

Oncolytic adenovirus Ad5/3- Δ 24 and chemotherapy for treatment of orthotopic ovarian cancer

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Abstract

Objective. Oncolytic adenoviruses capable of replication selectively in tumor cells are an appealing approach for the treatment of neoplastic diseases refractory to conventional therapies. The aim of this study was to evaluate the effect of dose and scheduling of a tropism-modified, adenovirus serotype 3 receptor-targeted, *Rb/p16* pathway-selective replication-competent adenovirus, Ad5/3- Δ 24, against human ovarian adenocarcinoma. As oncolytic viruses and chemotherapy can have synergistic interactions, the antitumor efficacy of Ad5/3- Δ 24 was also studied in combination with epirubicin and gemcitabine, common second-line treatment options for platinum-resistant ovarian cancer.

Methods. Orthotopic murine models of peritoneally disseminated ovarian cancer were utilized to compare survival of mice treated with either a single viral dose or weekly delivery. The lowest effective dose of intraperitoneal Ad5/3- Δ 24 was determined. Combinations of Ad5/3- Δ 24 and gemcitabine or epirubicin were studied *in vitro* as well as *in vivo*.

Results. Treatment outcome after administration of a single dose of Ad5/3- Δ 24 was as effective as delivery of several weekly doses. Our results also demonstrate that a single intraperitoneal injection of 100 viral particles significantly increased the survival of mice compared to untreated animals. Further, combining Ad5/3- Δ 24 with either gemcitabine or epirubicin resulted in greater therapeutic benefit than either agent alone.

Conclusion. These preclinical data suggest that Ad5/3- Δ 24 represents a promising treatment strategy for advanced ovarian cancer as a single agent or in combination with chemotherapy.

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Keywords: Oncolytic adenovirus; Virotherapy; Chemotherapy; Ovarian cancer; Orthotopic murine model

Introduction

Despite improvements in the treatment of ovarian carcinoma in the last three decades, it remains the leading cause of death from gynecological malignancies in developed countries [1]. Median survival has improved due to multi-modality

treatments often including a combination of primary cytoreductive surgery with platinum and taxane compounds. Nonetheless, early diagnosis continues to be challenging, and about 70% of patients present with disseminated disease for which the 5-year survival rate is approximately 30%. Therefore, novel treatment approaches such as targeted therapies are needed [2,3]. Ovarian cancer spreads preferentially throughout the peritoneal cavity (stage III) and, since the peritoneal cavity is easily accessible and offers a degree of compartmentalization, these patients represent an attractive target for regional therapy. Of note, there is growing evidence

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that patients with microscopic residual disease might benefit from intraperitoneal (i.p.) treatment [4].

Oncolytic adenoviruses represent an attractive modality for cancers refractory to conventional therapies [5–7]. Such viruses are modified to take advantage of tumor-specific alterations that allow viral replication preferentially in cancer cells. Replication causes oncolytic death of the cell, resulting in release of viral progeny to surrounding cells. Amplification of the virus leads to more efficient intratumoral penetration and spread within solid tumor masses when compared to replication-deficient adenovirus vectors, which are traditionally used in gene therapy approaches. One strategy to limit replication of oncolytic adenoviruses to tumor tissues is to engineer specific deletions into the viral genome. These deletions abrogate replication in normal cells but can be trans-complemented by tumor-specific factors. Ad5- Δ 24 (a.k.a. dl922–947) carries a 24-bp deletion in the constant region 2 domain of the adenoviral *E1A* gene [8,9]. Thus, the E1A protein is unable to bind the retinoblastoma (Rb) tumor suppressor protein for release of E2F and subsequent effective viral replication in non-cycling normal cells. Therefore, the virus replicates selectively in cells deficient in the *Rb/p16* pathway, including most if not all cancer cells including ovarian cancer cells [10,11]. Tumor specificity of these adenoviral mutants has been previously demonstrated [8,9].

