Experimental treatment of ovarian cancers by adenovirus vectors combining receptor targeting and selective expression of tumor necrosis factor

SELVA R. MURUGESAN1, MASAKI AKIYAMA2, DAVID A. EINFELD1, THOMAS J. WICKHAM1,3 and C. RICHTER KING1

1GenVec Inc, 65 West Watkins Mill Rd, Gaithersburg, MD 20878, USA; 2FUSO Pharmaceutical Industries, Ltd., 2-3-30 Morinomiya, Joto-ku, Osaka, 536-8523, Japan

Received May 8, 2007; Accepted June 25, 2007

Abstract. Ovarian cancer is the fourth most common cancer among women and existing treatment is not routinely curative. One new strategy for cancer therapy is the selective delivery of TNFα to tumors via adenovirus vectors. We have tested the combination of two modifications to adenovirus vectors designed to limit delivery to tumors, capsid modification and expression control. To target \( \alpha_v\beta_3/\beta_5 \) integrin receptors that are highly expressed in tumor and sparsely expressed in the epithelial layer of peritoneum, we modified the capsid fiber and penton base to remove native receptor binding and incorporated an RGD-4C motif in the fiber knob (Ad.PB*F*RGD). This vector exhibits effective gene transfer in all of the \( \alpha_v\beta_3/\beta_5 \)-positive ovarian cancer cells tested in vitro and in vivo. Importantly, the Ad.PB*F*RGD vector is able to transduce ovarian tumor nodules and avoid infecting the normal mesothelial cells that line the intraperitoneal space following intraperitoneal administration. To further increase selectivity, different promoters were incorporated into the capsid-modified vector to confer the expression of the hTNFα therapeutic gene. We analyzed both constitutive (CMV or RSV) and potentially tumor selective promoters (MUC-1, E2F or hTERT) in terms of efficacy, selectivity and safety, TNF-expressing Ad.PB*F*RGD vectors containing the MUC-1 promoter showed anti-tumor activity in two ovarian cancer xenograft models (Caov3 and Igr-ov1) with little evidence of toxicity or systemic TNF. The data indicate that combination of capsid modification and transcriptional regulation of expression is a promising strategy for development of a new ovarian cancer treatment.

