Treatment of Ovarian Cancer with a Tropism Modified Oncolytic Adenovirus

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Abstract

Ad5-Δ24RGD is an adenovirus that is selectively replication competent in cells defective in the Rb/p16 pathway, such as ovarian cancer cells. The fiber of Ad5-Δ24RGD contains an integrin binding RGD-4C motif, allowing Coxsackie adenovirus receptor-independent infection of cancer cells. Oncolysis of cell lines was similar to that of a wild-type control, and replication in primary tumor material was shown using a novel three-dimensional spheroid model. Finally, an orthotopic murine model of peritoneally disseminated ovarian cancer was used to test i.p. administration to tumor-bearing animals. Injection of the agent resulted in eradication of i.p. disease, whereas control animals expired (P < 0.0001). These results suggest that Ad5-Δ24RGD could be useful for treatment of ovarian cancer in humans.

Introduction

Ovarian adenocarcinoma is a leading cause of gynecological cancer mortality in Western countries. Due to the lack of an effective screening strategy and inconspicuous early symptoms, most patients are detected at an advanced stage, for which 5-year survival rates continue to be <30%. Despite aggressive surgical debulking and advances in chemotherapy, survival figures have improved minimally. CRADs take advantage of tumor specific changes that allow preferential replication in tumor cells. Replication causes oncolytic death of the cell and subsequent release of virions and subsequent infection of surrounding cells, resulting in efficient tumor penetration and amplification of effect. Importantly, the antitumor effect of CRADs is determined by the capability of the agent to infect tumor cells (1–3). Unfortunately, recent evidence suggests that the expression level of the CAR is highly variable and often low on primary ovarian cancer cells (4). This is concerning, because CAR expression appears to be the major factor determining infectivity with the most commonly used Ad serotype 5. In addition to ovarian cancer, frequent CAR deficiency has been shown for various other tumor types and could be a ubiquitous phenomenon (5). Because most normal epithelial tissues express CAR, the use of untargeted Ads may result in transduction of mainly nonmalignant cells. Nevertheless, even first-generation CRADs have shown some clinical utility (5, 6). This suggests that if infectivity and replicativity of the agents can be improved, significant improvements in clinical efficacy could be gained. In this study, we have used a replication-competent recombinant Ad (Ad5-Δ24RGD), which has a 24-bp deletion in the CR2 of the E1A gene (7). This domain of the E1A protein is responsible for binding the retinoblastoma tumor suppressor/cell cycle regulator protein, Rb, which allows Ad to induce S-phase entry. Therefore, viruses with this type of deletion are reduced in their ability to overcome the G1-S checkpoint and replicate efficiently only in cells where this interaction is not necessary, e.g., in tumor cells defective in the Rb/p16 pathway (8, 9). This pathway is inactive in almost all human tumors (10), including ovarian cancers (11, 12). To circumvent the CAR deficiency of primary human ovarian cancers, the fiber of Ad5-Δ24RGD was modified by incorporating an integrin binding RGD-4C motif into the HI loop. This infectivity enhancement has been shown to dramatically increase transduction of ovarian cancer cells (13). Because most ovarian cancer patients present with ascites, it is important to note that the RGD-4C modification allows partial escape from neutralizing antibodies regularly present in the ascites (13).

Materials and Methods

Cell Culture. Hey, OV-4, and SKOV3.ip1 ovarian adenocarcinoma cell lines are kind gifts from Dr. Timothy J. Eberlein (Harvard Medical School, Boston, MA) and Drs. Judy Wolf and Janet Price (both from The University of Texas M. D. Anderson Cancer Center, Houston, TX). PA-1 and A549 were obtained from American Type Culture Collection (Manassas, VA), and 293 was obtained from Microbix (Toronto, Canada). Cell lines were maintained in recommended conditions.