The efficacy of oncolytic adenoviruses can be compromised if they cannot enter target cells effectively [12]. Most adenoviral gene therapy strategies are based on serotype 5 (Ad5), which binds to the coxsackie-adenovirus receptor (CAR). Unfortunately, expression of CAR is frequently down-regulated in many types of advanced cancers including ovarian cancer [6], and may be a general phenomenon related to carcinogenesis. However, lack of CAR can be circumvented by substituting the knob domain of Ad5 with the corresponding domain of serotype 3 (Ad3). Utilization of such 5/3 fiber chimeras allows virus binding and entry through the Ad3 receptor, which is expressed to high degree on ovarian cancer cells [13]. The preclinical safety and normal tissue biodistribution of this approach seems to be comparable to wild type Ad5 virus [14].

Ad5/3- Δ 24 is an Rb binding deleted oncolytic adenovirus retargeted to the Ad3 receptor. It has been previously shown that this agent delivers a powerful antitumor effect to ovarian cancer cells *in vitro*, to clinical ovarian cancer specimens, and in orthotopic models of ovarian cancer [15,16]. However, when combined with gemcitabine for intraperitoneal treatment of SKOV3.ip1 carcinomatosis, certain schedules resulted in liver toxicity, while giving virus 24 h after gemcitabine resulted in dramatically enhanced survival [17]. Therefore, we sought to investigate if these were model or chemotherapy agent-specific findings. Also, for optimizing protocols for upcoming clinical trials, it is of interest if weekly administration is superior over a single dose. Each ovarian cancer cell infected *in vitro* with Ad5/3- Δ 24 rapidly produces thousands of new virions. Therefore, we performed *in vivo* dose de-escalation to test if virus amplification would translate into efficacy with very small doses, which might be promising with regard to clinical trials with patients with advanced ovarian cancer.

Materials and methods

Cells, viruses and chemotherapeutic agents

Human ovarian adenocarcinoma cell lines SKOV3.ip1 and Hey were provided by Dr. Janet Price and Dr. Judy Wolf (both from M. D. Anderson Cancer Center, Houston, TX). Human E1-transformed embryonal kidney cell line 293 was obtained from Microbix (Toronto, Canada) while human lung adenocarcinoma kidney cell line A549 was purchased from American Type Culture Collection (Manassas, VA). All cell lines were cultured in the recommended growth media with 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere at 37 °C and 5% CO₂.

Ad5/3- Δ 24 has been previously described [15]. Ad5/3luc1 is a serotype 3 receptor-targeted adenovirus containing a firefly luciferase transgene in the place of the deleted *E1* region and was used as a replication-deficient control [13]. Viruses were amplified on A549 or 293 cells, respectively, and purified on double caesium chloride gradients. The viral particle (VP) concentration was measured at 260 nm and plaque-forming units (pfu) were determined by standard plaque assay on both A549 and 293 cells. The titers for Ad5/3- Δ 24 were 2.2×10^{11} pfu/ml and 2.8×10^{11} pfu/ml, respectively, confirming the feasibility of using either cell line for functional titering. The ratio of VP/infectious particles was 6.1 and 11.3 for Ad5/3- Δ 24 and Ad5/3luc1, respectively.

Gemcitabine (Gemzar®) was purchased from Eli Lilly and Company (Indianapolis, IN). 40 mg/ml stock solution was prepared in 0.9% sodium chloride and this was further diluted in growth media immediately before use. Epirubicin (Farmorubicin®) 2 mg/ml solution was purchased from Pharmacia (Vantaa, Finland) and was diluted in growth media immediately before use.

Mice

For survival experiments, 3- to 4-week-old female C.B-17 SCID mice were obtained from Taconic (Ejby, Denmark) and quarantined for 2 weeks. All animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. The health of the mice was monitored daily and mice were sacrificed when there was evidence of pain or distress. All agents were diluted in 500 μ l growth media for i.p. injections and mock treated control mice received only growth media. The number of control mice was always same in the treatment groups if not otherwise stated.

