model for the Study of Oncolytic Adenovirus Vectors

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Abstract

Oncolytic adenoviruses represent an innovative approach to cancer therapy. These vectors are typically evaluated in immunodeficient mice with human xenograft tumors. However, in addition to being immunodeficient, this model is limited because normal and cancerous mouse tissues are poorly permissive for human adenovirus replication. This prompted us to search for a model that more accurately reflects the use of oncolytic adenoviruses in cancer patients. To this end, we developed a novel Syrian hamster model that is both immunocompetent and replication-permissive. We found that human adenovirus replicates well in Syrian hamster cell lines and confirmed replication in the lungs. Oncolytic adenovirus injection showed efficacy in three different hamster tumor models. Furthermore, i.t. oncolytic adenovirus injection resulted in suppression of primary and metastatic lesions, i.t. replication and necrosis, vector entrance into the bloodstream, replication in the liver and lungs, and anti-adenovirus antibody induction. Our findings show that the Syrian hamster is a promising immunocompetent model that is permissive to human adenovirus replication in tumors as well as normal tissues. Therefore, the Syrian hamster model may become a valuable tool for the field of oncolytic adenovirus vectors in which vector safety and efficacy can be evaluated. (Cancer Res 2006; 66(3): 1270-6)

Introduction

Cancer is a leading cause of death worldwide, and there is a critical need for novel cancer therapies. One treatment approach is the use of viral vectors, including those based on adenovirus serotype 5 (Ad5), which generally causes a mild respiratory illness in young children. Oncolytic (replication competent) Ad vectors kill cancer cells as part of the natural Ad life cycle, by replicating and releasing progeny virions, lysing the cancer cell in the process.

Ad replication is generally species specific, and human Ads replicate poorly in cells from most other species. For example, the natural site of Ad5 replication in humans is the lung, but attempts to detect Ad replication in the mouse lung have been unsuccessful (1, 2). Consequently, oncolytic Ad vectors are commonly evaluated in immunodeficient mice, which permit the growth of human xenograft tumors. However, this model does not accurately reflect how oncolytic Ad vectors might behave in humans because these mice lack an intact immune system and both normal and cancerous mouse tissues are poorly permissive for Ad replication (1–4). Therefore, there is a serious need for an immunocompetent animal model that is permissive for replication in malignant and normal tissues so that the toxicity, biodistribution, and antitumor efficacy of oncolytic Ad vectors can be accurately evaluated.

In contrast to most other species examined, the Syrian hamster is permissive for human Ad replication in the lung (2). Following intranasal inoculation of Ad5 into Syrian hamsters, virus yields increased in the lungs, and histopathology consistent with pneumonia was observed (2). Therefore, we examined the Syrian hamster as an immunocompetent, replication-permissive animal model for the evaluation of oncolytic Ad vectors.

Materials and Methods

Cell lines. Syrian hamster cell lines (Hak and DDT1 MF-2) and the human cell line A549 (American Type Culture Collection (ATCC), Manassas, VA) were maintained in DMEM containing 10% fetal bovine serum (FBS; ref. 5). Although Hak cells were originally derived from normal kidneys, tumors formed in all animals. Additionally, HaK tumors metastasized to multiple sites and were histologically characterized as adenocarcinomas. The Syrian hamster pancreatic carcinoma cell line, PC1 (Dr. Parviz Pour, University of Nebraska Medical Center), was maintained in RPMI 1640 containing 10% FBS (5).

Viruses and vectors. VRX-007 (6) is identical to Ad5, except the former lacks most of the E3 region (7) and overexpresses the E3 11.6k adenovirus death protein (ADP; ref. 8). Although HaK cells were originally derived from normal kidneys, tumors formed in all animals. Additionally, HaK tumors metastasized to multiple sites and were histologically characterized as adenocarcinomas. The Syrian hamster pancreatic carcinoma cell line, PC1 (Dr. Parviz Pour, University of Nebraska Medical Center), was maintained in RPMI 1640 containing 10% FBS (5).