Viruses. Ad5-Δ24RGD (Fig. 1) was created using a shuttle vector containing a 24-bp deletion in the CR2 region of E1A (7). The shuttle was co-transformed into Escherichia coli cells for homologous recombination with pVK503, an E3-containing rescue plasmid with the RGD-4C modification of the fiber. The virus was propagated on A549 cells. Ad5wtRGD was created by digesting pVK503 with PciI, followed by transfection into 293 cells, resulting in an otherwise wt Ad with the RGD-4C modification in the fiber. Ad5wtRGD and Ad5shcRGD (nonreplicating, luc-expressing, RGD-4C-modified virus) were propagated on 293 cells. All viruses were purified with double CsCl gradients using standard methods and titered with standard spectrophotometry and plaque assay. The presence of the E3 region and the RGD-4C modification was confirmed with PCR (primers: E3L2, 5'-CCTGAAACACCTGTGCTCACT-3'; E3R2, 5'-GCCACAGTTAGGCTCTGTA-3'; FiberUp, 5'-CAACCGTTGGTGGATTATG-3'; and FiberDown, 5'-GTGTAAGGAGATGTTGCAATA-3'). The presence of the 24-bp deletion in E1A and the absence of wt E1A were confirmed with PCR (primers: D24L1, 5'-GTCGCTGTGTTATGCTGC-3'; and D24R1, 5'-GCACCTGTTCTACCTCTGTC-3') followed by sequencing.

Cell Killing Assays. Cells were plated at 500,000 cells/well on 6-well plates and infected with 0, 0.1, 1, or 10 VP/cell for 1 h, followed by washing. Growth medium with 5% FBS was changed every other day. On day 10 (PA-1), day 14 (Hey and SKOV3.ip1), or day 17 (OV-4), respectively, crystal violet staining was performed as described previously (3). For the protein...
concentration-based oncolysis assay, cells were plated at 15,000 cells/well on 96-well plates and infected as described above. On day 8 (PA-1), day 15 (Hey and SKOV3.ip1), or day 16 (OV-4), plates were washed twice, and cells were lysed (Reporter Lysis Buffer; Promega, Madison, WI) and freeze-thawed once. Protein concentration was measured with the DC Protein Assay system (Bio-Rad Laboratories, Hercules, CA). The ANOVA F-test (SAS v.8.2; SAS Institute, Cary, NC) was performed to see if there were differences between the oncolytic potency of the viruses within each dose and cell line. If there was unequal distribution of the results, a two-sided t test was used to assess statistical significance in comparison with the nonreplicating virus.

Quantitative PCR. Ovarian adenocarcinoma primary cells were purified from malignant ascites samples as described previously (14). Analysis and creation of spheroids from primary ovarian carcinoma cells are detailed elsewhere (15). Briefly, purified unpassaged cells were incubated overnight in a 3% agar-coated flask on a rocker to form spheroids, i.e. three-dimensional clusters of cells. The spheroids were resuspended and infected with 1000 VP/cell Ad5-Δ24RGD. The next day, the spheroids were divided into five equal aliquots of 1×10^5 cells, which were collected daily. DNA was extracted with QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA), and quantitative PCR for the E1 gene was performed with Lightcycler methodology as described previously (13). To display the negative control, readings below the detection limit of the assay were set as 1, and other results are displayed relative to this control.

Therapeutic Ovarian Carcinoma Model. CB17 SCID mice (n = 11 mice/group) were infected with 1×10^5 SKOV3.ip1 cells i.p. on day 0. On days 4, 5, and 6, mice were injected i.p. with 1×10^10 or 5×10^9 VP of Ad5-Δ24RGD, Ad5lucRGD (nonreplicative control), or no virus. The virus was diluted with Opti-MEM into 1 ml in each case. Mice were inspected daily, and euthanasia was performed in case of discomfort or distress. Survival data were plotted into a Kaplan-Meier curve, and, using the LIFETEST procedure in SAS v.8.2, the Ad5-Δ24RGD group was compared with the other groups with the log-rank test. The distribution of the data best fit the Weibull model, which was used for individual comparisons between the Ad5-Δ24RGD groups and controls, using the χ² test of SAS v.8.2 LIFEREG procedure.