Comparison of single and weekly delivery of Ad5/3- Δ 24

To evaluate if treatment with a single dose of Ad5/3- Δ 24 differs from weekly delivery schedule, 3×10^6 Hey cells were injected i.p. on day 0, and mice ($n=12$) were treated with 1×10^8 VP i.p. on day 3, or same dose was given weekly on days 3, 10, 17 and 24. In another experiment, the tumors were established by injecting 1×10^7 SKOV3.ip1 cells i.p. on day 0, and treated with a single injection of 1×10^8 VP on day 10 ($n=10$), or same dose was administered weekly on days 10, 17, 24 and 31.

Determination of lowest effective dose of i.p. delivered Ad5/3- Δ 24

To determine the lowest effective dose of Ad5/3- Δ 24, mice were injected i.p. with 1×10^7 SKOV3.ip1 cells and carcinomatosis was allowed to develop. On day 10, control group ($n=12$) received growth media i.p. and treatment groups ($n=8$) were injected i.p. with 10^2 – 10^6 VP.

Ad5/3- Δ 24 in combination with epirubicin on ovarian cancer cells and on orthotopic murine model of ovarian cancer

Hey cells were seeded at 1.5×10^4 cells/well on 96-well plates. Next day, cells in triplicate were either infected with virus or treated with epirubicin or with a combination of both. Ad5/3- Δ 24 and Ad5/3luc1 were added at 0, 0.01, 0.1, 1, 10, 100 and 1000 VP/cell diluted in 50 μ l of growth media with 5% FBS. Epirubicin was diluted in 50 μ l of growth media with 5% FBS at final concentrations of 0, 0.0025, 0.025 and 0.25 μ g/ml. Cells were incubated at

37 °C and cell viability was measured 4 days after infection using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

To evaluate the efficacy of this combination *in vivo*, mice received 3×10^6 Hey cells i.p. On days 3, 10 and 17, 1 mg/kg epirubicin and 1×10^8 VP Ad5/3- Δ 24 was given i.p. either alone or in combination of both ($n=12$).

Ad5/3- Δ 24 in combination with gemcitabine on orthotopic murine model of ovarian cancer

In gemcitabine experiment, mice received 3×10^6 Hey cells i.p. Thereafter, 40 mg/kg gemcitabine and 1×10^8 VP Ad5/3- Δ 24 was administered i.p. alone or in a combination ($n=12$) on days 3, 10, 17 and 24. Tumors were fixed in 10% neutral-buffered formalin. Serial paraffin-embedded sections were taken and stained with H&E. Histopathology was scored blinded by an independent pathologist.

Statistical analysis

Chou and Talalay's median-effect method [18] was used to calculate combination index (CI) values under assumption of mutually nonexclusive drug interactions using S-PLUS 6.0 (Insightful Corporation, Seattle, WA). The combination index (CI) equation of <1 indicates synergism, 1 =additivity, and $CI > 1$ indicates antagonism. One-sample *t*-test was performed to determine whether the mean combination index values from separate experiments at multiple effects levels were significantly different from a value of 1.0. For all analyses, a *p* value of <0.05 was deemed statistically significant. Survival data was plotted into a Kaplan–Meier curve and groups were compared pair-wise with log-rank test using SPSS 14.0 (SPSS Inc, Chicago, IL).

Results

Comparison of single and weekly delivery of Ad5/3- Δ 24

Treatment with a single Ad5/3- Δ 24 injection was compared to multiple injections in orthotopic murine models of ovarian cancer. Peritoneally disseminated carcinomatosis was allowed to develop, and Ad5/3- Δ 24 was given i.p. either as a single or a weekly dose of 1×10^8 VP. Both treatment schedules resulted in significantly improved survival compared to untreated mice ($p < 0.001$) in both Hey and SKOV3.ip1 *in vivo* models (Fig. 1). However, administration of a single viral dose did not differ significantly from several doses in either model ($p=0.900$ and $p=0.654$, respectively). In aggressively growing Hey xenografts, the median survival of untreated mice was 23 days, while single and weekly virus treatments increased the median survival to 35 and 31 days, respectively. All mice died due to tumor progression in the peritoneal cavity. In the SKOV3.ip1 model, untreated mice achieved a median survival of 37 days, while single and weekly injection schedules enhanced the median survival to 58 and 75 days, respectively. Most of the treated mice died due to disease progression, but in a few cases there was evidence of hepatic toxicity in both treated groups, a known side effect from persistent virus replication. 1–2 mice/group were alive and healthy at the end of the experiment on day 136 in both virus-injected groups.

