Enhanced killing of pancreatic cancer cells by expression of fusogenic membrane glycoproteins in combination with chemotherapy

Dennis Hoffmann and Oliver Wildner

Department of Molecular and Medical Virology, Institute of Microbiology and Hygiene, Ruhr-University Bochum, Bochum, Germany

Abstract

Pancreatic cancer has a poor prognosis with an annual mortality rate close to the annual incidence rate. We evaluated whether the expression of measles virus fusogenic membrane glycoproteins (FMG) H and F will enhance chemotherapy. Using Chou-Talalay analysis, we showed in vitro in pancreatic cancer cells that the expression of FMG often synergistically enhances clinically relevant chemotherapy. Furthermore, cell fusion in combination with chemotherapy resulted in strongly enhanced Annexin V binding, an early marker for apoptosis, when compared with single treatment. We showed in an i.p. and s.c. pancreatic xenograft model that the administration of a replication-defective adenoviral vector Ad.H/F encoding tumor-restricted FMG in combination with gemcitabine significantly enhanced treatment outcome when compared with treatment with each compound individually. To improve tumor transduction efficiency, the Ad.H/F vector was also transcomplemented with an oncolytic replication-restricted adenovirus (Ad.COX×MK), resulting in significantly improved treatment efficacy. We assessed treatment efficacy by survival analysis or measuring growth, respectively. In the i.p. model, on day 120, three of eight animals treated with this novel triple therapy consisting of Ad.H/F, gemcitabine, and Ad.COX×MK were alive and tumor free. Treatment with Ad.H/F and Ad.COX×MK resulted in one long-term survivor. In all other treatment groups, there were no long-term survivors. The significantly improved therapeutic outcome of animals receiving the triple therapy was attributed to multiple factors, including most likely improved FMG expression throughout the tumor and enhanced sensitivity of the tumor cells to gemcitabine by adenoviral gene products but also FMG expression. Qualitatively similar results were obtained in a s.c. pancreatic xenograft model. [Mol Cancer Ther 2006;5(8):2013–22]

Introduction

Annually, ~32,000 patients in the United States and ~50,000 in Europe will develop and also die from pancreatic cancer (1, 2). Pancreatic cancer is extremely aggressive locally and frequently metastasizes to the liver and peritoneum. Complete surgical resection currently offers the only potential for long-term survival. For patients with advanced or metastatic cancer of the pancreas, the most active chemotherapeutic agents are gemcitabine and 5-fluorouracil (5-FU), which have a response rate of 15% to 20% and 10% to 20%, respectively (3, 4). The thymidylate synthetase inhibition of 5-FU is enhanced by leucovorin (LV; ref. 5). Currently, gemcitabine is the new standard of care in pancreatic cancer. It has shown activity in pancreatic cancer refractory to 5-FU (6) and offers a slightly better survival than 5-FU (4). In addition, irinotecan (CPT-11; ref. 7) and combination chemotherapy regimens with infusional 5-FU/LV and oxaliplatin, known as FOLFOX (8), have been evaluated in clinical trials. However, minimal benefit in survival has been shown for these agents as responses are usually partial remission and of short duration.

In this study, we evaluated in vitro by using Chou-Talalay analysis (9) and in two pancreatic xenograft models whether the expression of measles virus fusogenic membrane glycoproteins (FMG) H and F, encoded by a replication-defective adenoviral vector Ad.H/F, will enhance chemotherapy.

The intratumoral injection of replication-defective viral vectors results in low tumor transduction efficiency because the dispersion of replication-deficient vectors is largely confined close to the initial site of injection (10). Several factors are responsible for this phenomenon, including the interconnection of tumor cells by tight junctions; high density of cellular receptors and binding of viral particles by these receptors effectively removes them from the intracellular space, decreasing the driving gradient for particle diffusion (11).

Previously, it has been shown by several groups that replication-competent vectors, or replication-defective vectors rendered replication-competent by transcomplementation of missing genes pivotal for vector replication, have the potential to overcome poor tumor transduction...
efficiency (12–16). Thus, we transcomplemented the FMG-encoding vector with the oncolytic replication-restricted adenovirus vector Ad.COX•MK (17). In cells transduced with both the replication-defective vector encoding FMG and the replication-competent oncolytic vector, the replication-defective vector will be transcomplemented to allow vector amplification and intratumoral spread, resulting in improved tumor transduction (12–16, 18, 19). In addition, oncolytic adenoviruses are also potent chemosensitizers (20).

