Gastric cancer currently ranks second in global cancer mortality (1). Most patients are either diagnosed at an advanced stage, or develop relapse after apparently curative operation (2). When metastatic, gastric cancer remains incurable, and despite some recent advances, chemotherapy provides only a modest increase in survival, often with significant side effects (3). Therefore, new approaches are critical for the treatment of this common and deadly disease. Gene therapy with adenoviruses is a promising modality for the treatment of advanced cancers refractory to other therapies. Importantly, the safety and efficacy of the approach has been recently validated in landmark randomized trials (4–6). However, a limiting factor for the most frequently used serotype 5 adenoviruses (Ad5) is dependence on the coxsackie-adenovirus receptor (CAR), which is variably expressed in most advanced cancers (7). This has also been studied in the context of gastric cancer cell lines, which were shown to have low to moderate expression of CAR (8). Native Ad5 tropism can be modified to circumvent CAR deficiency in cancer cells. Transductional targeting of adenoviruses aims at enhanced transduction of the target cell, e.g., by incorporating targeting moieties into the fiber knob region. Incorporation of an Arg-Gly-Asp (RGD)-containing peptide in the HI loop of the fiber knob allows the virus to utilize αvβ3-class integrins for binding and internalization (9). These integrins are frequently highly expressed in advanced gastric cancers (10). Adenoviruses with a COOH-terminal polylysine tail (11, 12) bind to heparan sulfates, often expressed to a high degree in gastric cancer (13). Substitution of the entire fiber knob was used in the construction of Ad5/3, an Ad5 vector that features a chimeric fiber with the adenovirus serotype 3 (Ad3) knob

Infectivity-Enhanced Adenoviruses Deliver Efficacy in Clinical Samples and Orthotopic Models of Disseminated Gastric Cancer

Lotta Kangasniemi,1,2 Tuula Kiviluoto,3 Anna Kanerva,1,2,4 Mari Raki,1,2 Tuuli Ranki,1,2 Merja Sarkioja,1,2 Hongju Wu,7 Frank Marini,8 Krister Höckerstedt,8 Helena Isoniemi,8 Henrik Alfthan,6 Ulf-Håkan Stenman,6 David T. Curiel,7 and Akseli Hemminki1,2

Abstract

Purpose: Metastatic gastric cancer remains a common and devastating disease without curative treatment. Recent proof-of-concept clinical trials have validated gene therapy with adenoviruses as an effective and safe modality for the treatment of cancer. However, expression of the primary coxsackie-adenovirus receptor is variable in advanced cancers, and therefore, the use of heterologous receptors could be advantageous.

Experimental Design: Here, we used capsid-modified adenoviruses for increasing the transduction and subsequent antitumor efficacy. 5/3 chimeric viruses have a serotype 3 knob which allows binding to a receptor distinct from coxsackie-adenovirus receptor. The fiber of Ad5LucRGD is modified with an integrin-targeted motif. Polylysine motifs, pK7 and pK21, bind to heparan sulfates. Oncolytic adenoviruses replicate in and kill tumor cells selectively. Gastric cancer cell lines and fresh clinical samples from patients were infected with transductionally targeted viruses. Capsid-modified oncolytic adenoviruses were used in cell killing experiments. To test viral transduction and therapeutic efficacy in vivo, we developed orthotopic mouse models featuring i.p. disseminated human gastric cancer, which allowed the evaluation of biodistribution and antitumor efficacy in a system similar to humans.

Results: Capsid modifications benefited gene transfer efficiency and cell killing in gastric cancer cell lines and clinical samples in vitro and in vivo. Modified oncolytic adenoviruses significantly increased the survival of mice with orthotopic gastric cancer.

Conclusions: These preclinical data set the stage for the clinical evaluation of safety and efficacy in patients with disease refractory to current modalities.
domain (14). The receptor for Ad3 is currently unknown, although some candidates have been proposed and CD46 seems to be emerging as a likely main receptor candidate (15–17). However, it is unknown if CD46 would also be the receptor for 5/3 chimeric viruses. Nevertheless, it is intriguing that CD46 has been reported to be highly expressed in advanced gastric cancers (18).