Determination of lowest effective dose of intraperitoneally delivered Ad5/3- Δ 24

The lowest effective i.p. dose of Ad5/3- Δ 24 was evaluated in mice with orthotopic SKOV3.ip1 tumors. 10^8 VP had been

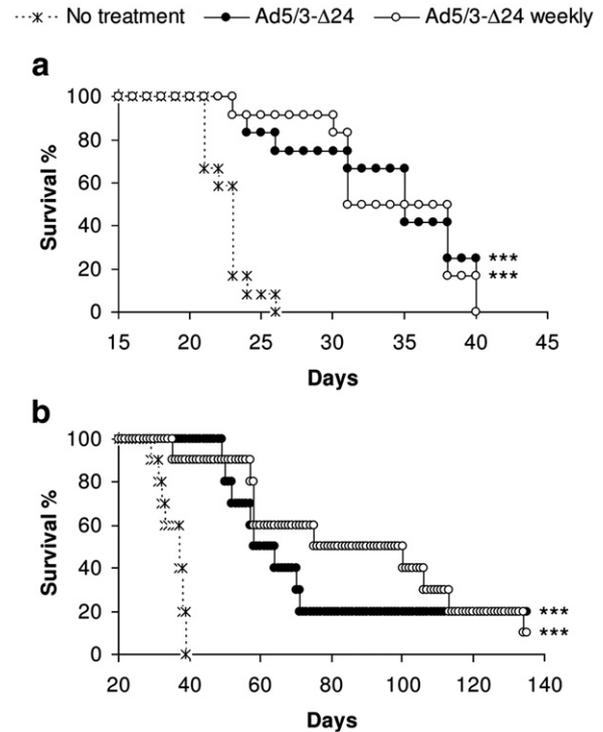


Fig. 1. Comparison of single versus multiple doses of Ad5/3- Δ 24 in (a) Hey or (b) SKOV3.ip1 models of intraperitoneally (i.p.) disseminated ovarian cancer. Tumor-bearing mice received single or weekly injections of Ad5/3- Δ 24 i.p. Both treatment schedules result in significant survival compared to untreated mice ($***p < 0.001$). Demonstrating effective *in vivo* replication of the agent following a single injection, antitumor efficacy was not improved with weekly dosing.

found effective (Fig. 1) and therefore dose de-escalation started from 10^6 and was continued to 10^2 . Although not significant, there seemed to be a trend towards dose-dependency since treatment with the highest dose 10^6 VP resulted in highest survival rates and median survival of 83 days (Fig. 2). However, even the lowest viral dose 10^2 VP with median survival of 54 days was enough to increase the survival of mice significantly compared to untreated animals ($p=0.001$). Slight variations in median survival did not result in different long-term survival as treatment with all doses enhanced the survival significantly compared to untreated animals.

Ad5/3- Δ 24 in combination with epirubicin on ovarian cancer cells

In order to determine the interaction between Ad5/3- Δ 24 and anthracycline epirubicin *in vitro*, cytotoxicity of the combination treatment was compared to single agent treatment of established ovarian adenocarcinoma cells. Cells were treated either with variable concentrations of virus, epirubicin or a combination of both, and cell viability was measured with MTS assay. Ad5/3luc1 was used as a replication-deficient control and it did not cause oncolysis (Fig. 3a). Ad5/3- Δ 24 infection of cells with 0.1 VP/cell resulted in 88% cell survival, whereas 0.25 μ g/ml epirubicin resulted in 63% viability (Fig. 3b). However, when both agents were administered together at these doses, the amount of living cells decreased to