Intratumoral expression of fusogenic glycoproteins lead to syncytia formation of infected cells with adjacent cells, thereby increasing the dispersion of viruses throughout the tumor, lateral spread of the transgene, virus release, and immunogenicity of tumor cells (21–25). For cancer gene therapy, glycoproteins from HIV-1 (23), gibbon ape leukemia virus (22, 24), and measles virus (25) have been evaluated. In measles virus, the hemagglutinin (H) protein mediates attachment to its receptor on the target cell (CD46 or CD150; refs. 26, 27) and thus triggers conformational changes in the virus fusion (F) glycoprotein (28). This leads to a biologically active fusogenic form of the F protein that interacts with the host cell membrane, causing virus-cell or cell-cell fusion (29). Both H and F proteins are necessary for fusion to occur.

To improve safety and therapeutic index of replication-competent adenoviral vectors, it has been attempted to restrict vector replication to tumor cells. One approach is based on the replacement of viral promoters by tumor/tissue–specific promoters, which are critical for replication. In this transcriptional targeting strategy, initially only the adenovirus E1A gene has been placed under the transcriptional control of a tumor-specific promoter (30–33). Recent studies showed enhanced tumor-restricted adenoviral replication and lysis by placing the viral E1A and E4 genes under the transcriptional control of heterologous promoters (17, 34–36).

To our knowledge, this is the first report analyzing the interaction of viral fusogenic glycoproteins and chemotherapy for the treatment of cancer. To enhance in vivo transduction efficiency, we also transcomplemented the replication-defective FMG-encoding vector with the oncolytic replication-restricted adenovirus vector Ad.COX•MK. This novel triple therapy had the highest antineoplastic efficacy.

Materials and Methods

Cells and Cell Culture

The permanent human cell lines cfPac1, Panc1, 293, and HeLa were obtained from American Type Culture Collection (Manassas, VA).

We established short-term cultures of pancreatic cancer cells, designated PPC#1, PPC#2, and PPC#3, from patients who underwent surgery for treatment of histologically confirmed pancreatic cancer. The polyclonal cell lines PPC#1, PPC#2, and PPC#3, which constitutively express firefly luciferase, were generated by transduction with an murine leukemia virus–based retrovirus encoding luciferase (GC Luc3 EN; ref. 37) and subsequent neomycin selection.

Viruses

The replication-defective adenoviral vector Ad.Luc encoding firefly luciferase (pGL3basic, Promega, Madison, WI) driven by the hCMV-IE promoter was generated with the AdEasy system (38). In the tumor-restricted, replication-competent adenovirus Ad.COX•MK, the adenoviral E1 and E4 gene products, which are essential for viral replication, are transcriptionally targeted by the cyclo-oxygenase-2 (−327/+59; ref. 39) and midkine (MK−1,009/+25; ref. 40) promoter, respectively, as described previously (17).

The replication-defective adenoviral vector Ad.H/F encodes the measles virus FMG under the transcriptional control of the tumor-specific human telomerase reverse transcriptase gene promoter (−548/+5; ref. 41). For this, the CMV-IE promoter of the measles virus H and F bicistronic expression plasmid pGC-H/IRE/F (kindly provided by Roberto Cattaneo, Mayo Clinic College of Medicine, Rochester, MN; ref. 42) was replaced with the human telomerase reverse transcriptase promoter from the plasmid pBTdel-548 (kindly provided by Izumi Horikawa, National Cancer Institute, NIH, Bethesda, MD; ref. 41) and inserted into the multiple cloning site of pAdTrack (38), creating pAdTrack human telomerase reverse transcriptase H/F. Infectious viral vector was generated with the AdEasy-1 system, as described previously (38).

The reference strain VR-5 (American Type Culture Collection) was used as wild-type adenovirus type 5. The vectors Ad.Luc and Ad.H/F were amplified in 293 cells; the Ad.COX•MK vector and Ad5 in HeLa cells were purified as described previously (43). The vector Ad.H/F was generated and produced in the presence of the synthetic fusion inhibitory peptide Z-D-Phe-Phe-Gly-OH (10 μmol/L; Bachem AG, Bubendorf, Switzerland; ref. 44).