Introduction

There is a compelling need for new approaches to the treatment of ovarian cancer. It is estimated that 22,430 new cases of ovarian cancer will be diagnosed and 15,280 deaths from this cancer will occur in 2007 in the United States of America (1). There have been many improvements in the treatment of newly diagnosed ovarian cancer, however, most patients still recur and die of the disease. Improvement of the treatment of ovarian cancer will require efficacy against a disease that is disseminated within the peritoneal cavity. Metastatic spread outside the peritoneum is uncommon. Treatment of ovarian cancer involves surgical removal of intraperitoneal tumor masses (2) and chemotherapy to diminish disseminated cancer. Complete elimination of detectable tumor occurs in ~60% of patients (3) and five-year survival rates of approximately 50% have been reported (4). Unfortunately, these treatments do not routinely remove all occult disease as relapse from ovarian cancer is very common, >75%, and primary disease failure nearly always occurs within the peritoneal cavity (5). Treatment of relapsed disease can involve surgery and chemotherapy. Chemotherapy for both primary and relapsed disease has been delivered either systemically or into the peritoneal cavity. Experimental therapies for ovarian cancer have the common goal of decreasing disease burden in the peritoneal space and include the addition of new cytotoxic agents, immune stimulation agents, and antibody based treatments (6). The confinement of ovarian cancer to the peritoneal space suggests that adenovirus vectors could provide a new treatment approach (7,8). Delivery to the peritoneal space places the vector at high concentrations in close proximity to the target disease. As a result, approaches utilizing adenovirus vectors have undergone clinical testing as treatments for ovarian cancer (5,9). The most advanced clinical studies involved vectors containing an expression cassette for the normal p53 gene (5,10). Delivery of p53 protein to cancer cells having mutated p53 was expected to allow ovarian cancer cells to respond to apoptotic stimuli. Vectors that express a single chain antibody to erbB2 (11), a herpes virus TK gene (12,13), the mda-7 gene
Since systemic delivery of recombinant TNF protein results in and necrotic to tumor cells as well as causing destruction of tumor vasculature and activation of immune responses (16). Since systemic delivery of recombinant TNF protein results in significant dose limiting toxicities caused by vascular leakage (17), the only clinical application of recombinant TNF protein involves isolated limb perfusion for treatment of soft tissue sarcoma (18). The clinical activity of recombinant TNFα protein supports the concept of delivering a gene for TNF to tumors where it can be expressed and secreted causing the needed bystander effect without leading to significant systemic exposure to TNF. Such a strategy would require that expression be confined to the site of disease to limit systemic toxicities. A simple approach to confining TNF exposure is to directly inject into tumors an adenovirus vector containing the TNFα under a radiation inducible promoter (19,20). Phase I testing of this agent indicates that direct injection into tumors was well tolerated with little evidence of systemic exposure to TNF. Partial or complete responses were seen in tumors derived from the breast, colon, pancreas, melanoma, rectum, lung and soft tissue sarcoma (21). In phase II testing in pancreatic cancer, at the maximum tolerated dose, approximately 60% of patients saw tumor shrinkage, improved survival, longer times of progression free survival and lower tumor markers. A further conclusion derived for preclinical and clinical testing of adenovirus vector expressing TNFα is that anti-tumor responses are mediated by only a relatively small number of cells transduced in the tumor. Studies in animal models indicate that only ≤5% of cells contain an active gene following direct transduction in the tumor. TNF is directly apoptotic to tumor cells as well as causing destruction of tumor vasculature and activation of immune responses (16). Since systemic delivery of recombinant TNF protein results in significant dose limiting toxicities caused by vascular leakage (17), the only clinical application of recombinant TNF protein involves isolated limb perfusion for treatment of soft tissue sarcoma (18). The clinical activity of recombinant TNFα protein supports the concept of delivering a gene for TNF to tumors where it can be expressed and secreted causing the needed bystander effect without leading to significant systemic exposure to TNF. Such a strategy would require that expression be confined to the site of disease to limit systemic toxicities. A simple approach to confining TNF exposure is to directly inject into tumors an adenovirus vector containing the TNFα under a radiation inducible promoter (19,20). Phase I testing of this agent indicates that direct injection into tumors was well tolerated with little evidence of systemic exposure to TNF. Partial or complete responses were seen in tumors derived from the breast, colon, pancreas, melanoma, rectum, lung and soft tissue sarcoma (21). In phase II testing in pancreatic cancer, at the maximum tolerated dose, approximately 60% of patients saw tumor shrinkage, improved survival, longer times of progression free survival and lower tumor markers. A further conclusion derived for preclinical and clinical testing of adenovirus vector expressing TNFα is that anti-tumor responses are mediated by only a relatively small number of cells transduced in the tumor. Studies in animal models indicate that only ≤5% of cells contain an active gene following direct intra-tumoral injection (manuscript in preparation). These results suggest that the secreted nature of the TNF combined with TNF’s breadth of biological activities might provide the increases in potency required for application in ovarian cancer. However, the systemic toxicity of TNFα is likely to require that expression must be confined to the site of disease.

Improvements in selectivity of adenovirus vectors can be accomplished by manipulating the capsid proteins that bind to cellular receptors and facilitate cell entry (8,22). The primary receptor for adenovirus is the coxsackie adenovirus receptor (CAR) (23). CAR binding can be ablated through mutations of the fiber knob (24,25). Secondary interactions occur through the penton base through binding to integrins (26) that can be ablated through removal of an RGD sequence in the penton (27). In order to target ovarian cancer cells we incorporated into the vector capsid a short peptide motif that binds a specific set of cell surface proteins, \( \alpha_5\beta_3 \) integrins, that are frequently expressed in ovarian and other cancers (28,29).

A second approach to improving selectivity is to control gene expression through the choice of promoter (7,8,30). Promoters can confer tumor selectivity according to tissue type (e.g. MUC-1), through cell cycle dependency (e.g. E2F), or by favoring immortalized cells (e.g. TERT). Expression of MUC-1 is high in many adenocarcinomas including those derived from the breast, stomach, colon and ovary (31-33). Prevalence of expression of MUC-1 in ovarian cancers has been estimated as >90% (34). E2F is elevated in ovarian (35) and brain (36) cancers. Likewise, hTERT is expressed in most human cancers (37) but inactive in normal somatic cells. The application of tumor selective promoters in adenovirus vectors has been used to generate replication-selective adenoviruses (38-40).

In the current study we explored the combination of capsid modification and promoter selectivity for delivery of TNFα in an animal model of ovarian cancer. Our results indicate that such vectors can induce anti-tumor responses at doses where there is limited expression of TNF in normal tissue and no evidence of toxicity. We conclude that this combination of targeting strategies is a promising approach to the generation of new ovarian cancer treatments.