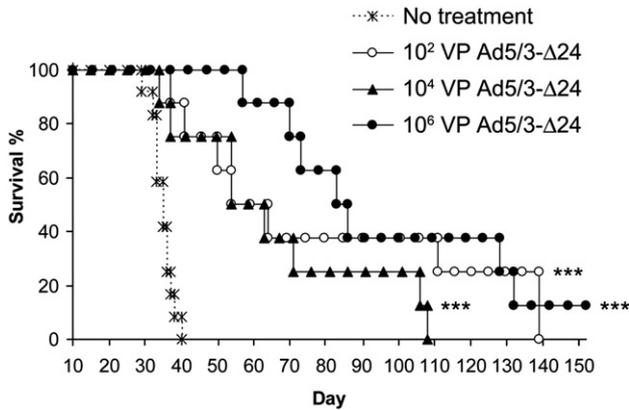


Fig. 2. Determination of lowest effective dose of Ad5/3-Δ24 in SKOV3.ip1 orthotopic model of ovarian cancer. Mice with intraperitoneally (i.p.) disseminated tumors received low doses of Ad5/3-Δ24 i.p. Even 10² VP resulted in significantly greater therapeutic benefit compared to untreated mice (***) ($p < 0.001$). Demonstrating effective *in vivo* replication of the agent, survival was not dependent on dose.

37%. Synergism was quantitated with the Chou and Talalay’s median-effect method [18] and combination resulted in significantly improved cancer cell killing compared to single treatments ($p = 0.002$).

Ad5/3-Δ24 in combination with epirubicin on orthotopic murine model of ovarian cancer

To study if Ad5/3-Δ24 and epirubicin work synergistically also *in vivo*, SCID mice with advanced ovarian cancer were treated either with single agents or the combination (Fig. 4). All treatment schedules resulted in significantly improved survival ($p < 0.001$) compared to untreated mice, which had a median survival of 25 days. Treatment with Ad5/3-Δ24 or epirubicin alone enhanced the survival to 32 or 37 days, respectively. There was no significant difference between single therapies ($p = 0.565$). However, the combination resulted in greater efficacy compared to Ad5/3-Δ24 ($p = 0.030$) or epirubicin ($p = 0.022$) alone. Median survival increased to 42 days and two mice were alive at the end of the experiment on day 50.

Ad5/3-Δ24 in combination with gemcitabine on orthotopic murine model of ovarian cancer

We have previously demonstrated synergy between Ad5/3-Δ24 and pyrimidine analogue gemcitabine [17]. In this study, combination of these agents was tested in a more aggressive, orthotopic Hey ovarian carcinoma model (Fig. 5a). All treatment schedules resulted in significantly enhanced survival ($p < 0.001$) compared to untreated mice, whose median survival was 23 days. Animals receiving Ad5/3-Δ24 and gemcitabine as a single treatment had an improved median survival of 31 and 38 days, respectively, and these treatments did not differ significantly ($p = 0.096$). The combination resulted in superior antitumor efficacy compared to Ad5/3-Δ24 ($p < 0.001$) or gemcitabine ($p = 0.012$) alone and median survival increased to 54 days.