The bioactivity of the Ad.Luc vector was determined on 293 cells by bioluminescence and expressed as gene transfer units (GTU)/mL (45). The titer (GTU/mL) of the Ad.H/F vector, which encodes green fluorescent protein by a second transcription unit, was determined on cfPac1, Panc1, PPC#1, PPC#2, PPC#3, and 293 cells in the presence of the synthetic fusion inhibitory peptide by flow cytometry (46). The bioactivity of the Ad.COX•MK was determined by TCID50 (45) using cfPac1 and 293 cells.

Viral Transduction Efficiency

Cells were seeded at a concentration of 1 × 10^3 per well in 100 μL into 96-well flat-bottomed culture plates. Twelve hours later, cell monolayers were transduced with a defined amount (3 × 10^9 GTU) of Ad.Luc, resulting in 30% of the maximum obtainable luciferase activity in 293 cells. Twenty-four hours posttransduction, cell monolayers were washed with PBS and luciferase assay was carried out as described above.
Analysis of Combined Drug Effects
Cells were treated with serial dilutions of each drug individually or with a fixed ratio of both drugs simultaneously at doses that corresponded to $\frac{1}{2}, \frac{3}{2}, \frac{2}{3}, \frac{1}{2}, 1$, and $1 \frac{1}{2}$ times the respective ED$_{50}$. When the two drugs were administered at a fixed ratio, the dose of the combination required to produce fractional survival ($f$) could be separated into the components ($D_1$ and $D_2$) of drugs 1 and 2, respectively. For each level of cytotoxicity ($f = 0.95, 0.90, \ldots, 0.05$), a variable called the combination index (CI) was calculated using the software CalcuSyn (47) according to the Chou and Talalay equation (9, 48):

$$CI = \frac{(D_1)}{(D_x)} + \frac{(D_2)}{D_x} = 1 \frac{1}{(DRI)_1} + 1 \frac{1}{(DRI)_2}$$

CI < 1 indicates synergy, whereas a CI = 1 shows an additive effect, and CI > 1 indicates antagonism (9, 48). In this equation, the dose reduction index (DRI) defines the extent of drug dose reduction possible in a combination for a given degree of effect compared with the dose of each drug alone (48, 49). When the concentration-effect relationship follows the principle of mass action, the median-effect plot should be linear. Linear correlation coefficients ($r$) were generated for each curve to determine the applicability of the data to this method of analysis. In all synergy experiments, $r$ was >0.9.

Thirty-six hours after treatment, cytotoxicity of the cfPac1-Luc3 and Panc1-Luc3 cell lines were determined by bioluminescence. For cells not genetically engineered to express luciferase, cytotoxicity was analyzed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), as recommended by the manufacturer.

Analysis of Apoptosis
Fourteen hours after treatment, Annexin V binding and caspase-3/7 activity of Panc1 or cfPac1 cells was analyzed with the Annexin V-FITC Apoptosis Detection kit I (BD, Mansfield, MA) or Caspase-Glo 3/7 assay (Promega), respectively, according to the instructions from the manufacturer.

Bioluminescence Imaging
To assess the cancer treatment response, nude mice were anesthetized (ketamine/xylazine 95 and 12 mg/kg i.p., respectively), and K$^+$ D-luciferin (150 mg/kg body weight; BD) was injected i.p. 10 minutes before imaging using a photon counting system (Hamamatsu Photonics, Herrsching am Ammersee, Germany) and HPD-LIS 2.6 software (Hamamatsu Photonics). An integration time of 10 minutes was used for luminescent image acquisition.

Animal Studies
Six- to eight-week-old female athymic nude (BALB/c nu/nu) mice were obtained from Janvier (Le Genest-St-Isle, France). For the i.p. pancreatic cancer model, $1 \times 10^6$ viable cfPac1-Luc3 cells were injected in 1 mL PBS into the peritoneal cavity. Six days later, animals were assigned randomly to groups of eight animals. One animal of each group underwent bioluminescence imaging to confirm peritoneal carcinomatosis at this time point. One day later, therapy was initiated. Animals treated with Ad.H/F vector or Ad.COX•MK, alone or in combination with gemcitabine, received per treatment cycle $2 \times 10^5$ TCID$_{50}$ or GTU determined on cfPac1, respectively. Animals treated with Ad.H/F in combination with Ad.COX•MK received per treatment cycle $1 \times 10^5$ TCID$_{50}$ plus $1 \times 10^7$ GTU (determined on cfPac1) alone or in combination with gemcitabine. All viral vectors were administered i.p. on day 0 and day 2 in 3 mL 4% (w/v) polyethylene glycol (Sigma-Aldrich, St. Louis, MO) in PBS. For the s.c. xenograft model, mice received s.c. $1 \times 10^7$ cfPac1-Luc3 cells in 100 µL into the hind flanks. When the tumors reached a volume of ~200 mm$^3$, animals were randomly assigned to treatment groups ($n = 6$). Viral vectors were given intratumoral at the same dose as in the i.p. model on 3 consecutive days in 100 µL PBS. At least weekly, minimum and maximum perpendicular tumor axes were measured using vernier calipers, and tumor volume was calculated using the formula $l \times w^2 \times 0.5$. The skin thickness of 0.4 mm was subtracted from the measurements.