### Materials and methods

#### Animals

Six week-old, immunocompetent female BALB/c or immunodeficient athymic nude, nu/nu, mice weighing 19-22 g were purchased from Harlan, USA, and were acclimated for 1 week, while caged in groups of five. The animals were housed in micro-isolator cages; each individually ventilated with HEPA supply and HEPA exhaust and maintained under conventional conditions. The animals were provided with a diet of regular global rodent chow (2018), purchased from Harlan, Teklad and sterilized or autoclaved water ad libitum throughout the experiment. The GenVec animal facility operates in accordance with the NIH Guide for the Care and Use of Laboratory Animals and complies with the PHS (Public Health Service) Policy on Humane Care and Use of Laboratory Animals. All of the cancer experimental procedures were conducted according to GLPs (Good Laboratory Practices) and were in compliance with the NIH guidelines and PHS policy for the care and welfare of animals.

#### Cell culture

Human ovarian cancer cells, A2780, Igr-ov1, Ovarc-4, Ovarc-5 and Ovarc-8 were obtained from NCI, Frederick, MD. The PA-1, ES2, Ovarc3 and Caov3 ovarian cancer cell lines were obtained from American Type Culture Collection, Manassas, VA. These cell lines were passaged 3-4 times before being used in experiments. A2780, Igr-ov1, Ovarc-3, Ovarc-4, Ovarc-5 and Ovarc-8 were cultured in RPMI-1640 (Cambrex, Walkersville, MD), whereas PA-1, ES2, and Caov3 were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 2 mM glutamine and antibiotics (100 μg/ml of streptomycin, and 100 U/ml of penicillin). All the cultures were maintained at 37°C in a 5% CO₂ atmosphere.

#### Construction of recombinant adenovirus vectors

All the vectors described in this study are based on Ad5 with deletions in the E1, E3 and E4 regions. The deletion in E3 comprises the native XbaI fragment while the E4 region has been replaced with a transcriptionally inert spacer (41,42). The AdL and AdGFP vectors express luciferase and GFP, using the CMV promoter and the SV40 early polyA sequence, with the expression cassette replacing nucleotides 356-3327 of the E1

---

Promoters can confer tumor selectivity according to tissue type (e.g. MUC-1), through cell cycle dependency (e.g. E2F), or by favoring immortalized cells (e.g. TERT). Expression of MUC-1 is high in many adenocarcinomas including those derived from the breast, stomach, colon and ovary (31-33). Prevalence of expression of MUC-1 in ovarian cancers has been estimated as >90% (34). E2F is elevated in ovarian (35) and brain (36) cancers. Likewise, hTERT is expressed in most human cancers (37) but inactive in normal somatic cells. The application of tumor selective promoters in adenovirus vectors has been used to generate replication-selective adenoviruses (38-40).

In the current study we explored the combination of capsid modification and promoter selectivity for delivery of TNFα in an animal model of ovarian cancer. Our results indicate that such vectors can induce anti-tumor responses at doses where there is limited expression of TNF in normal tissue and no evidence of toxicity. We conclude that this combination of targeting strategies is a promising approach to the generation of new ovarian cancer treatments.
region and oriented with transcription directed toward the left end of the vector genome. The CMV promoter consists of nucleotides (-583) to (+167) relative to the transcription start site (Accession number X03922) and has a downstream splice acceptor prior to the transgene. The TNF vectors carry the gene for human TNFα (42) and differ from AdL and AdGFP in having an E1 deletion that extends from 356-3510 of Ad5.

Alternative promoters were inserted upstream of TNF by replacing nucleotides (-583) to (+74) of the CMV promoter, using the native SpeI and EagI sites at these positions in the E1-replacement shuttle plasmid. The human telomerase reverse transcriptase (TERT) and E2F promoters were generated by PCR amplification of genomic DNA from ARPE-19 cells (ATCC, CRL-2302). The primers ACTAGTCCCTCAGGACC GGCGTCGCCAC and CCGCGCGGGCCAGGGTTC were used to amplify nucleotides (-182) to (+68), relative to the transcription start site of TERT (AF516106) with flanking SpeI and EagI sites (added nucleotides underlined). Nucleotides (-219) to (+57) of the E2F promoter (AF516106) were amplified with flanking SpeI and NotI sites using the primers ACTAGTCCCTCAGGACC GGCGTCGCCAC and CCGCGCGGGCCAGGGTTC. PCR amplification of the MUC-1 promoter sequence flanked by SpeI and XhoI sites has been described previously (39). The SpeI-XhoI(blunt) fragment, carrying nucleotides (-690) to (+38) of the MUC-1 promoter (AL713999) was isolated and ligated into the SpeI-EagI(blunt) shuttle plasmid. The SpeI-EagI(blunt) shuttle was also used for insertion of the RSV promoter as an SpeI-EcoRV fragment. The RSV fragment consists of nucleotides (-340) to (+34) relative to the transcription start site of the promoter (L291299) followed by a 63-base spacer derived from the RSV expression plasmid.