Replication-deficient vectors may be of use when tumor burden is small or when combined with another treatment modality (4, 5). However, when faced with advanced metastatic masses, tumor penetration is key to efficacy and cannot be easily achieved with replication-deficient viruses due to poor intratumoral diffusion (19). To overcome this obstacle, conditionally replicative oncolytic adenoviruses (CRAds) were constructed. Ad5-Δ24 (also called d1922-947) contains a 24-bp deletion in the constant region 2 of E1A. Thus, the expressed protein is unable to bind the retinoblastoma protein for the induction of S phase (20, 21). Therefore, the virus replicates selectively in cells deficient in the retinoblastoma/p16 pathway. Most advanced human tumors, including gastric cancers, are deficient in this crucial pathway (22–24). Importantly, in contrast with earlier CRAds such as the prototype dl1520 (25), the constant region 2 deletion does not reduce replication of the agent in tumor cells (21).

S.c. tumors have been widely used in cancer research due to the ease of inoculation and tumor measurement. Nevertheless, human cancers are rarely located s.c. and it is well-established that the tumor environment has an effect on growth and behavior, including response to antitumor agents. Furthermore, it has been shown that orthotopic tumors, growing in the correct organ or region, resemble the primary clinical tumor more accurately with regard to treatment sensitivity, than the same cells grown s.c. (26). Gastric cancer typically spreads i.p., more accurately with regard to treatment sensitivity, than the same cells grown s.c. (26). Gastric cancer typically spreads i.p., more accurately with regard to treatment sensitivity, than the same cells grown s.c. (26). Gastric cancer typically spreads i.p., more accurately with regard to treatment sensitivity, than the same cells grown s.c. (26).

Materials and Methods

Cells and tissues. A549 human lung adenocarcinoma cells and 293 embryonal kidney cells were from the American Type Culture Collection (Borås, Sweden). Six gastric cancer cell lines, comprising three intestinal (MKN-7, MKN-28, and MKN-74), two diffuse (KatoIII and MKN-45), and an adenosquamous type (MKN-1) were used (from Dr. Hiroshi Yokozaki). KatoIII is from American Type Culture Collection. All lines were cultured as recommended. Fresh gastric cancer samples were obtained with signed informed consent and ethical committee permission from patients undergoing surgery at Helsinki University Central Hospital. For transportation, the tissue was kept on ice in RPMI 1640 with 20% FCS, 200 IU/mL penicillin, 200 μL/mL streptomycin, and 2 mmol/L L-glutamine. Sample processing was started within an hour from surgery. The size of the available sample usually allowed only one dose of virus to be investigated.

Adenoviruses. Replication-competent and -incompetent viruses (Table 1) were propagated on A549 and 293 cells, respectively, and purified on cesium chloride gradients. The viral particle concentration was determined at 260 nm, and standard plaque assay on 293 cells was done for infectious particles.

Gene transfer assays. Cells were infected with replication-deficient viruses for 30 minutes at room temperature in 200 μL of growth medium with 2% FCS. Cells were washed once and complete medium was added. After 24 hours, incubation at 37°C, luciferase (Luciferase Assay System, Promega, Madison, WI) or β-gal (Galacto Light Plus, Tropix, Bedford, MA) assays were done.

Human gastric cancer samples were cut into 200 μg pieces and homogenized. Samples were washed twice with RPMI 1640 containing 2% FCS and infected with 2.5 × 10^7 viral particles in 50 μL of growth medium. After 1 hour of infection at 37°C, the medium was replaced with 2 mL of growth medium. Tissue homogenates were lysed by adding 200 μL of Cell Culture Lysis Buffer (Promega), or with lysis buffer for luminescent β-galactosidase reporter system 3 (BD Biosciences, Palo Alto, CA). Samples were freeze-thawed thrice and supernatant was analyzed for luciferase or β-gal activity. Protein concentration was determined by using a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA).

