EVALUATION OF A SELECTIVELY ONCOLYTIC ADENOVIRUS FOR LOCAL AND SYSTEMIC TREATMENT OF CERVICAL CANCER

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Treatment options for disseminated cervical cancer remain inadequate. Here, we investigated a strategy featuring Ad5-Δ24RGD, an oncolytic adenovirus replication-competent selectively in cells defective in the Rb-p16 pathway, such as most cervical cancer cells. The viral fiber contains an α5β2 and α5β3 integrin-binding RGD-4C motif, allowing coxsackie-adenovirus receptor-independent infection. These integrins have been reported to be frequently upregulated in cervical cancer. Oncolysis of cervical cancer cells was similar to a wild-type control in vitro. In an animal model of cervical cancer, the therapeutic efficacy of Ad5-Δ24RGD could be demonstrated for both intratumoral and intravenous application routes. Biodistribution was determined following intravenous administration to mice. Further preclinical safety data were obtained by demonstrating lack of replication of the agent in human peripheral blood mononuclear cells. These results suggest that Ad5-Δ24RGD could be useful for local or systemic treatment of cervical cancer in patients with disease resistant to currently available modalities.

Key words: adenovirus; cervical cancer; oncolytic; biologic therapy; gene therapy; conditionally replicating adenovirus; virus replication; peripheral blood mononuclear cells

Cervical cancer, caused by human papillomavirus infection and considered a sexually transmitted disease, affects more young women than any other type of gynecologic cancer. The American Cancer Society estimates about 13,000 first diagnoses and about 4,100 deaths of invasive cervical cancer for 2002 in the United States. Early stages are well treatable with a combination approach of surgery and radiotherapy, but since most types of cervical cancer are highly resistant to current chemotherapy regimes, treatment of more advanced stages is often unsatisfactory. Thus, women diagnosed with advanced-stage cervical cancer still have a poor prognosis.1

Conditionally replicating adenoviruses (CRADs)2 take advantage of tumor-specific changes, allowing preferential replication in tumor cells. Replication causes oncolytic death of the cell, release of virions and subsequent infection of surrounding cells, resulting in efficient tumor penetration and amplification of effect. Therefore, the antitumor effect is caused by replication of the agent per se. Consequently, limiting replication to tumor cells is important to minimize side effects. The antitumor effect of CRADs is determined by their capability to infect tumor cells. Unfortunately, recent evidence suggests that the expression level of coxsackie-adenovirus receptor (CAR) is highly variable and often low in many human tumors.3 As most epithelial-derived normal tissues express CAR, use of untargeted adenovirus (Ad) may result in transduction of mainly nontarget cells. Nevertheless, even first-generation CRADs have shown some clinical utility.4 This suggests that if the capacity of the agents to transduce cancer cells could be improved, significant progress in clinical efficacy might be gained.

In this study, we have used a CRAD (Ad5-Δ24RGD) that has a 24 bp deletion in the constant region 2 (CR2) of the EIA gene.5 This domain of the EIA protein is responsible for binding the retinoblastoma (Rb) tumor suppressor/cell cycle regulator protein, 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., tumor cells defective in the Rb-p16 pathway, or in rapidly cycling normal cells featuring phosphorylation of Rb.6–8 This pathway may be inactive in all human tumors.9

To circumvent the CAR-deficiency frequently seen on human tumor cells, the fiber of Ad5-Δ24RGD was modified by incorporating an α5β2 and α5β3 integrin-binding arginine-glycine-aspartic acid (RGD-4C) motif into the HI loop of the knob domain.5 This expands the tropism of the virus from CAR to these integrins.10 RGD was initially identified due to its homing to tumor vasculature.11 Subsequently, it has been shown that RGD, and RGD-modified Ads, also bind effectively to α5β3 and α5β4 integrins expressed on the tumor cells themselves.12 Further, this tropism expands.

Abbreviations: Ad, adenovirus; CAR, coxsackie-adenovirus receptor; CR2, constant region 2; CRAD, conditionally replicating adenovirus; IL-2, interleukin 2; PBMC, peripheral blood mononuclear cell; PHA-P, phytohemagglutinin-P; Rb, retinoblastoma; RGD-4C, arginine-glycine-aspartic acid; VP, viral particle.