In both tumor models, gemcitabine (125 mg/kg/d) was given i.p. daily for 5 consecutive days starting on day 4 in 3 mL 0.9% NaCl solution.

Statistical Methods and Median-Effect Analysis
The statistical software package SPSS 13 (SPSS, Inc., Chicago, IL) was used for data analysis with indicated tests. Analysis of combined effects of multiple drugs, described by Chou and Talalay (9), was done with the software CalcuSyn 2.0 (Biosoft, Cambridge, United Kingdom).

Results
Efficient In vitro Transduction of Pancreatic Cancer Cells with Ad5-based Vectors
In this study, we analyzed whether the efficacy of chemotherapy can be enhanced by expression of measles virus FMGs (H and F). Because our strategy relies on the transduction of pancreatic cancer cells by an adenoviral vector to express measles virus FMGs, we first analyzed the transduction efficiency of permanent pancreatic cancer cell lines and of short-term cultures of pancreatic cancer cells with a replication-defective adenoviral vector encoding firefly luciferase (Ad.Luc). Cell monolayers were transduced with a defined amount ($3 \times 10^5$ GTU) of Ad.Luc, resulting in 30% of the maximum obtainable luciferase activity in 293 cells 24 hours posttransduction. The ED$_{50}$ was chosen to minimize double transductions and saturation effects. As shown in Fig. 1, transduction of the established pancreatic cancer cell lines (cfPac1 and Panc1) and the short-term pancreatic cancer cell cultures (PPC#1, PPC#2, and PPC#3) with Ad.Luc resulted in $4.59 \times 10^5 \pm 1.6 \times 10^6, 5.78 \times 10^6 \pm 2.3 \times 10^5, 2.67 \times 10^6 \pm 2.2 \times 10^5, 3.60 \times 10^5 \pm 3.8 \times 10^5$, and $2.17 \times 10^6 \pm 2.8 \times 10^5$ RLU/10$^5$ cells, respectively. By Student’s $t$ test, transduction efficiency of short-term pancreatic cancer cultures with the Ad5-based vector was significantly lower than that of permanent pancreatic cancer cell lines ($P < 0.05$).
Synergism between FMG Expression and Chemotherapy In vitro

Next, we determined in vitro whether the expression of measles virus H and F encoded by a replication-defective adenovirus vector will enhance chemotherapy. To minimize unwanted effects, the expression of H and F was transcriptionally driven by the human telomerase reverse transcriptase gene promoter, which is tumor specific, as previously reported (50, 51). The activity of the human telomerase reverse transcriptase promoter was similar in all tested pancreatic cancer cell lines (data not shown).

The Ad.H/F doses of transduction were adjusted based on the titer determined by GFP for each cell line individually. The ED₅₀ concentrations (fraction of affected cells is 0.5) were determined for each drug (Table 1). For Ad.H/F, the 6:1 ratio of particles to GTU was used to calculate the molarity (6.022 × 10²³ viral particles = 1 mol (45)). This provided the basis for the appropriate ratio of Ad.H/F and chemotherapeutic drugs. The ratio was kept constant when the drugs were studied in combination. The interaction of the drugs was analyzed with the software CalcuSyn (47). The dose-effect curves and combination index plots are shown in Figs. 2 and 3. In the combination index plots, values >1 indicate antagonistic drug effects, whereas those <1 are considered synergistic. A combination index of 1 suggests additive effects of both drugs. The effects of drug combinations on the ED₅₀ concentrations are presented in Table 2. For example, in cfPac1-Luc3 cells treated with Ad.H/F and gemcitabine, the dose of Ad.H/F and gemcitabine needed to achieve the ED₅₀ was reduced 44.32- and 119.77-fold, respectively, when compared with single-agent treatment. More striking results were obtained with Ad.H/F and FOLFOX in Panc1-Luc3 and cfPac1-Luc3. In both tested permanent pancreatic cancer cell lines, the expression of FMG resulted in enhanced cytotoxicity of the used chemotherapeutic drugs (Fig. 3A).