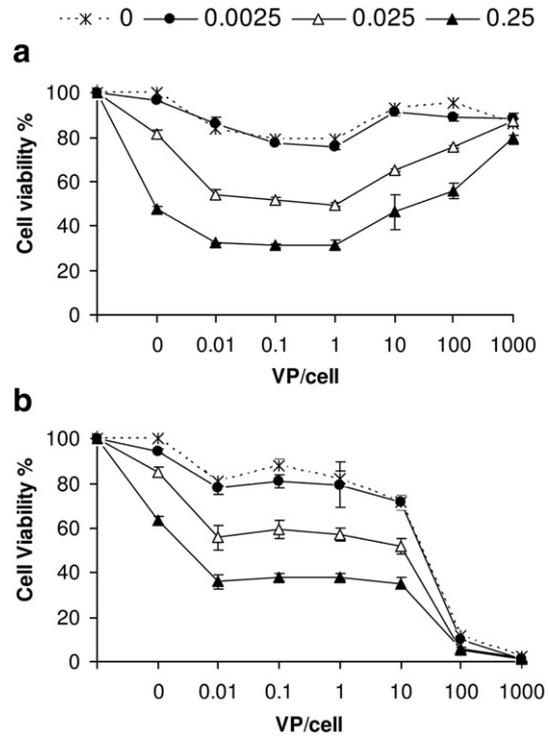


Fig. 3. Combination of Ad5/3-Δ24 and epirubicin *in vitro*. Viability of Hey ovarian cancer cells after infection with (a) Ad5/3-luc1 (replication-deficient control) or (b) Ad5/3-Δ24 in combination with epirubicin. Cells were infected at indicated doses of virus with epirubicin and viability was measured with MTS assay. Combination of Ad5/3-Δ24 and epirubicin resulted in significant synergy ($p = 0.002$). The OD₄₅₀ values of uninfected cells were set as 100%. Data are presented as means ± SD of triplicate experiments.

Tumors were harvested and analyzed histopathologically. Generally, changes such as apoptosis, diffuse necrosis and fibrotic strias were more prominent in the treated tumors (Fig. 5c), while the group injected with growth medium only

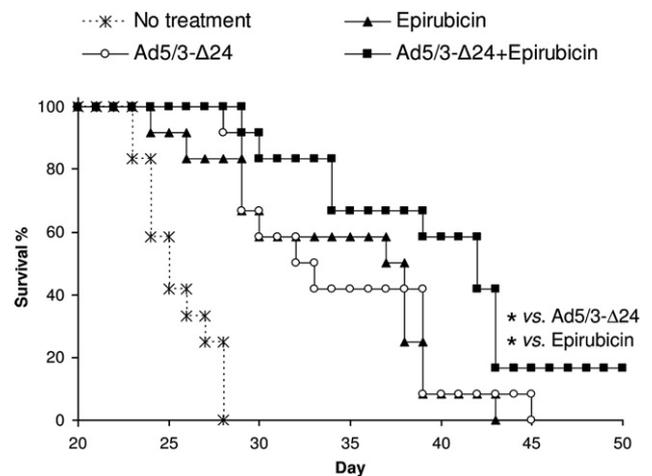


Fig. 4. Antitumor efficacy of Ad5/3-Δ24 and epirubicin in an orthotopic Hey model of intraperitoneally (i.p.) metastatic ovarian cancer. Mice were treated with a total of three i.p. injections of epirubicin and Ad5/3-Δ24 alone or in combination. The combination enhanced survival of mice in comparison to either treatment alone (* $p < 0.05$).