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modification can mediate dramatic improvements in gene transfer to cancer cells in vitro and in vivo, with subsequent therapeutic gain.\textsuperscript{13–18} Some integrins such as α\textsubscript{v} and α\textsubscript{v}β\textsubscript{3} have been reported downregulated in cervical cancer.\textsuperscript{19} In contrast, the expression of α\textsubscript{v} and α\textsubscript{v}β\textsubscript{3} integrins is retained in cervical carcinogenesis, with frequent overexpression seen in advanced tumors.\textsuperscript{20–22} This could be related to the invasive potential of the tumors, perhaps by association to matrix metalloproteinase 2.\textsuperscript{2,23,24} For potential systemic application, it is important to note that RGD-modified Ads partially avoid preexisting neutralizing antibodies.\textsuperscript{14,25}

Here, we studied the efficacy of Ad5-Δ24RGD on cervical cancer substrates, including a panel of cell lines and animal models. Further, we attempted to obtain preclinical safety data by performing murine biodistribution studies and toxicity evaluations on human peripheral blood mononuclear cells (PBMCs).

\section*{MATERIAL AND METHODS}

\subsection*{Cell culture}
Caski, Hela, SiHa and C33A cervical cancer cell lines and the human lung adenocarcinoma cell line A549 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The 293 human-transformed embryonal kidney cell line was purchased from Microbi (Toronto, Canada). All cell lines were maintained in a humidified 37°C atmosphere containing 5% CO\textsubscript{2}. MTS assay was performed on days 4 and 6. Results are measured as follow-up. The viruses were diluted with OptiMEM in each case. Mice were inspected daily and euthanasia of the complete group was performed in case of high tumor load, evident pain or distress. Tumor size is shown as percentage of size on day of first viral injection. Bars indicate the standard error. Studies were approved by the University of Alabama at Birmingham Institutional Review Board.

\subsection*{Biodistribution for Ad5-Δ24RGD}
C33A tumors were inoculated in nude mice as above (n = 5/group). At day 21, 5 × 10\textsuperscript{10} VPs Ad5-Δ24RGD diluted in 200 μl OptiMEM was injected i.v. into each mouse. On days 1 and 7 after injection, liver, spleen, kidneys, heart, lungs and tumors were collected and snap-frozen with dry ice/ethanol. DNA was extracted and E4 copy number was determined as above.

\subsection*{Statistical analysis}
Analysis of the tumor size data was performed using a repeated measures growth model with PROC MIXED (SAS version 8.02). The tumor size data was log-transformed for normality. The effects of treatment group, time and the interaction of treatment group and time were evaluated by F-tests. Baseline tumor size was included as a covariate in all models. The a priori planned comparisons of adjusted differences in predicted treatment means were computed by t-statistics averaged over all time points. For all analyses, a 2-sided p value of < 0.05 was deemed statistically significant.

\subsection*{Transduction assay}
Cervical cancer cells were infected with 200 VPs/cell for 1 hr, followed by removal of the growth medium and washing to remove any unbound viruses. Twenty-four hours later, luciferase assay was performed as reported.\textsuperscript{29}

\subsection*{Crystal violet cell-killing assay}
Cells were infected with the indicated amount of virus for 1 hr and viability was assessed with the crystal violet assay when the most oncolytic virus had achieved almost complete cell killing, as reported.\textsuperscript{37} C33A cells were analyzed 2 days and others cell lines 3 days after infection.

\section*{RESULTS}

\subsection*{Ad5-Δ24RGD has oncolytic activity in cervical cancer cells in vitro}
In all 4 cell lines, the mitochondrial activity-based MTS cell-killing assay showed oncolysis of Ad5-Δ24RGD and Ad300wt
Therapeutic effect of i.t. injected Ad5-Δ24RGD in cervical cancer

Advanced C33A tumors were allowed to grow. Then, mice were randomized into 6 groups and treated with 3 i.t. doses of either 10^7, 10^8, or 10^10 VPs of Ad5-Δ24RGD, 10^9 VPs of the E1-deleted Ad5lucRGD or no virus in 200 µl OptiMEM. Treatment with the nonreplicating Ad5lucRGD or without virus did not show an effect on tumor growth (Fig. 2a). Ad5-Δ24RGD at 10^10 VPs gave a significantly improved therapeutic effect over Ad5lucRGD and mock (both p < 0.0001). The same was true when the dose was 10^9 VPs (p < 0.0001 vs. Ad5lucRGD and p = 0.0004 vs. mock) or 10^8 VPs (p = 0.0002 vs. Ad5lucRGD and p = 0.0190 vs. mock).