The AdL.PB*F*HA vector has the penton base sequence HAIRGDTF replaced with TSYPYPDVPDYASS and an insertion of SRGFKSGYDPYDPYDA between Gly504 and Asp504 of the fiber protein (H1 loop) as described previously (43). In addition, the fiber knob has the mutations S408E, A415G, E416G and K417G to disable CAR binding. Vectors carrying an RGD motif in fiber have an insertion of CDCRGDCFC in the HI loop of the fiber knob together with the above CAR binding mutations. The GFP, luciferase, and TNF expressing RGD vectors do not have an HA epitope in the penton base, but only the SpeI linker that replaces HAIRGDTF with TS.

All vectors were expanded using 293-orf6 cells as previously described (43). Vectors were purified using 3 successive CsCl gradient centrifugation runs and dialyzed into formulation buffer. Purity and activity were monitored using standard methods (21). All preparations were active showing pu/FFU ratios <32.

In vitro gene transfer assays. Established human ovarian cancers such as PA-1, Ovcar-5, Caov3, Igr-ov1 and A2780 cells were cultured in 24-well plates (1x105 cells/well) at 37°C. These cell lines were infected with AdL (native tropism), AdL.PB*F* (CAR and integrin binding ablated) or AdL.PB*F*RGD (CAR and integrin binding ablated plus RGD-4C motif inserted in the fiber) vectors at 1x109 pu/well for 1 h. Following 1 h incubation at 37°C, unbound virus in each well was removed, washed and replaced with 1 ml complete medium (DMEM or RPMI, 10% FBS, 10 mM HEPES). After the cells were incubated overnight in the incubator at 37°C, 5% CO2, the cells were lysed with cell culture lysis buffer (Promega, Madison, WI) and the lysate was analysed using a luciferase assay system (Promega Corp, Madison, WI). The luciferase activity was measured in a TR717 multichannel luminesimeter (Applied Biosystems, Bedford, MA) and expressed as RLU (relative light units)/20 μl lysate. All of the experiments were carried out in triplicate.

In vivo fluorescence microscopy. Human ovarian cancer Caov3 or Igr-ov1 cells (1x107 in 500 μl serum free medium) were injected into the intraperitoneal cavity of athymic nude mice. After the tumor cells formed well-defined nodules on the surface of the various organs in the abdominal cavity, the vector administration was initiated. The GFP gene (controlled by CMV promoter) containing AdGFP (1x1011 pu, GFP marker), and AdGFP.PB*F*RGD (1x1010 pu) were injected into the intraperitoneal space of tumor bearing mice. Following 48-h vector injection, mice were sacrificed. The abdominal cavity of each mouse was carefully opened and examined for tumor nodules. Then, the GFP expression in tumor, kidney, liver, lung, spleen, peritoneal membrane, mesentery and diaphragm was detected using a Leica (MZFLIII) fluorescence microscope with a separate filter for GFP. The images of GFP activity in each group were documented by a Spot RT digital camera (Diagnostic Instruments, Inc.).

In vivo measurement of luciferase marker expression. For the in vivo luciferase expression study, 2x1010 pu of either AdL or AdL.PB*F*RGD in 500 μl volume was administered to the peritoneal cavity of Caov3 tumor bearing mice. One day following administration the animals were sacrificed and the tumor nodules, liver, spleen, kidneys, peritoneum and lungs were snap-frozen in liquid nitrogen. The frozen tissue was ground and a portion was lysed in cell culture lysis reagent (Promega). Luciferase activity in the supernatant was determined using a Leica (MZFLIII) fluorescence microscope with a separate filter for GFP. The images of GFP activity in each group were documented by a Spot RT digital camera (Diagnostic Instruments, Inc.).