Immunofluorescence. Cells were plated onto coverslips and infected 1 to 2 days later. Cells were fixed (3.7% paraformaldehyde in PBS), permeabilized (methanol containing 4% dimethylsulfoxide), and stained (pool of E1A monoclonals, DBP antiserum, fiber monoclonal). Secondary antibodies were goat anti-rabbit IgG FITC-conjugate and goat anti-mouse IgG rhodamine-conjugate (Cappell/ICN, Aurora, OH).

Western blots. Monolayers were infected and at each time point were washed thrice with cold PBS and frozen in radioimmunoprecipitation assay suspension buffer (11). Ten micrograms of protein were run on a 10% SDS-polyacrylamide gel, transferred to polyvinylidene fluoride membranes, and incubated with rabbit serum against Ad5 late proteins (ATCC; ref. 12).

Single-step growth curves. Cells were plated onto 35-mm dishes and infected 1 to 2 days later. At 1 hour after infection, monolayers were washed thrice, and at the indicated time points, both monolayer and medium were harvested (5).

Animals. Syrian (Golden) hamsters (Mesocricetus auratus; 4–5 weeks old) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals were allowed access to food and water ad libitum. Studies were approved by the Animal Care Committee (Saint Louis University) and done in accordance with institutional and federal regulations.
S.c. tumor efficacy studies. Tumors were formed by s.c. injection of cells (HaK, 2 × 10^3; DDT1 MF-2, 1 × 10^3; or PC1, 1 × 10^6) into the hindflank(s) of hamsters (5). Established tumors were injected i.t. Tumor volumes were monitored with digital calipers.

Endotracheal instillation. Ad5 was administered via endotracheal instillation into anesthetized hamsters (5). Lungs were harvested, minced, frozen and thawed thrice, homogenized, sonicated, centrifuged, and titered by plaque assays on A549 cells.

I.t. replication and biodistribution. Animals with single HaK tumors received one i.t. injection of 2.5 × 10^11 viral particles of VRX-007 (± 1 × 10^10 TCID_{50} infectious units), 2.5 × 10^11 viral particles of Ad-GFP (± 1 × 10^9 TCID_{50} infectious units), or vehicle. At the indicated time points, animals were sacrificed and organs were collected. Organs were homogenized, frozen and thawed thrice, sonicated, centrifuged, and titered by TCID_{50} assays on 929 cells (5).

Evaluation of the immune response. Sera were obtained from hamsters before and 13 to 14 days after i.t. injection of virus. Immunofluorescence, immunoprecipitation, and Western blots were done on Ad5-infected cells using these sera. Virus neutralization activity was determined by serially diluting the sera (1:2), incubating with VRX-007 [1 × 10^5 plaque-forming units (pfu)] for 1 hour in 96-well plates, and then seeding A549 cells in the wells. At 8 days after infection, monolayers were fixed and stained with crystal violet. Crystal violet was extracted with 30% acetic acid, and absorbance was quantitated at 560 nm. Neutralizing antibody titers were determined by the dilution that inhibited cytopathic effects by ≥75%.

Statistical analysis. To compare two treatments, a Student’s t test was used. Kaplan-Meier survival analysis and log-rank statistics were used to evaluate survival curves. For all experiments, a significance level of P < 0.05 was considered significant.

Results

Expression of Ad genes in Syrian hamster cell lines. We initially evaluated the ability of the oncolytic Ad vector VRX-007 to infect Syrian hamster cancer cell lines. VRX-007 (6) is identical to Ad5, except the former lacks most of the E3 region (7) and overexpresses ADP (8). VRX-007 infected HaK cells (Syrian hamster renal cancer cell line) and A549 cells (highly permissive human cancer cell line) with similar efficiency, even at low multiplicities of infection (Fig. 1A). In another experiment with HaK and two additional Syrian hamster cancer cell lines [PC1 (pancreatic carcinoma) and DDT1 MF-2 (ductus deferens leiomyosarcoma)], VRX-007 infected the majority of the cells (i.e., they expressed the Ad E1A and DBP proteins at 24 hours after infection), and progression into late infection was observed (Ad fiber expression at 48 hours; Fig. 1B). The level of late protein expression in the hamster cell lines was comparable with that seen in A549 cells (Fig. 1C).