Because permanent cell lines are often in continuous culture for several years and thus likely to have genetic aberrations, we verified our data in short-term cultures of primary pancreatic cancer cells (Figs. 2B and 3B). Two of the three primary cell cultures (PPC#2 and PPC#3) treated with Ad.H/F, in combination with gemcitabine or 5-FU/LV, showed synergy.

Increased Signs of Apoptosis after FMG Expression and Chemotherapy

For this, Panc1 cells were transduced with Ad.F/H at a multiplicity of infection of 5 (determined on Panc1 cells) and incubated with gemcitabine (25 nmol/L). Fourteen hours later, we analyzed early apoptotic events by Annexin V binding and caspase-3/7 activity (Fig. 4B) assays. As shown in Fig. 4A, incubation of the cells with Ad.H/F or gemcitabine resulted in 9.9 ± 0.5% and 6.5 ± 0.4% Annexin V–positive cells, respectively. When the cells were treated with Ad.H/F and gemcitabine, 58.3 ± 1.6% of the cells were Annexin V positive. As shown in Fig. 4B, incubation of the cells with Ad.H/F or gemcitabine resulted in ~1.2-fold increased caspase-3/7 activity when compared with untreated cells. Treatment of the cells with Ad.H/F and gemcitabine resulted in 2.01-fold increased caspase-3/7 activity.

Using plasmid DNA–encoding FMG, qualitatively similar results were obtained (data not shown). Transduction of the cells with Ad.Luc or Ad.H/F in the presence of the synthetic fusion inhibitory peptide (44) had no effect on the binding of Annexin V or caspase-3/7 activity (data not shown).

Fourteen hours postinfection in both apoptosis assays, the addition of Ad.COX-MK to the above-described treatment significantly changed neither Annexin V binding nor caspase-3/7 activity. Similar results were obtained using cfPac1 cells (data not shown).

FMG Expression and/or Transcomplementation Enhance In vivo Transduction

We evaluated, by bioluminescence imaging, whether the i.p. transduction efficiency of tumor cells can be enhanced by transcomplementation of the replication-defective

Table 1. ED₅₀ of single agents

<table>
<thead>
<tr>
<th></th>
<th>cfPac1</th>
<th>Panc1</th>
<th>PPC#1</th>
<th>PPC#2</th>
<th>PPC#3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad.H/F</td>
<td>208.01</td>
<td>91.73</td>
<td>559.29</td>
<td>46.73</td>
<td>559.29</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>7.71</td>
<td>4.42</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>76.96</td>
<td>91.00</td>
<td>&gt;1,000</td>
<td>46.73</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>5-FU + LV</td>
<td>65.12</td>
<td>71.25</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
<td>597.47</td>
</tr>
<tr>
<td>FOLFOX (5-FU/LV + Ox)</td>
<td>8.57</td>
<td>8.98</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE: All concentrations are given in nmol/L; the 6:1 ratio of particles to GTU was used to calculate the molarity (6.022 × 10²³ viral particles = 1 mol) of Ad.H/F (45).

Abbreviation: ND, not determined.
Ad.Luc vector with the replication-restricted Ad.COX•MK (Fig. 5A). On day 7, the luciferase activity Ad.Luc + Ad.COX•MK was 73.7-fold enhanced when compared with Ad.Luc alone. In both sets of experiments, the i.p. administered dose of luciferase encoding vector resulted in vitro in an ED_{50} of luciferase activity in the initial number of injected cfPac1 cells.

Treatment with FMG-Encoding Vector and Chemo-therapy Results in Enhanced In vivo Treatment Efficacy

To determine whether the synergy observed in the in vitro cytotoxicity assays would also result in an enhanced in vivo treatment efficacy, we evaluated this treatment combination in an i.p. and s.c. cfPac1-Luc3 pancreatic xenograft model in nude mice.