Biodistribution. Female 3- to 4-week-old NMRI nude mice were purchased from Taconic (Ejby, Denmark) and injected with 1 × 10^7

Table 1. Viruses used in the experiments

<table>
<thead>
<tr>
<th>Virus</th>
<th>EIA</th>
<th>Reporter gene</th>
<th>Fiber</th>
<th>Main receptor</th>
<th>Ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5Luc1</td>
<td>Deleted</td>
<td>Luciferase</td>
<td>Wild-type</td>
<td>CAR</td>
<td>4</td>
<td>(14)</td>
</tr>
<tr>
<td>Ad5/3Luc1</td>
<td>Deleted</td>
<td>Luciferase</td>
<td>Serotype 3 knob</td>
<td>Probably CD46</td>
<td>13</td>
<td>(14)</td>
</tr>
<tr>
<td>Ad5LucRGD</td>
<td>Deleted</td>
<td>Luciferase</td>
<td>RGD motif in the HI loop</td>
<td>uαvβ2 integrins</td>
<td>53</td>
<td>(9)</td>
</tr>
<tr>
<td>Ad5/GL</td>
<td>Deleted</td>
<td>GFP + luciferase</td>
<td>Wild-type</td>
<td>CAR</td>
<td>11</td>
<td>(11)</td>
</tr>
<tr>
<td>Ad5/pK7</td>
<td>Deleted</td>
<td>GFP + luciferase</td>
<td>7 polylysines at the COOH terminus</td>
<td>Heparan sulfates</td>
<td>25</td>
<td>(11)</td>
</tr>
<tr>
<td>Ad5RGD.pK7</td>
<td>Deleted</td>
<td>GFP + luciferase</td>
<td>RGD motif in the HI loop and 7 polylysines at the COOH terminus</td>
<td>uαvβ2 integrins, heparan sulfates</td>
<td>38</td>
<td>(11)</td>
</tr>
<tr>
<td>Ad5LacZ</td>
<td>Deleted</td>
<td>lacZ</td>
<td>wild-type</td>
<td>CAR</td>
<td>12</td>
<td>(12)</td>
</tr>
<tr>
<td>Ad5pK21LacZ</td>
<td>Deleted</td>
<td>lacZ</td>
<td>21 polylysines at the COOH terminus</td>
<td>Heparan sulfates</td>
<td>10</td>
<td>(12)</td>
</tr>
<tr>
<td>Ad5/3-Δ24</td>
<td>24 bp deletion</td>
<td>—</td>
<td>Serotype 3 knob</td>
<td>Probably CD46</td>
<td>10</td>
<td>(43)</td>
</tr>
<tr>
<td>Ad5-Δ24RGD</td>
<td>24 bp deletion</td>
<td>—</td>
<td>RGD motif in the HI loop</td>
<td>uαvβ2 integrins</td>
<td>11</td>
<td>(47)</td>
</tr>
<tr>
<td>Ad5-Δ24E3</td>
<td>24 bp deletion</td>
<td>—</td>
<td>Wild-type</td>
<td>CAR</td>
<td>8</td>
<td>(47)</td>
</tr>
<tr>
<td>Ad300wt</td>
<td>Wild-type</td>
<td>—</td>
<td>Wild-type</td>
<td>CAR</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

1 Ratio of total viral particles to infectious particles, describes effectiveness of virus packaging.

2 24 bp deletion in the constant region 2 of the adenoviral E1A protein abrogates binding of retinoblastoma protein in normal cells. Retinoblastoma is inactivated during tumorigenesis in tumor cells, and therefore, the virus replicates selectively in tumor cells.
MKN-45 cells i.p. after quarantine. Eighteen days later, mice were randomized (n = 4-5/group) and injected i.p. with transductionally targeted replication-deficient viruses at 1 × 10^9 viral particles in 500 μL of MEM. Forty-eight hours later, selected organs were collected and snap-frozen. The tissue samples were homogenized and lysed with Cell Culture Lysis Buffer, freeze-thawed, and supernatant was analyzed for transgene expression and protein content as with the clinical samples. Animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland.

Cell viability. Gastric cancer cells were seeded at 1 × 10^4 cells/well on 96-well plates and cultured overnight. Cells in triplicate were infected in growth medium with 2% FCS. After 1 hour, medium was changed to 5% FCS which was replaced every other day. Ten to 14 days after infection, cell viability was measured with MTS assay (Promega).