Therapeutic effect of i.v. delivered Ad5-Δ24RGD

To mimic treatment of metastatic and disseminated disease, advanced C33A tumors were established in both flanks of mice. Then, mice were injected i.v. either with a single dose of 10^10, 3 doses of 3 x 10^10, or a single dose of 10^11 VPs of Ad5-Δ24RGD (Fig. 2b). The controls were a single dose of 10^11 VPs of Ad5lucRGD and no virus. At the lower single dose, Ad5-Δ24RGD had an undramatic but statistically significant effect on tumor growth (p = 0.0002 vs. Ad5lucRGD and p = 0.0125 vs. mock). The triple dose gave a more pronounced therapeutic effect over no virus, Ad5lucRGD or the lower single dose (all p < 0.0001). The same was true for the higher single dose (all p < 0.0001). There was no difference in efficacy between the triple and the higher single dose (p = 0.3681). Therefore, we were able to achieve therapeutic benefit utilizing systemic treatment in a murine model of disseminated cervical cancer.

Biodistribution of Ad5-Δ24RGD

Eighteen hours after i.v. injection of Ad5-Δ24RGD, virus was detected mainly in liver, spleen (35% of the liver dose), lungs (25%) and tumor (14%), whereas heart (1.8%) and kidneys (1.4%) were infected with a lower amount (Fig. 3). The liver-to-tumor ratio of virus copies was 7:1. On day 7 after injection, a 558-fold relative amplification of the virus within the tumor was found, suggesting i.t. viral replication in vivo. Human Ads do not productively replicate in nonhuman cells and therefore it was an expected finding to see lack of replication of the agent in murine tissues.30 In the liver, the virus DNA increased 3.5-fold (not significant), which may reflect a low degree of DNA multiplication without virion production.

Ad5-Δ24RGD in PBMCs

Freshly obtained PBMCs displayed no reduction in viability 4 or 6 days after infection with up to 1,000 VPs/cell of Ad5-Δ24RGD (Fig. 4a and b). This dose corresponds with a 10^13 VPs i.v. dose for a human adult with a blood count of 4.3 x 10^11 leukocytes/liter, typical for cancer patients after chemotherapy,31 and could therefore correlate with exposure in a clinical trial context. In contrast, the cervical cancer cells that were included as controls were killed (Fig. 4c and d).

Ad5-Δ24RGD does not replicate in PBMCs but inhibits mitogenesis

To mimic in vivo activation, PBMCs were infected in vitro with Ad5-Δ24RGD or controls and simultaneously stimulated with recombinant human IL-2 or PHA-P (Fig. 5a and b). Tritium incorporation was used as a marker of proliferation. On day 4, IL-2 and PHA-P had caused significant proliferation regardless of virus infection, although Ad5-Δ24RGD-infected cells expanded slightly.
less. By day 6, these differences were increased, indicating a possible toxic effect of Ad5-Δ24RGD. To determine if this was due to virus replication, virus copy number was determined in stimulated and unstimulated cells (Fig. 5c, d and e). No replication of any of the viruses was detected.

**Effect of RGD-4C modification on infectivity and killing of cervical cancer cells**

In 2 out of 4 cervical cancer cell lines, the RGD-4C-modified virus allowed increased gene transfer (Fig. 6). However, in one line, Ad5Luc1 was better. This suggests that loss of CAR expression may not be a ubiquitous phenomenon in cervical cancer cell lines and should be studied further with clinical specimens. For comparison of the oncolytic potency of Ad5-Δ24RGD to the isogenic virus with a wild-type serotype 5 capsid (Ad5-Δ24E3), a crystal violet cell-killing assay was performed. In all cases, the oncolytic potency of Ad5-Δ24RGD was comparable or superior to Ad5-Δ24E3 and the wild-type control virus Ad300wt (Fig. 7).