Ad.Luc vector with the replication-restricted Ad.COX•MK (Fig. 5A). On day 7, the luciferase activity Ad.Luc + Ad.COX•MK was 73.7-fold enhanced when compared with Ad.Luc alone. In this experiment, the i.p. administered dose of luciferase-encoding vector resulted in vitro in an ED_{50} of luciferase activity in the initial number of injected cfPac1 cells. The ratio of replication-competent to replication-defective was 1:1, as in the transcomplementation of Ad.H/F.

Administration of the Ad.Luc vector with an equal infective dose of Ad.H/F resulted in a 61.2-fold enhanced photon emission when compared with the respective luciferase-encoding vector alone Fig. 5B. In both sets of experiments, the i.p. administered dose of luciferase encoding vector resulted in vitro in an ED_{50} of luciferase activity in the initial number of injected cfPac1 cells.

Figure 2. Dose-effect curves. Permanent pancreatic cancer cell lines cfPac1-Luc3 and Panc1-Luc3 (A) and short-term cultures of pancreatic cancer cells, PPC#1, PPC#2, and PPC#3 cells (B), were exposed in vitro for 36 h to irinotecan (Irin), gemcitabine (Gem), 5-FU/LV (6:1), and FOLFOX (LV/5-FU + Ox; 2:5:8:0.5), alone or in combination with a replication-defective adenoviral vector encoding measles virus H and F (Ad.H/F) at a constant molar ratio. Cytotoxicity was assessed by bioluminescence.

Figure 3. Analysis of combined drug effects. To quantitate the interaction between the treatments, CI values versus cytotoxicity plots were generated for the permanent pancreatic cancer cell lines (A) and short-term cultures of pancreatic cancer cells (B) from the median-effect plots (25). For these curves, CI < 1 defines a synergistic interaction and CI > 1 defines antagonistic drug effects. The straight line at CI = 1 represents the additive effects of both drugs. The CI values for the ED_{50} are indicated.
In a pilot toxicity study, nude mice received one i.p. injection of $2 \times 10^8$ GTU Ad.H/F. One week after treatment, we did not observe clinical signs suggestive of bowel perforation or any other toxicity. When exploratory laparotomy was done, we did not observe peritoneal adhesions or macroscopic signs of hepatitis.

For the i.p. tumor model, animals were treated with the Ad.H/F vector alone or in combination with gemcitabine, and/or the replication-restricted oncolytic adenovirus Ad.COX•MK (Fig. 6A and B). As shown in Table 3, untreated animals had a median survival of 19 days. Animals receiving Ad.H/F or gemcitabine had an improved survival (42 and 31 days, respectively; log-rank test, $P < 0.001$). Administration of Ad.H/F and gemcitabine resulted in a significantly improved survival when compared with single-agent treatment (47 days, $P < 0.001$).

To improve the transduction efficiency of the replication-defective Ad.H/F vector, we transcomplemented this vector with the replication-restricted adenovirus Ad.COX•MK. Administration of this vector alone resulted in an enhanced survival ($P < 0.001$) due to its oncolytic effect, which was further improved by combination with Ad.H/F ($P \leq 0.014$). The best treatment outcome was observed with the triple therapy ($P \leq 0.041$). At the end of the observation period (day 120), three of eight animals treated with this novel triple therapy, consisting of Ad.H/F, gemcitabine, and Ad.COX•MK, were alive and tumor-free. Treatment with Ad.H/F and Ad.COX•MK resulted in one long-term survivor. In all other treatment groups, there were no long-term survivors. The only sign of toxicity we observed was some weight loss in the group of animals that received the triple therapy. However, none of the animals died.

In addition, we evaluated the antineoplastic efficacy of this treatment regimen in a s.c. pancreatic xenograft model. As shown in Fig. 7 and Table 3, on day 28, the median tumor volume of untreated animals was 1,625 mm$^3$. The median tumor volume of animals treated with gemcitabine, Ad.H/F, and Ad.COX•MK alone was 1,347, 1,009, and 1,187 mm$^3$, respectively. When compared with untreated animals, the tumor volume was significantly reduced ($P \leq 0.046$). The combination of Ad.H/F with gemcitabine showed a significantly reduced tumor volume compared with treatment with the individual compounds and untreated animals ($P \leq 0.001$). The median tumor volume of animals treated with the triple therapy was 343 mm$^3$.