In vivo survival. Female Fox Chase severe combined immunodeficiency mice (Taconic) were purchased at 4 to 5 weeks of age. MKN-28 cells (5 × 10^5) were injected i.p. into mice randomized into five treatment groups (n = 15). Ten days later, mice received a single i.p. injection of 1 × 10^9 viral particles in 500 μL of MEM. Serum samples for hCG were collected from the tail vein 50 days after injecting the cells (n = 2-6). hCG concentration was quantitated with a time-resolved immunofluorometric assay based on free hCG-specific monoclonal antibodies.
Liver explants. Fresh normal human liver samples were obtained and transported as tumor samples above. Precision-cut, 200-μm-thick slices (1000 Plus Sectioning System, Vibratome, St. Louis, MO) were infected with CRAds at 10⁷ viral particles and incubated at 37°C. At the indicated time points, tissue was homogenized, freeze-thawed three times, and infectious particles were determined by TCID₅₀. During slicing, tissue was kept on ice in William’s medium E (Sigma, St. Louis, MO) containing 25 mmol/L d-glucose and 50 μg/mL gentamicin.

Statistical analysis. Two-tailed Student’s t test was used to compare the differences between CRAds and control viruses. Survival was analyzed according to Kaplan-Meier with SPSS 11.5 for Windows.

Results

Capsid modifications increase gene transfer to gastric cancer cell lines. We compared a panel of adenoviruses in six gastric cancer cell lines representing three different histologic groups (Fig. 1A). In CAR-positive nonmalignant 293 cells, differences in gene transfer were mostly ≤2-fold (Fig. 1B). With the intestinal gastric cancer cell lines, gene transfer with Ad5.pK7 was increased up to 24-fold in MKN-7 and 5-fold in MKN-28, compared with the wild-type Ad5 capsid virus. Transductional efficacy with Ad5pK21LacZ was increased up to 479-fold in MKN-7, 35-fold in MKN-28, and 52-fold in MKN-74; with Ad5lucRGD, the respective values in these cell lines were 34-, 8-, and 3-fold, and with Ad5/3luc1 it was 5-, 9-, and 3-fold.

In the diffuse-type cell lines, gene transfer with Ad5.pK7 was increased 17-fold in KatoIII and 75-fold in MKN-45 compared with the virus with the Ad5 capsid. With Ad5pK21LacZ, relative increases in transduction were up to 15-fold in KatoIII and 71-fold in MKN-45. With Ad5lucRGD, the respective values in these cell lines were 8- and 10-fold, and with Ad5/3luc1, 5- and 7-fold. Ad5RGD.pK7 increased adenovirus gene level in
MKN-45 up to 3-fold, but not in KatoIII cells. In adenosquamous MKN-1 cells, gene transfer was increased up to 52-fold with Ad5pK21LacZ and 29-fold with Ad5.pK7 compared with Ad5. Ad5/3luc1 and Ad5lucRGD increased gene transfer up to 3- and 6-fold, respectively.

**Capsid modifications increase gene transfer to primary gastric cancer specimens.** Ad5.pK7 increased gene transfer in six out of the seven samples up to 32-fold (Fig. 2). Ad5/3luc1 and Ad5lucRGD increased transduction in all the samples up to 256-fold and 198-fold, respectively. Ad5RGD.pK7 increased gene transfer in three out of seven samples, and up to 10-fold compared with Ad5. Ad5pK21LacZ was tested on two samples, and it was 11- and 15-fold more effective than the respective control Ad5lucZ.

**Capsid modifications enhance gene transfer in vivo.** Relative to Ad5, Ad5/3luc1 increased gene transfer to tumors, spleen, heart, and lungs, but not to liver or kidneys (Fig. 3A). Ad5.pK7 augmented the transduction of tumors but not of other organs. Ad5pK21LacZ or Ad5RGD.pK7 did not affect gene transfer significantly. Reduced transduction of tumor was seen with Ad5lucRGD. When comparing marker gene level in the tumors versus the liver, Ad5RGD.pK7 resulted in 11 times more activity in tumor tissue than in the liver (Fig. 3B).