**DISCUSSION**

Malignancies resistant to current treatment modalities require novel interventions. A promising experimental approach is oncolytic virotherapy with CRADs, which has shown encouraging results in recent trials, chiefly in the context of head and neck cancer.2 These developments have led to a phase 3 trial utilizing a combination approach with chemotherapy.3 However, for cervical cancer, no such progress has yet been reported. In this study, we have utilized a CRAD, which was infectivity-enhanced with an RGD-4C motif, allowing binding to integrins, which have been reported frequently highly expressed in advanced cervical cancers.20–22 Previously, this agent has been evaluated for treatment of peritoneally disseminated ovarian cancer and a phase 1 trial is in development.27 Our preliminary results suggest that the RGD-4C modification may be beneficial in many cases of cervical cancer (Fig. 6). However, this could be studied further with clinical specimens. However, it would be useful if also Ad5-Δ24E3 is proven effective for treatment of cervical cancer patients. Our preliminary data suggests that this virus may also have antitumor activity on cervical cancer substrates (Fig. 7). The availability of 2 CRADs with different capsids would be beneficial to patients, as it would facilitate retreatment without interference due to neutralizing antibodies.25,28

Cervical cancers are rarely sensitive to chemotherapy.33 In particular, advanced stages are mostly resistant to chemotherapy and...
radiation. Thus, although the majority of cases are treatable locally, options are limited when local therapy fails. The disease relapses both locally and at distant sites. Therefore, we evaluated both i.t. and systemic administration. The replication selectivity of Ad5-Δ24RGD is based on a defective Rb-p16 pathway. It has been suggested that most if not all human cancer are defective in this pathway, which regulates the G1/S cell cycle checkpoint. With regard to cervical cancer, the mechanism of dysregulation of this pathway has been investigated. Since the E7 protein of high-risk human papillomavirus types is known to inactivate Rb functionally, most cervical cancer cells should allow replication and oncolysis by Ad5-Δ24RGD. Lack of replication in normal tissues may be a major factor determining the side effect profile of CRADs in humans. It has been shown previously that replication of viruses featuring the 24 bp CR2 deletion are attenuated in nonproliferating normal cells.

In this study, we show oncolysis with Ad5-Δ24RGD in cervical cancer cell lines (Fig. 1). In 3 out of 4 cell lines, the oncolytic potency of Ad5-Δ24RGD was similar to a wild-type Ad, included as a positive control, while on C33A cells, wild type was slightly more potent. Ad5-Δ24RGD was also compared to Ad5-Δ24E3, which is otherwise identical, but has a serotype 5 capsid. Similar or improved oncolytic potency was seen on all cell lines studied (Fig. 7). This is in accord with findings of other investigators and our previous studies, demonstrating similar replicativity of CR2-deleted and wild-type adenoviruses.

The ultimate preclinical tests of experimental therapeutics are in vivo models. Here, we utilized a murine model to mimic locally recurrent advanced cervical cancer, a form of disease that would probably be accessible to local injection. Interestingly, even the smallest dose injected gave a therapeutic effect in this aggressive model. The larger doses resulted in slightly higher efficacy (Fig. 2a). Converted weight/weight into a human dose, 107 VPs would be approximately 2 × 1010, well below the 1 × 1013 VPs of another CRAD that has been administered i.t. to humans without dose-limiting side effects.

The most difficult form of cervical cancer to treat is disseminated disease. For such applications, systemic administration is necessary. Therefore, we evaluated i.v. injection of Ad5-Δ24RGD into mice bearing multiple tumors (Fig. 2b); i.v. injection of 1010 VPs Ad5-Δ24RGD resulted in a slight reduction in tumor growth. This is in accord with previous studies, where 1–2 × 1010 VPs has been suggested as the threshold for uptake by the Kupffer cells of the liver. In other words, only doses larger than this result in effective circulation of virus. Fittingly, 1011 VPs resulted in significant reduction of tumor growth, irrespective of whether a single or a split dose was used. These data suggest that Ad5-Δ24RGD can successfully infect tumor cells and replicate in vivo, even when the virus is injected i.v. It is worth noting that none of the mice died or displayed visual evidence of toxicity after i.t. or i.v. viral injection.