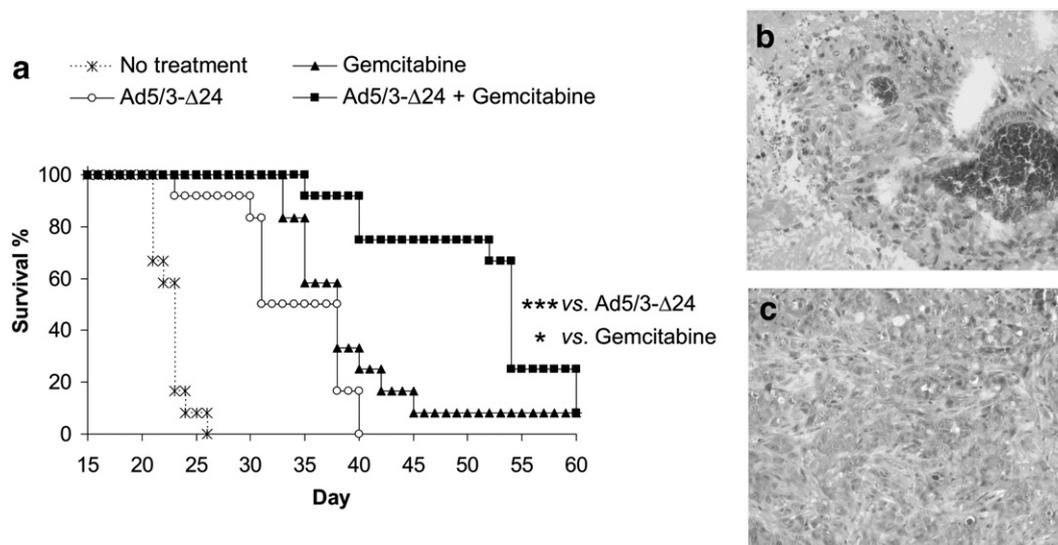


Fig. 5. Antitumor efficacy of combination of Ad5/3-Δ24 and gemcitabine in orthotopic Hey model of intraperitoneally (i.p.) metastatic ovarian cancer. Tumor-bearing mice received a total of four i.p. injections of gemcitabine and Ad5/3-Δ24 alone or in combination. (a) The combination treatment resulted in significantly improved survival in comparison to the single agents (* $p < 0.05$, *** $p < 0.001$). Tumors were harvested and analyzed histopathologically. The group injected with growth medium only showed tumor necrosis (b), while the necrotic changes were more prominent in the treated tumors (c).

showed reticular necrosis and necrotic foci (tumor necrosis) (Fig. 5b).

Discussion

Due to theoretical safety concerns, most gene therapy strategies in recent decades have been based on viruses that are unable to proliferate in infected cells. Although replication-deficient viruses expressing therapeutic transgenes have provided high *in vitro* and *in vivo* preclinical efficacy and good clinical safety data, trials have demonstrated that the utility of these agents may be limited when faced with advanced and bulky disease [19]. Nevertheless, even early generation replication-deficient adenoviruses have demonstrated efficacy in randomized trials, when given in the adjuvant setting [20], or combined with another modality [21]. Viruses that replicate and spread specifically inside the tumor have been suggested as a way to improve penetration of and dissemination within solid tumor masses [5–7]. With regard to gynecological cancer, replication-competent adenoviruses were tested in patients already more than 50 years ago [22].

The clinically most widely studied oncolytic adenovirus, ONYX-015 (a.k.a. *dl1520*) [23], carries deletions in *E1B*, exhibits reduced binding of p53 and replicates selectively in tumor cells. ONYX-015 has been evaluated in dozens of human trials with excellent safety data and some evidence of efficacy [24]. With regard to ovarian cancer, 16 patients enrolled in phase 1 trial received from one to four cycles of ONYX-015 on 5 consecutive days at doses from 1×10^9 pfu to 1×10^{11} pfu [25]. One patient developed dose-limiting, grade 3 abdominal pain and diarrhea. Nevertheless, the maximum tolerated dose was not reached, and the “maximum affordable dose” was 10^{11} VP. However, there were no clinical or radiological responses in any patients, which may relate to low replicativity and oncolytic potency of the virus [24,26].

Importantly, it has been shown in the context of oncolytic viruses, that the infectivity of target cells is a main determinant of oncolytic potency [12]. Unfortunately, ovarian cancer is similar to many other cancers in that CAR expression levels are highly variable and often low [6]. Thus, the lack of responses seen with ONYX-015 in ovarian cancer may result from the combination of low replicativity, low oncolytic potency and low infectivity due to variable CAR level. A contributing factor could be the frequent presence of malignant ascites, which often contains preexisting anti-Ad5-neutralizing antibodies [27]. Therefore, infectivity enhancement, retargeting to non-CAR receptors and avoidance of neutralizing antibodies may be useful for improving efficacy of oncolytic adenovirus therapy for ovarian cancer.

In this study, we utilized Ad5/3-Δ24 for treatment of advanced ovarian cancer in various murine models. The tumor specificity of Ad5/3-Δ24 is based on the inability of the modified E1A protein to bind cellular Rb. In normal cells, this interaction is required for effective virus replication [28,29]. Binding of Rb by E1A releases E2F, which activates the viral *E2* promoter and several cell cycle regulatory genes in order to initiate viral replication [30]. However, in tumor cells, where the *Rb/p16* pathway is inactive, there is abundant E2F available and E1A-Rb binding is not necessary. It has been suggested that most human cancers, including ovarian cancer, are deficient in this crucial pathway that regulates the G₁-S checkpoint [10,11]. Further, Ad5/3-Δ24 incorporates the Ad3 knob in the Ad5 fiber, and therefore, the virus binds to the Ad3 receptor highly expressed on ovarian cancer cells [13]. Finally, Ad5/3 chimeric fiber allows partial escape from neutralizing antibodies [14]. Of note, Ad5/3-Δ24 is under development for human trials.