**Discussion**

In this study, we analyzed whether the efficacy of chemotherapy can be enhanced by expression of measles virus FMGs (H and F).

Because this strategy relies on the efficient transduction of tumor cells, we analyzed adenoviral transduction...
We were able to show in vitro that short-term cultures of pancreatic cancer cells can be sufficiently transduced with an Ad5-based vector. This is important for the clinical applicability of this strategy because short-term cultures of human melanoma cells for instance are difficult to transduce with an Ad5-based vector in contrast to long-term cultures (52). Yet, the transduction efficiency of short-term cultures of pancreatic cancer cells when compared with established cell lines (cfPac1 and Panc1) was significantly reduced by up to 2.7-fold, respectively.

Furthermore, synergistic cell killing of pancreatic cancer cells expressing the measles virus FMGs, in combination with chemotherapeutic drugs used clinically for the treatment of pancreatic cancer, was observed in vitro over most of the cytotoxic dose range by the median-effect principle described by Chou and Talalay (9). Permanent cell lines are often in continuous culture for several years and thus likely to have genetic aberrations. Additionally, the pancreatic cancer cell lines cfPac1 (53) and Panc1 (54) were established before gemcitabine was available. Thus, the drug resistance pattern differed significantly between the permanent and the short-term pancreatic cell cultures. Nevertheless, we confirmed in two of the three short-term cultures of pancreatic cancer cells synergistic toxicity of cell fusion induced by measles virus FMGs and chemotherapy.

In cells treated with gemcitabine, the Annexin V binding was similar to those expressing FMG. However, when cells were treated with Ad.H/F and gemcitabine, the Annexin V binding rose by 5-fold. One conceivable explanation for the synergistic toxicity observed in vitro is the proapoptotic effect of the measles virus FMGs. This observation might be supported by another study demonstrating that expression of gibbon ape leukemia virus FMGs causes syncytia formation with bioenergetic cell death (55). However, another study using gibbon ape leukemia virus reported a nonapoptotic mechanism of syncytial-mediated cell killing (21).

We assessed in an i.p. and s.c. pancreatic xenograft model, using a constitutively luciferase-expressing human tumor cell line cfPac1-Luc3, whether the in vitro findings could be confirmed in vivo. In the i.p. model, we used survival as the primary therapeutic end point. One day before treatment, bioluminescence imaging was carried out to assure peritoneal carcinomatosis. Despite the moderate sensitivity of the permanent pancreatic cancer cell line cfPac1 to gemcitabine, we used this drug as the chemotherapeutic agent because gemcitabine is used as first-line therapy for patients with advanced pancreatic cancer.

Previously, it has been shown by several groups in s.c. tumor models that the fraction of transduced tumor cells will be greatly enhanced when replication-defective viral vectors will be rendered replication competent in cells.
transduced with viruses or mutants that transcomplement each other for missing gene products pivotal for vector replication and thus allowing in situ vector production and transduction of neighboring tumor cells (12–16). Using bioluminescence imaging, we showed that rendering the replication-defective Ad.Luc vector replication competent by transcomplementation resulted in enhanced i.p. tumor transduction efficiency. This strategy of transcomplementation is important for adenoviral vectors because most of the conditionally replication-competent vectors cannot carry a therapeutic gene due to size constraints. In our Ad.COX•MK vector, the ~6.4 kb FMG expression cassette would have clearly exceeded viral packaging capacity. Thus, we transcomplemented the FMG-encoding vectors with the oncolytic replication-restricted adenovirus Ad.COX•MK. However, because oncolytic adenoviruses can enhance the efficacy of chemotherapeutic agents (20), as also observed in both of our tumor models, this transcomplementation approach might improve chemotherapy by itself independently of the FMG expression. On the other hand, chemotherapeutic agents will also inhibit viral replication (56, 57). Furthermore, without using chemotherapy, several groups reported previously that the intratumoral expression of FMGs enhances the efficacy of virotherapy (24, 58). We were able to confirm these findings in both of our tumor models. However, although the replication-defective Ad.H/F vector can only transduce a small fraction of cells in a given tumor, the administration of Ad.H/F in combination with gemcitabine was significantly more efficacious in both tumor models than treatment with the individual compounds. Thus, we were able to support our in vitro data in the adenoviral system. In the i.p. tumor model, the survival of animals treated with Ad.H/F in combination with the oncolytic adenovirus was significantly more efficacious when compared with animals treated with the individual compounds, confirming previous results (24). When the treatment with Ad.H/F and Ad.COX•MK was combined with gemcitabine, the number of tumor-free, long-term survivors was thrice higher.