**Capsid modifications increase the oncolytic potential of CRAds in vitro.** Three isogenic CRAds were available, with capsids identical to the replication-deficient viruses. In all of the cell lines, Ad5/3-Δ24 was the most oncolytic (Fig. 4): with well-differentiated MKN-28 and MKN-7 cells, as well as with poorly differentiated MKN-45 cells. Wild-type virus was the next best and Ad5-Δ24E3 was third. With well-differentiated MKN-74 cells, Ad5-Δ24E3 displayed nearly the same cell-killing capacity as Ad5/3-Δ24, and wild-type virus was the third most effective. In poorly differentiated KatoIII and adenosquamous MKN-1, there was no significant difference between Ad5/3-Δ24 and wild-type, which was the second most effective after Ad5/3-Δ24. With MKN-7, MKN-45, MKN-74, and MKN-1 cells, Ad5-Δ24RGD displayed only modest oncolytic activity. A CRAd with a polylisine modification of the fiber and a 24 bp deletion in E1A would have been of interest for comparison, but unfortunately has not been described.

**Capsid-modified CRAds increase survival of severe combined immunodeficiency mice bearing orthotopic gastric cancer.** Median survival was 37 days in the nontreated (mock) group and 40 days in the group treated with replication-deficient Ad5luc1 (Fig. 5A). With the CRAds, median survival was 64 days with both Ad5-Δ24E3 and Ad5-Δ24RGD, and 67 days in the group receiving Ad5/3-Δ24. There was no significant difference in survival between the mock mice versus the Ad5luc1-treated mice (P > 0.25). All CRAds prolonged the survival significantly both compared with the mock (P < 0.0005) as well as with the Ad5luc1-treated mice (P < 0.05). Despite a trend favoring Ad5/3-Δ24, there was no significant difference between the mice treated with Ad5-Δ24E3, Ad5/3-Δ24, or Ad5-Δ24RGD. Serum hCG concentration is a prognostic factor in patients with gastric cancer (28). Mean hCG values were lower in CRAd-treated mice (not significant, Fig. 5B).

**Liver replication assay.** Human liver explants were infected with CRAds and control viruses (Fig. 5). For most viruses, no replication was seen. However, functional titers of Ad5/3-Δ24 increased ~3-fold, which suggests a low but detectable level of virus replication.

**Discussion**

There is an urgent need for new approaches for the treatment of metastatic gastric cancer. Using adenoviruses that enter through non-CAR receptors, we were able to increase gene transfer to gastric cancer cell lines (Fig. 1A). There was no obvious systematic relationship between histologic tumor classification and the best capsid modification. However, Ad5.pK7 seemed to be rather effective in the poorly differentiated samples. Also, Ad5pK21LacZ increased gene transfer to all gastric cancer cell lines studied and Ad5/3luc1 was more effective than Ad5 in most lines.
An important part of the study was the analysis of clinical samples fresh from patients (Fig. 2). As it is increasingly accepted that established cell lines differ from the initial clinical tumors (29), it was not completely unexpected to see a different profile of gene transfer in comparison with the cell lines. Although many of the capsid modifications were more effective than Ad5, Ad5/3luc1 emerged as the “virus of choice.” Moreover, given the variation between samples, the results suggest that for clinical intervention with adenovirus gene therapy, it might be useful to analyze the tumor before selecting a virus for treatment.

In vivo, in a model that closely resembles human metastatic disease, Ad5/3luc1 and Ad5.pK7 increased gene transfer to i.p. disseminated tumors (Fig. 3A). Ad5/3luc1 also showed increased gene transfer to the spleen, heart, and lungs. Importantly, as the liver is the most important organ with regard to side effects (30), neither virus increased hepatic gene transfer. More importantly, none of the CRAds replicated effectively in liver explants (Fig. 6). Although Ad5/3-Δ24 levels increased to ~3-fold, this is a rather ineffective replication compared with permissive cells, which can amplify the input dose up to 10^10-fold (31). Nevertheless, liver toxicity should be monitored carefully in human trials.