Here, we demonstrate that treatment with multiple injections of Ad5/3-Δ24 did not significantly improve results over a single injection in orthotopic models of peritoneally disseminated ovarian cancer (Fig. 1). Further, utilizing dose de-escalation, we

found out that even a single viral dose of 100 VP increased the survival of mice significantly compared to untreated animals, and coming down million-fold from 10^8 did not reduce efficacy of the treatment (Figs. 1 and 2). These findings seem consistent with potent amplification of the agent. However, the survival curves appear to favor the higher dose somewhat. Although not demonstrated before *in vivo*, even a small amount of virus can amplify exponentially to high degree and kill large numbers of tumor cells. In theory, the oncolytic process would be expected to continue as long as target cells persist. Nevertheless, given the different tumor characteristics, these findings might have been different for other cancer cell lines. Further, the statistical power behind these conclusions was limited.

A powerful approach for further increasing the efficacy of virotherapy is utilization of oncolytic viruses in combination with conventional anticancer therapies in a multimodal anti-tumor approach. There are several preclinical studies suggesting enhanced and even synergistic cell killing and antitumor activity when oncolytic adenoviruses and chemotherapy have been combined [17,31–40], and the safety of the approach in humans has been demonstrated [21,41–46]. Furthermore, the toxicity spectrum of viruses is different from that of chemotherapeutics, and therefore combination treatment may not automatically lead to increased toxicity. Moreover, there is no cross resistance between adenoviruses and chemotherapy which may reduce development of resistant clones. In contrast, molecular reasons for synergy have been suggested, which might allow utilizing a lower dose of chemotherapy, thus retaining efficacy. Thus, gene therapy differs from chemotherapy with regard to mechanism and side effects, providing a possibility for additive or synergistic interactions. Although the mechanism of augmented therapeutic effects of combining oncolytic virotherapy and chemotherapy is still unknown, several hypotheses can be put forth. Firstly, expression of adenoviral E1A protein may sensitize cells to chemotherapy-mediated cell killing [34,47]. Secondly, chemotherapeutic agents may enhance the level of virus replication [34]. Finally, chemotherapeutic agents may affect the receptor status of the target cells, or each agent may work independently on different cell populations.

Most patients with advanced epithelial ovarian cancer will relapse after first-line platinum/taxane-based chemotherapy. Thus, given the most severe unmet clinical need of these heavily pretreated patients, we tried to model the situation of this patient population, and chose gemcitabine and epirubicin in our experiments as examples of commonly used agent for treatment of platinum-resistant ovarian cancer [48].

We first evaluated the efficacy of Ad5/3- Δ 24 and epirubicin on ovarian cancer cells and in an animal model of advanced ovarian cancer. The combination of epirubicin and Ad5/3- Δ 24 resulted in decreased viability of ovarian cancer cells (Fig. 3) and a significant survival benefit compared to single treatments (Fig. 4).

We have previously demonstrated that combination of Ad5/3- Δ 24 and gemcitabine delivers a synergistic effect to ovarian cancer cells both *in vitro* and *in vivo* [17]. However, enhanced efficacy was sometimes associated with increased liver toxicity. To investigate the generalizability of these findings, we eval-

uated the combination of Ad5/3- Δ 24 and gemcitabine in more aggressive Hey cell model of peritoneally disseminated ovarian cancer. The combination resulted in significant increase in survival compared to single agents, and hepatic toxicity was circumvented by using lower doses of gemcitabine (Fig. 5a).

In summary, the combination of Ad5/3- Δ 24 with epirubicin or gemcitabine appears promising. However, clinical trials are needed to confirm the data in humans. Promising safety data seen here, and in previous publications [21,41–43,45,46], suggests that clinical evaluation of the approach is feasible.

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