Results

Ad5-Δ24RGD Replicates in and Kills Ovarian Carcinoma Cells. In all cell lines, the crystal violet-based cell killing assay showed replication of Ad5-Δ24RGD and Ad5wtRGD, followed by oncolytic death of cells (Fig. 2A). In the adenocarcinoma lines (Hey, SKOV3.ip1, and Ov-4), the CRAD replicated to a similar degree as the virus containing the wt early genes (Ad5wtRGD). For PA-1, a teratocarcinoma line, the oncolytic effect of Ad5wtRGD was slightly stronger than that of Ad5-Δ24RGD. Ad5lucRGD did not cause oncolysis. The crystal violet findings were confirmed with a quantitative assay based on protein concentration, which reflects the amount of cells left after replication and oncolysis (Fig. 2B). When Ad5-Δ24RGD (10 VP/cell) was used for infection of Hey, SKOV3.ip1, PA-1, and OV-4 cells, 11.2% (P < 0.0001), 46.2% (P < 0.0001), 73.0% (P = 0.0908), and 46.7% (P = 0.0028) of cells remained alive (as compared with uninfected wells). For Ad5wtRGD, the positive control, the results were similar [13.7% (P < 0.0001), 22.7% (P < 0.0001), 28.0% (P = 0.0325), and 51.9% (P = 0.0295)]. Therefore, Ad5-Δ24RGD has similar oncolytic potential to an E1 wt virus.

Replication of Ad5-Δ24RGD in Ovarian Cancer Primary Cell Spheroids. Ovarian cancer primary cell spheroids provide a useful three-dimensional model for assessing replicativity of CRADs. More importantly, they provide a convenient means of maintaining primary cells alive in culture, without the confounding effect caused by clonal selection pressure involved in passaging and adaptation to cell culture. Spheroids were collected 1 through 5 days after infection, and quantitative PCR was performed to detect viral copies (Fig. 3). One day after infection, 1.13 copies/well were detected, and the number grew exponentially to 1,036, 19,336, 402,000, and 4,296,000 copies on days 2, 3, 4, and 5. Thus, Ad5-Δ24RGD infects and replicates in primary unpassaged ovarian cancer cells.

Therapeutic Effect of Ad5-Δ24RGD in an i.p. Model of Ovarian Cancer. We used a well-established murine model of i.p. carcinogenesis and treated the mice with three i.p. doses of 1×10^9 VP of Ad5-D24RGD, the nonreplicative Ad5lucRGD, or no virus (Fig. 4a). Median survival was 64, 45, and 36 days, respectively, and the mean survival times for Ad5lucRGD and no virus were 45.7 and 37.6 days, respectively. Statistical analysis with the log-rank and χ² tests indicated that survival was significantly better in animals treated with Ad5-Δ24RGD (P < 0.0001). A smaller dose of the viruses (5×10^8 VP/day) was also investigated (Fig. 4b). The median survival was not reached for Ad5-Δ24RGD. For Ad5lucRGD and no virus, the median survival was 40 and 36 days, and the means were 41.9 and 37.6 days, respectively. All mice in the control groups expired before day 60. All mice treated with 5×10^8 VP of Ad5-Δ24RGD survived until at least day 61. The log-rank and χ² tests confirmed that survival was significantly better in animals treated with Ad5-Δ24RGD (P < 0.0001).

Interestingly, none of the mice treated with Ad5-Δ24RGD showed evidence of i.p. disease after treatment. Instead, many developed s.c. tumors at the site where tumor cell injection had been performed, which eventually necessitated sacrifice of the animals. All animals in the control groups expired or were sacrificed due to i.p. tumor growth.

Discussion

Treatment of malignancies resistant to traditional modalities requires novel approaches. Gene therapy applications in which viral or nonviral vehicles are used for gene transfer have shown tremendous promise in preclinical studies, but inefficient tumor transduction has often precluded significant clinical benefit. To address this crucial aspect, CRADs are emerging as a powerful way to improve tumor penetration and amplify the delivered dose. However, another obstacle has become evident. Unlike normal epithelial tissues and many cell lines, primary tumors seem to express variable and often very low amounts of the requisite receptor, CAR. Because CAR levels appear to be the major factor determining binding and subsequent infectivity, low CAR may translate into low tumor transduction and low CRAD efficacy. The relationship between infectivity and oncolytic potency has been confirmed in experimental models (1–3). Therefore, it seems likely that to achieve a significant antitumor effect, it is necessary to direct gene therapy agents to targets more prevalent in tumor tissue. In this study, we have used the well-characterized RGD-4C motif, which binds the αvβ3 group of integrins and allows dramatically increased transduction of primary ovarian cancer cells (13).
on i.p. inoculation of the agent. This is due to the fact that the disease usually presents at an advanced stage, typically with peritoneal metastasis, which has the worst prognosis. The i.p. administration and readministration can be conveniently achieved with a catheter, and the peritoneal cavity allows a degree of compartmentalization, which may have contributed to the excellent safety profile observed in Phase I studies (5). The peritoneal cavity could also allow effective dissemination of a CRAD, resulting in good transduction of i.p. tumor masses. However, these patients usually have malignant ascites, which contain anti-Ad neutralizing antibodies. It is important to note that the RGD-4C modification of the fiber allows Ad to partially escape preexisting anti-Ad antibodies, which could improve transduction of tumor tissue (13).