There is limited data available on i.v. administration of CRADs to humans, but the trials that have been completed suggest good safety. The lack of demonstration of efficacy may be related to the low replicativity of the agents used, perhaps compounded by variable expression of CAR on target cells. In contrast, Ad5-Δ24RGD features replicativity similar to wild type enhanced infectivity via metastasis-associated integrins and independence from CAR. Considering the high prevalence of circulating neutralizing antadenovirus antibodies, the capacity of RGD-4C-modified Ads to avoid preexisting neutralizing antibodies partially may be useful. It is not known if the threshold effect associated with i.v. administration to mice applies to humans, or what the threshold dose might be. Obviously, this is of utmost importance for systemic application of CRADs and needs to be studied.

For performing a clinical trial, the best available preclinical assays should be used for evaluation of not only the efficacy but also the safety of agents. Comprehensive murine toxicity studies with human serotype CRADs are not optimal, as human Ads do not productively replicate in mice. Nevertheless, biodistribution can be assessed. Eighteen hours after i.v. injection, the liver to tumor copy number ratio was 7:1. Six days later, a 558-fold i.t. copy number increase was seen (Fig. 3). The dose of virus was chosen to allow direct comparison to previous experiments that were performed with E1-deleted Ads. Our results are well in accord with the data obtained with the RGD-4C-modified vector. As expected, the liver is the preferred organ for viral infection after i.v. injection. In contrast to i.p. injection, where spleen and liver have a similar viral uptake, only 35% of the liver dose reached the spleen after i.v. injection. Whereas lung infection is insignificant after i.p. injection, 25% of the liver dose was found there after i.v. injection. More relevant toxicity studies could be performed if syngeneic model systems can be developed. However, such systems would not be applicable to human viruses. Nevertheless, the respective nonhuman viruses could be constructed.

Of special interest for possible human applications of Ad5-Δ24RGD is the effect on human leukocytes. Ad serotype 5-based agents do not effectively infect PBMCs in vivo, although transduction can be forced with high titers in vitro. However, the RGD modification could increase the infectivity of PBMCs as these cells have been reported to express the relevant integrins. Ad capsids induce clonal increases in various PBMC lineages and there is migration of PBMCs to the infection site. Thus, if PBMCs can be infected and sustain replication, there may be potential for hematologic toxicity. In the experiments performed...
here, we used 1,000 VPs/cell, an amount of virus corresponding to about 10^{11} VPs i.v., similar to a CRAD dose tested in clinical trials.2

Importantly, we did not see any decrease in the viability of PBMCs following infection with Ad5-Δ24RGD (Fig. 4). Further, no replication of the agent was seen in PBMCs regardless of stimulation (Fig. 5c and d). However, our results suggest that Ad5-Δ24RGD does interfere with IL-2- or PHA-P-mediated stimulation (Fig. 5a and b). This was especially evident on day 6. The effect may be dependent on E1 expression in the cells, as the E1-deleted Ad5lucRGD did not produce similar results. Further, as the isogenic control virus with a wild-type adenovirus 5 capsid (Ad5Δ24E3) did not cause this effect, it seems likely that RGD-4C is required for entry. Despite probably entering PBMCs, Ad5-Δ24RGD was not able to replicate in or kill the cells, perhaps due to an intact Rb-p16 pathway or other cell-type-specific reasons (Figs. 4 and 5c, d and e). Nevertheless, these experiments were designed as a preliminary investigation and more comprehensive studies are clearly required. Furthermore, while information from preclinical experiments such as here yields interesting and important data, clinical studies will ultimately determine the safety and efficacy of novel agents.

In conclusion, we were able to demonstrate the oncolytic potency of the replication-competent adenovirus, Ad5-Δ24RGD, for treatment of cervical cancer. Ad5-Δ24RGD was administered locally and systemically and therapeutic efficacy was seen with amounts of virus (converted into human doses) that are amenable to clinical testing and have proven safe in trials with other CRADs. These results suggest that Ad5-Δ24RGD may be a useful agent for testing in human trials with cervical cancer patients suffering form disease refractory to current treatment modalities.

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