Ad replication in vitro. When Ad replication was examined in a single-step growth curve, the burst size at 4 days in HaK cells was only 7-fold less than in human A549 cells (Fig. 1D, top). VRX-007 replicated well in the hamster cell lines (PC1, HaK, and DDT1 MF-2), yielding more than three logs of virus growth (Fig. 1D, bottom). Therefore, human Ad is able to infect, express viral proteins, and replicate in Syrian hamster cell lines at levels comparable with human cells.

Oncolytic Ad efficacy in hamster tumor models. After showing Ad replication in vitro, we tested the ability of oncolytic Ad vectors to inhibit s.c. hamster tumor growth. Established HaK tumors were injected with VRX-007, wild-type Ad5, UV-inactivated VRX-007, or vehicle. VRX-007 and Ad5 reduced the mean tumor growth rate (Fig. 2A, left) and increased animal survival (Fig. 2A, right) compared with UV-inactivated VRX-007 and vehicle. One animal treated with VRX-007 was cured and remained tumor free >5 months after treatment. Interestingly, we observed considerable peritumoral inflammation in animals that were injected with VRX-007 or Ad5, which is reflected in the peak of tumor volumes at about 1 week, that resolved by about 2 weeks after injection (Fig. 2A, left). Virus capsid was not responsible for this inflammation, as it was not observed in tumors injected with UV-inactivated VRX-007. A similar acute tumor swelling was observed in a clinical trial following administration of an oncolytic Ad vector in cancer patients (13).

Primary s.c. HaK tumors metastasized to the lungs, kidneys, and renal lymph nodes. In addition to suppressing the growth of primary tumors (Fig. 2B, top left), VRX-007 suppressed metastasis (Fig. 2B, top right and bottom), as exemplified by a 75% reduction of tumor burden in the lungs (data not shown). In fact, the lungs from some VRX-007–treated animals contained no metastases. Histopathologic examination confirmed a significant reduction in metastasis to the lungs (P = 0.001) and the kidneys (P = 0.02) by VRX-007 (Fig. 2B, top right).

Other hamster tumor models were also examined. VRX-007 significantly reduced the growth of DDT1 MF-2 tumors (P = 0.001; Fig. 2C). Necropsy of animals in this study revealed that animals injected with VRX-007 had smaller tumors and fewer lung metastases. With a third hamster cell line, PC1, VRX-007 suppressed tumor growth (P < 0.05) despite the fact that initial tumor volumes were ~1 mL (Fig. 2D).

Examination of tumor pathology and immune response following i.t. oncolytic Ad injection. To learn more about the mechanism of tumor suppression by Ad, we examined the histology of tumors at study termination. Histopathologic examination of s.c. HaK tumors revealed areas of necrosis in many tumors injected with VRX-007 or Ad5 but not with UV-inactivated VRX-007 or vehicle (Fig. 3A). These necrotic areas had a central area of primarily acellular debris (Fig. 3A, rightmost image in tumor injected with VRX-007), immediately surrounded by degenerating tumor cells and neutrophils, and more peripherally surrounded by macrophages and fibroblasts.

We also evaluated the immune response following i.t. Ad injection. Anti-Ad antibodies were present in sera from animals injected with VRX-007 and Ad5 (Fig. 3B). Based on the staining patterns observed (± 1-β-D-arabinofuranosylcytosine), the humoral immune response was directed against both early and late Ad proteins. We also found that hamsters generated both anti-hexon and anti-fiber antibodies (Fig. 3C). When these sera were tested for neutralizing antibodies, the Ad neutralization titers ranged from 1/64 to 1/256.

Ad replication in Syrian hamster lungs. Because replication in the natural site of infection is a key feature of this model, we confirmed the historical observation of Ad5 replication in Syrian hamster lungs (2). Following endotracheal instillation of Ad5, we observed more than four orders of magnitude of virus growth in the lungs between days 1 and 3, and by day 9, virus clearance had begun (Fig. 3D).