The replicative selectivity of Ad5-Δ24RGD (Fig. 1) is based on incapability of binding Rb, and therefore replication is expected to occur preferentially in cells where S-phase induction is not required, such as cells defective in the Rb/p16 pathway. This pathway may be faulty in all human cancers (10), including ovarian adenocarcinomas (11, 12). Previously, it has been shown that replication of CR2-deleted viruses is attenuated in nonproliferating normal cells (8, 9). Interestingly, abrogation of replication was also demonstrated when Rb was reintroduced into otherwise permissive cells (8).

In this study, we show replication of Ad5-Δ24RGD in ovarian cancer cell lines (Fig. 2). The assays used here measure both replication and subsequent oncolysis because the readout corresponds with the remaining amount of live cells. The variable numbers of live cells at termination of the assays reflect the opposite effects of viral oncolysis on one hand and cell replication on the other. In vivo, cell turnover is likely to be slower, and viral dispersion dynamics are likely to be different due to the three-dimensional nature of solid tumors. Therefore, it is difficult to draw definite conclusions from the differences seen in the antitumor effect between the cell lines. Instead, the main finding is that replication and oncolysis were seen in each cell line. Impressively, the potency of Ad5-Δ24RGD was very similar to that of Ad5wtRGD, which is wt except for the fiber modification. This virus was chosen as the positive control to avoid bias due to differences in infectivity (and subsequent oncolytic potency) caused by nonidentical fibers. These findings are in accord with those of other investigators who have studied the effect of the CR2 deletion on replicativity and oncolytic potency (9). In cancer cell lines, a CR2-deleted virus demonstrated similar superior replicativity when compared with wt Ad (9). Also, the CR2-deleted virus was compared with the E1B55K-deleted dl1520 virus, which has been used extensively for treatment of patients. Although there are some promising results in

![Fig. 2. A, replication of Ad5-Δ24RGD kills ovarian carcinoma cells. Cells were infected for 1 h with 0, 0.1, 1, or 10 VP/cell Ad5lacRGD (nonreplicative negative control), Ad5-Δ24RGD, or Ad5wtRGD (positive control for replication). On day 10 (PA-1), day 14 (Hey and SKOV3.ip1), or day 17 (OV-4), respectively, plates were washed and stained with crystal violet. Each experiment was performed in triplicate wells. B, Ad5-Δ24RGD causes oncolysis of ovarian carcinoma cells. Cells were infected for 1 h with 0, 0.1, 1, or 10 VP/cell Ad5lacRGD, Ad5-Δ24RGD, or Ad5wtRGD. On day 8 (PA-1), day 15 (Hey and SKOV3.ip1), or day 16 (OV-4), protein concentration was determined. The protein concentration of cells infected with 0 VP/cell was set at 100%. Error bars, ±1 SD resulting from quadruplicate experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.]

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combination with chemotherapy (6), the evidence showing replication of dl1520 in tumor tissue has been anecdotal, and the single agent clinical efficacy has been marginal, possibly due to the low replicativity of the agent. In contrast, the CR2 deletion does not seem to affect replicativity to a significant degree, and it will be interesting to see if this leads to higher therapeutic efficacy.