Furthermore, we observed by bioluminescence imaging an enhanced photon emission when the Ad.Luc vector was coadministered with the replication-defective Ad.H/F vector. However, due to the syncytia formation, the relationship of photon emission and transduction efficiency might be separated. A conceivable explanation for this might be that 24 hours after transduction, fused cells can produce higher luciferase amounts than single cells. Similarly, this might be true for therapeutic genes. Furthermore, due to the syncytia formation, fewer cells

### Table 3. *In vivo* efficacy study

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>cfPac1-Luc3 xenografts</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I.p.</td>
<td>S.c.</td>
</tr>
<tr>
<td></td>
<td>Mean survival ± SD (d)</td>
<td>Mean tumor volume ± SD (mm³)¹</td>
</tr>
<tr>
<td>Untreated</td>
<td>19.3 ± 0.3</td>
<td>1,665 ± 103</td>
</tr>
<tr>
<td>Ad.H/F</td>
<td>41.4 ± 0.5</td>
<td>998 ± 158</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>30.9 ± 0.4</td>
<td>1,336 ± 77</td>
</tr>
<tr>
<td>Ad.H/F + gemcitabine</td>
<td>46.8 ± 1.3</td>
<td>726 ± 117</td>
</tr>
<tr>
<td>Ad.COX•MK</td>
<td>32.3 ± 0.8</td>
<td>1,208 ± 150</td>
</tr>
<tr>
<td>Ad.COX•MK + Ad.H/F</td>
<td>61.6 ± 2.1</td>
<td>675 ± 81</td>
</tr>
<tr>
<td>Ad.COX•MK + gemcitabine</td>
<td>45.0 ± 0.7</td>
<td>937 ± 119</td>
</tr>
<tr>
<td>Ad.COX•MK + Ad.H/F + gemcitabine</td>
<td>91.3 ± 0.7</td>
<td>372 ± 73</td>
</tr>
</tbody>
</table>

*Animals alive and tumor-free (determined by bioluminescence imaging) at the end of the observation period (120 days) are defined as long-term survivors.

¹At day 28.

**Figure 7.** S.c. pancreatic cancer tumor model. cfPac1-Luc3 xenografts were grown s.c. to volumes of ~200 mm³. Groups of mice (n = 6) were treated with a replication-defective adenovirus encoding measles virus H and F, gemcitabine, and a replication-restricted oncolytic adenovirus Ad.COX•MK alone or in combination. Tumors were measured at day 28. **Points,** tumors. **Horizontal lines,** median values.
within the tumor have to be genetically modified. This is an extension of previous findings demonstrating enhanced viral spread and oncolytic efficacy when FMGs are expressed (24, 58).

Thus, the significantly improved therapeutic outcome of animals receiving the triple therapy was attributed to multiple factors, including, most likely, improved FMG expression throughout the tumor, enhanced sensitivity of the tumor cells to gemicitabine by adenoviral gene products, and FMG expression. Qualitatively similar results were obtained in a s.c. pancreatic xenograft model.

In summary, intratumoral expression of measles virus FMGs can enhance the efficacy of chemotherapy. This effect can be enhanced by the multimodal process of combination with oncolytic viruses. Because it has been previously reported that fusogenic membrane proteins can enhance the immunogenicity of tumor cells (21), this approach might be even more effective in an immuno-competent host.

Acknowledgments

We thank Klaus Uberla for creating an inspiring environment and providing constant support; Andris Filling (Klinik für Allgemeine und Transplantationschirurgie, Essen, Germany); Cathrin Walter (Innere Klinik-Tumorforschung, Universitätshôpital, Essen, Germany) and Wibeke Bayer for critical review of the manuscript; and Matthias Dobbelstein and German P. Horn for helpful discussions and advice.

References


44. Richardson CD, Scheid A, Choppin PW. Specific inhibition of paramyxovirus and myxovirus replication by oligopeptides with amino acid sequences similar to those at the N-termini of the F1 or HA2 viral polypeptides. Virology 1980;105:205–22.


Enhanced killing of pancreatic cancer cells by expression of fusogenic membrane glycoproteins in combination with chemotherapy

Dennis Hoffmann and Oliver Wildner