Oncolytic Ad replication and biodistribution following i.t. injection. To assess i.t. vector replication, a single i.t. injection of VRX-007 (or replication-defective control, Ad-GFP) was administered, and virus yields from tumors, blood, liver, and lungs were determined. In tumors, Ad-GFP was steadily cleared, whereas VRX-007 remained elevated for 1 week at nearly 10^8 infectious units per tumor (Fig. 4A). At 14 days, there was approximately four orders of magnitude more VRX-007 per tumor than the replication-defective virus. Taken together with...
the evidence of i.t. necrosis and efficacy with replicating (but not UV-inactivated) viruses, it seems that oncolytic vector injection leads to i.t. viral replication and subsequent necrotic cell death of tumor cells.

We also assessed the biodistribution of VRX-007 and Ad-GFP after i.t. injection (Fig. 4B-D). Following i.t. injection, both vectors entered the bloodstream and reached distant sites at 1.5 hours after injection. Both viruses were essentially cleared from the bloodstream by 1 day after injection, with the exception of one animal (at 4 days) injected with VRX-007. In both the liver and lungs, the replication-defective virus was essentially cleared by 1 day, whereas VRX-007 remained elevated, suggesting replication. Thus, following i.t. injection, replication occurred in the liver and lungs as well as in the tumor.

Discussion

The field of oncolytic Ad vectors is in need of a permissive immunocompetent animal model because the use of immunodeficient mice bearing human xenograft tumors fails to fully assess the safety of replicating vectors as well as the effect of the immune system on the vector or the tumor. This need is reflected in the