Previous studies with Ads have suggested that there is a disconnect between transduction efficacy in preclinical versus human studies. An important reason for this may be the highly variable expression of CAR in primary tissue. Therefore, unpassaged and purified primary tumor material has been used as the most stringent template for testing novel approaches. A problem with primary cells is that they tend not to stay alive very long in vitro, and thus analysis of replication and oncolysis is difficult. We have developed an ovarian primary cell spheroid model that allows purified cancer cells to stay alive for at least a month (15). Also, the three-dimensional structure of spheroids may resemble in vivo tumors better than two-dimensional cell line cultures. Here, we tested the replication of Ad5-Δ24RGD on spheroids and observed an exponential increase in VP as a function of time (Fig. 3). This suggests that Ad5-Δ24RGD can infect and replicate in primary ovarian cancer cells.

The ultimate preclinical test of an experimental therapeutic is an orthotopic animal model. Here, we used a murine model of peritoneally metastatic ovarian cancer, and we performed i.p. injections of the virus, as would be offered in a human trial (Fig. 4). Mice treated with Ad5-Δ24RGD displayed healing of i.p. disease. However, some mice relapsed with s.c. tumors. This could have been caused by a small number of tumor cells contaminating the needle tract during the injection of the cells. The virus probably had little access to the s.c. tissue and therefore could not eradicate these cells. It should be noted that no cures or long-term survival has previously been reported for this aggressive model of ovarian cancer.

With the lowest viral dose used, we saw 100% survival for up to 61 days, when all animals in the control groups had expired (Fig. 4). The only other CRAD that has been used for treatment of an ovarian cancer animal model is dl1520 (16). Two days after i.p. inoculation of A2780 tumors, 1 x 10^6 plaque-forming units (2 x 10^10 VP) were injected daily for 5 days, resulting in 40% of mice showing no evidence of i.p. disease. Five doses of 4 x 10^8 VP of dl1520 i.p. into mice bearing OvCAR3 tumor resulted in resolving of i.p. tumors in four of four cases, but the follow-up period was only 42 days. Although direct comparison is difficult due to different experimental design, the in vivo efficacy of Ad5-Δ24RGD could compare favorably with that of dl1520.

The dose used here was 5 x 10^8 VP daily for 3 days, which would equal approximately 1 x 10^12 VP for a 60-kg human (w/w). dl1520 has been administered to humans i.p. with doses ranging from a daily dose of 1 x 10^11 to 1 x 10^13 VP for 5 consecutive days (17). Although the final data are currently unavailable, the preliminary results suggest that patients with bulky tumors (>2 cm) experienced dose-limiting side effects at 1 x 10^12 VP x 5, whereas patients with nonbulky tumors tolerated 1 x 10^13 VP x 5 without toxicity. Although comparisons between mouse and human data should be avoided because human Ads do not replicate in murine tissues to any significant degree, these figures suggest that the oncolytic potency of Ad5-Δ24RGD is sufficient to merit a human trial.

Recently, it has been demonstrated that gene therapy is a feasible way of achieving clinical benefits in patients (18–20). Interestingly, these findings have come from the seemingly unrelated fields of genetic disease on one hand and acquired vascular diseases on the other. What these success stories have in common is the rational approach investigators have had in developing the gene delivery tools. Thus, the clinical breakthroughs were based on advances in vector development. The approach described in this study combines two promising fields, replication-competent viruses and infectivity enhancement. Together, these powerful means to increase tumor transduction could help achieve similar breakthroughs in the field of cancer gene therapy.

In conclusion, we have used a novel replication-competent agent, Ad5-Δ24RGD, for treatment of ovarian cancer models. The infectivity of the virus has been enhanced for CAR-independent infection of tumor cells. We observed replication and oncolytic potency similar to those of a wt control virus (both viruses were fiber modified). Using a highly novel three-dimensional spheroid model, we detected an exponential increase in the amount of Ad5-Δ24RGD gene copies, suggesting efficient replication. Finally, we used a stringent orthotopic murine model of peritoneally metastatic ovarian cancer and saw significant improvement in survival of the animals. In fact, all animals displayed complete eradication of i.p. disease. These results suggest that Ad5-Δ24RGD could be an effective agent for treatment of ovarian cancer and that the toxicity should be evaluated in a Phase I trial.
Also, considering that Rb/p16 pathway abnormalities in tumors seem to be ubiquitous (10), the agent could prove useful for other types of tumors as well.

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References

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