Promising gene transfer and biodistribution data rationalized analysis of the respective CRAds for oncolytic activity (Fig. 4). Three isogenic Δ24-type CRAds were available. Each has an identical 24-bp deletion in the constant region 2 of E1A, which allows selective replication in retinoblastoma/p16 pathway-deficient tumor cells. Also, each virus has an intact E3 region, which is important for effective oncolysis (32). The viruses only differ with regard to the capsid. Ad5/3-Δ24 was consistently the most efficient. Moreover, these data suggest that it might be of interest to create CRAds featuring pK7 or pK21.

Most patients who die of gastric cancer have i.p. disseminated disease. Therefore, we developed orthotopic murine models to allow the analysis of CRAds (Figs. 3 and 5). Treatment was given i.p. to maximize local activity whereas limiting systemic exposure. I.p. adjuvant chemotherapy has been found to increase survival for patients undergoing resection of stage III gastric cancer (33). The survival of mice treated with the CRAds was significantly improved compared with controls (P < 0.05; Fig. 5A). Although there seemed to be a trend favoring Ad5/3-Δ24 over the other CRAds, the difference was not statistically significant. All mice in the control groups died due to rapid disease progression. Analysis of βhCG secreted from the tumor cells suggested a smaller tumor load in animals treated with the CRAds (Fig. 5B). Analysis of markers could allow sensitive and noninvasive detection of antitumor activity in this model as it requires only a small blood sample. However, this approach...
needs further optimization and would benefit from more frequent sampling at various time points.

These data set the stage for clinical testing of the agents. Gastric cancer may be a good disease for intervention with CRAds, due to the low effect and often significant toxicity of currently available agents. Also, frequent confinement of the disease to the peritoneal cavity creates a rationale for i.p. treatment. If confirmed, it would be useful that a number of CRAds have activity in human gastric cancer. Treatment with adenoviruses induces a neutralizing antibody response (34). Although this does not prevent readministration, particularly in the context of locoregional delivery, it could reduce the efficacy of subsequent cycles of therapy (35). However, neutralizing antibodies are conformation-sensitive, and it has been shown that even slight changes in the fiber allow escape from preexisting neutralizing antibodies (36–39). Thus, subsequent treatments could be given with viruses featuring different capsids.

A recent randomized phase III clinical trial validated the utility of CRAds for the treatment of squamous cell cancer of the head and neck or esophagus (6). Adding H101 to cisplatin and 5-fluorouracil chemotherapy increased the overall response rate from 39.6% to 78.8% (P < 0.0001). H101 is a CRAd that lacks the E1B55K protein, which may confer selectivity to tumor cells dysregulated in the p53/p14ARF pathway, and is closely related to dl1520, which has been previously evaluated in a number of phase I and II trials (40). In particular, promising activity was seen in a phase II trial when dl1520 was combined with cisplatin and 5-fluorouracil (41). Therefore, the phase III results with H101 are well in accord with earlier data with dl1520. Nevertheless, dl1520 is a prototype agent which is rather attenuated even in tumor cells, when compared with wild-type adenovirus (25, 40). In contrast, the 24-bp deletion in Δ24-based agents does not attenuate but may in fact increase replication of the virus (21), which could predict superior efficacy versus H101/dl1520, as already shown preclinically (21, 42).

The oncolytic potency of the viruses used here is further increased by capsid modification–mediated infectivity enhancement (40, 43). If preclinical data has predictive power, it is possible that this generation of CRAds could yield further improvements in efficacy (43–46). Moreover, it is important to note that the side effect profiles of agents currently used for treatment of metastatic cancers, including radiation therapy, chemotherapy, hormonal therapies, small molecular inhibitors, and monoclonal antibodies, are mostly different from the side effect profiles of oncolytic viruses. This could facilitate combination treatments with increased efficacy without increases in side effects (6, 31).

In summary, we have done a preclinical evaluation of various capsid-modified adenoviruses for the treatment of gastric cancer. Promising activity and favorable biodistribution was seen. Next, clinical evaluation is needed for the analysis of the safety and efficacy of these agents in humans. Eventually, such developments could translate into increased treatment options for patients with gastric cancer.

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