Figure 1. Human adenovirus is able to infect, express early and late adenovirus proteins, and replicate in Syrian hamster cell lines at levels comparable with infections of human A549 cells. A, Syrian hamster cell line HaK and the human cell line A549 were infected with VRX-007 at multiplicities of infection (MOI) of 5, 20, and 100 pfu/cell. At 24 hours after infection monolayers were fixed and immunostained for the early Ad protein, E1A; nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) to determine the proportion of cells that were infected. Similar results were obtained with wild-type Ads (data not shown). B, Syrian hamster cell lines (PC1, HaK, and DDT1 MF-2) were infected with VRX-007 at 50 pfu/cell. At 24 hours after infection, cells were fixed and immunostained for the early Ad proteins E1A and DNA-binding protein (DBP), and the nuclei were stained with 4',6-diamidino-2-phenylindole. At 48 hours after infection, cells were fixed and immunostained for DNA-binding protein and fiber (a late Ad protein that is a component of the capsid), and the nuclei were stained with 4',6-diamidino-2-phenylindole. Ad5 infections were conducted with similar results (data not shown). C, Syrian hamster cell lines (PC1, HaK, and DDT1 MF-2) as well as human A549 cells were infected with 100 pfu/cell of VRX-007 or mock infected. Cells were harvested at 1, 2, and 3 days after infection, and a Western blot was done on the lysates using a rabbit polyclonal serum against adenovirus late proteins. D, top, Syrian hamster HaK cell line and the human A549 cell line were infected with VRX-007 at a multiplicity of 100 pfu/cell. Dishes were harvested at 0, 1, 2, 4, and 6 days after infection, and virus yield was determined by plaque assay of the lysates on A549 cells. The burst size at 4 days after infection was 1,065 pfu/cell in HaK cells and 7,553 pfu/cell in A549 cells. Bottom, three Syrian hamster cell lines were infected with VRX-007 at a multiplicity of 100 pfu/cell, and dishes were harvested at 0, 2, 4, and 6 days after infection. The virus recovered was titered by plaque assay of the lysates on A549 cells.
Figure 2. Growth of Syrian hamster cell lines HaK, DDT1 MF-2, and PC1 as s.c. tumors is suppressed by i.t. injection of the oncolytic Ad vector VRX-007. A, established HaK tumors (mean initial volume = 270 μL; n = 12 per group) were injected with 1 × 10^10 pfu of VRX-007, wild-type Ad5, UV-inactivated VRX-007, or vehicle for six consecutive days. Left, mean fold increase in tumor volume of each treatment group is shown through day 43, when the animals in the vehicle and UV-inactivated VRX-007 treatment groups were sacrificed due to tumor burden. At 43 days, many vehicle-injected tumors had a discharge that caused an apparent decrease in tumor volume. Right, a survival curve (3 mL cutoff volume) is shown for the duration of the study. The vehicle and UV-inactivated VRX-007 groups were not significantly different from each other based on log-rank analysis of the survival curves (P > 0.05). The groups treated with VRX-007 and Ad5 were each significantly different from both vehicle and UV-inactivated groups (P < 0.01), while not being significantly different from each other (P > 0.05). B, established HaK tumors (mean initial volume = 490 μL; n = 18 per group) were injected with 1 × 10^10 pfu of VRX-007 or vehicle for six consecutive days. A second round of treatment (three injections of 1 × 10^10 pfu/dose) was initiated 16 days after the start of the first round of treatment. Top left, points, mean fold increase in tumor volume; bars, SE. The suppression of tumor growth by VRX-007 was statistically significant beginning on day 8, as determined by a Student’s t test (P < 0.02). Bottom, the degree of metastasis to the lungs was examined for both groups. At necropsy of all animals remaining at day 36, the lungs (except one lobe that was fixed for histopathologic analysis) were inflated with an India ink solution to visualize metastases (20); normal lung tissue stained black and the metastases remained white. Top right, the degree of pulmonary and renal metastasis for animals injected with vehicle or VRX-007. Boxplots were generated using SPSS statistical software. Solid black line, median; box, interquartile range (containing 50% of the values); whiskers, range of values, excluding outliers. Outliers are defined as cases with values that lie between 1.5 and 3 box lengths from the edge of the box (*). Extreme outliers are defined as cases with values that lie more than three box lengths from the edge of the box (**). C, established DDT1 MF-2 tumors (mean initial volume = 680 μL; n = 9 per group) were injected with either VRX-007 (3 × 10^9 pfu/injection; n = 10 tumors) or vehicle (n = 6 tumors) for six consecutive days. Points, mean fold increase in tumor volume; bars, SE. The growth of tumors injected with VRX-007 or vehicle was significantly different, as determined by a Student’s t test (P = 0.001). D, established PC1 tumors (mean initial volume = 950 μL; n = 9 per group) were injected with VRX-007 (1 × 10^10 pfu/injection) or vehicle for six consecutive days. Points, median fold increase in tumor volume; bars, SE. The suppression of tumor growth by VRX-007 was statistically significant at 13 days after injection (P < 0.05).
recent interest in developing a permissive immunocompetent animal model (12, 14–17).

We have shown that Syrian hamster cell lines supported Ad replication and the production of infectious progeny, with yields within 7-fold of human cells. Other Syrian hamster cancer cell lines exist, and it seems likely that Ad will replicate in many of these as well. Although some replication has been reported in vitro in murine, cotton rat, and canine cell lines, the yields are at least two orders of magnitude less than permissive human cell lines (12, 14–16, 18). In fact, the burst with wild-type Ad in these murine cells was <1 pfu/cell (4).

The oncolytic Ad, VRX-007, exhibited considerable antitumor efficacy in three hamster tumor models. Furthermore, VRX-007

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**Figure 3.** I.t. injection of VRX-007 resulted in i.t. necrosis and induction of an anti-Ad immune response. A, histopathologic analysis was done at 36 days after injection for HaK tumors from the experiment shown in Fig. 2B. Left, a representative vehicle-injected tumor; right, a tumor injected with VRX-007. Original magnifications, ×40 (left) and ×400 (right). Open arrowheads, outer skin surface; closed arrowheads, outer edge of the s.c. tumor; arrows, area of necrosis and inflammation. The necrotic response shown in the tumor injected with VRX-007 was seen in 6 of 18 tumors. These necrotic areas were within areas of viable tumor, not in the central necrotic area that most tumors contained. B and C, established DDT1 MF-2 tumors were injected with VRX-007, Ad5, or vehicle for two consecutive days (5 × 10⁹ pfu/dose), and serum was obtained before vector injection (preimmune) and 13 to 14 days after injection. B, Ad5-infected cells were infected with Ad5 [multiplicity of infection = 50, ±1-β-D-arabinofuranosylcytosine (ara-C) as indicated], fixed and permeabilized at 1 day after infection, and incubated with sera obtained from hamsters in this experiment. Anti-hamster IgG-Alexa Fluor 488 was used to visualize the results by immunofluorescence microscopy. The addition of 1-β-D-arabinofuranosylcytosine inhibits Ad DNA replication; therefore, only early Ad genes are expressed. C, left, lysates of Ad5-infected (I) or uninfected (U) A549 cells were electrophoresed and transferred to a membrane. The membrane was subsequently cut into strips, and a Western blot was done with sera obtained from animals injected with VRX-007, Ad5, or vehicle as indicated. Right, lysates of Ad5-infected (I) or uninfected (U) KB cells were immunoprecipitated with either fiber or hexon monoclonal antibody, and then a Western blot was done with serum from an animal that was injected with VRX-007. D, wild-type Ad5 (1 × 10⁷ pfu in 100 μL total volume) was administered to Syrian hamsters by endotracheal instillation. Hamsters were sacrificed, and lungs were harvested on 1, 3, and 9 days after instillation (n = 2 for 1 and 3 days after instillation, n = 1 for 9 days after instillation). Lungs were homogenized and titered by plaque assay on A549 cells, and the yields are reported as the total virus yield (pfu) per animal.
replicated in hamster tumors in vivo. Notably, efficacy and i.t. necrosis were observed with VRX-007 or Ad5 but not UV-inactivated virus, illustrating the importance of viral replication in antitumor efficacy. The level of Ad replication in murine and cotton rat tumors was several orders of magnitude lower (12, 15). Furthermore, the limited antitumor efficacy seen in the murine model seems to be immune mediated rather than due to viral replication (15, 17).

Our finding that Ad replicated in two sites of anticipated vector replication, liver and lungs, in the Syrian hamster after i.t. injection has not been observed in other animal models (4, 12), making the Syrian hamster model most able to evaluate vector safety. Although limited replication was reported in the liver of CBA mice after i.v. administration of lethal doses of Ad (3), other mouse strains (C57BL/6) do not support Ad replication in the liver (4), and efforts to show replication in the mouse lung have been entirely unsuccessful (1, 2). To our knowledge, the Syrian hamster (2) and cotton rat (19) are the only two species that support Ad replication in the lungs. It is crucial that an
animal model for oncolytic Ad vectors is permissive for replication in normal tissues to examine possible toxicities caused by vector replication.

The Syrian hamster model is advantageous over other models examined because it is the most permissive for human Ad replication both in vitro and in vivo. This is reflected in the antitumor efficacy and i.t. replication observed in the hamster model. Additionally, because normal tissues are also permissive, this model can assess vector safety. In addition, the availability of numerous hamster cancer cell lines and the ease of animal handling are advantages over the cotton rat model.

In conclusion, we have shown that human Ad replicates in Syrian hamster cancer cell lines and also confirmed replication in the lungs. Furthermore, i.t. oncolytic Ad injection resulted in suppression of primary and metastatic lesions, replication and necrosis in the tumor, vector entrance into the bloodstream, replication in the liver and lungs, and induction of an anti-Ad immune response. Many of these events are likely to occur in humans who have been administered oncolytic Ad vectors, such as VRX-007. This model may lead to a better understanding of the interactions between Ad vectors and the host, which may facilitate vector development and improve the efficacy of oncolytic Ad therapy. The Syrian hamster is a promising model for the evaluation of oncolytic Ad vectors and could become a great benefit to the field of Ad cancer therapy.

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References

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