In vitro quantitation of TNFα. Human ovarian carcinoma (PA-1, Caov3, and Igr-ov1) cell lines were seeded in 48-well plates (1x105 cells/well). The cells were infected with vectors, AdCMV.TNF.PB*F*RGD, AdRSV.TNF.PB*F*RGD, AdE2F.TNF.PB*F*RGD, AdMUC-1.TNF.PB*F*RGD and AdHTERT.TNF.PB*F*RGD at 1x109 pu/well for 24 h at 37°C, 5% CO2. The control wells received medium only. Then, the cells were lysed using cell culture lysis reagent (1x) (Promega) for 30 min at room temperature followed by a freeze-thaw cycle. The hTNFα concentrations were determined using a hTNFα ELISA kit (Pierce, Rockford, IL) with a level of detection of <2 pg/ml.

Development and treatment of peritoneal tumor model of human ovarian cancer. Tumor cells, Caov3 or Igr-ov1, (1x107 cells) in a volume of 500 μl serum-free medium containing 10 mM HEPES were introduced into the peritoneal cavity of female athymic nude mice (8-10 weeks). Three mice were sacrificed 4 or 5 days after cell inoculation and a
was performed by two sample independent t-test. *Statistically significant (RLU) per 20 μl lysate. Each bar represents the mean value of triplicates and for 1 h. Luciferase activity was measured and reported as relative light units.

The serum was separated and stored at -80˚C until further analysis. The hTNFα concentrations in the serum and peritoneal lavage were measured by ELISA using a Quantikine hTNFα enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) with a level of detection of <4.4 pg/ml.

**Statistics.** Data are presented as mean values ± standard deviation. Statistical analyses were performed with Origin 7.5 software (Origin Laboratories, Northampton, MA). A Shapiro-Wilk normality test was performed to determine whether the samples followed a normal distribution. For normally distributed data, the means of treatment groups were compared by one-way ANOVA for more than two groups. Tukey multiple comparisons were used to specify which groups differ at the predetermined p<0.05. Two-sample independent t-test was also employed to compare means between two groups. If the data were not normally distributed, the Wilcoxon signed-rank test was performed. In all cases, p-values of <0.05 were considered significant.

**Results.**

**Targeting adenovirus vectors using RGD-4C.** Adenovirus vectors containing mutations within the fiber knob and penton base to prevent binding to native receptors have been previously reported (43). Using such doubly ablated vectors as a base, we introduced the peptide CDCRGDCFC (RGD-4C) into the HI loop of the fiber knob to alter the primary receptor specificity to αvß3/5 integrins. To determine if the RGD-4C ligand permits vector transduction of established human ovarian cancer cells, we tested a panel of cell lines (PA-1, Caov3, Igr-ov1, A2780 and Ovarc-5). Fig. 1 demonstrates that the AdL vectors, possessing wild-type receptor binding, easily transduce all cell lines. Doubly ablated vectors (AdL.PB*F*) lacking native receptor binding have significantly less ability to transduce these cells as measured by luciferase activity. This vector was shown to be fully functional for entry into cells through testing on a cell line containing an artificial receptor capable of binding the fiber knob. Addition of the RGD-4C motif into the doubly ablated vector (AdL.PB*F*RGD) restored the ability significantly (p<0.05) to transduce ovarian cancer cell lines (Fig. 1).

**Intraperitoneal models of human ovarian cancer.** Human ovarian cancer forms extensive solid tumor carcinomatosis in the peritoneal space. To model this disease we examined the timing and morphology of tumor growth by human cell lines in nude mice. PA-1, Ovarc3, Caov3, SW626, A2780, Igr-ov1, Ovarc4, Ovarc-5 and Ovarc8, were assessed in nude mice on day 4, 8 and 15 after tumor cell inoculation (1x10⁶ cells/ 500 μl). Solid tumor nodules were found on the surface of the peritoneum, intestine, liver, diaphragm, pancreas, omentum and mesentry in each mouse (data not shown). Ascites was not found in any animals during the observation period. The PA-1, Caov3 and Igr-ov1 developed many fine nodules (~2 mm) between 4 and 8 days after tumor cell inoculation. The cell lines Igr-ov1 and Ovarc-5 grew very quickly with 4- to 12-mm tumor nodules at 15 days post implant. With all the tumor cell lines, no metastases were detected in organs outside the peritoneal cavity. Based on this observation we...
selected one slower growing tumor line Caov3 and one fast growing line Igr-ov1 for further testing. These results indicated that established tumor nodules of approximately 2 mm should be present at 4 or 5 days post tumor cell inoculation.

Selective gene delivery by RGD-4C Ad vectors in the peritoneal cavity. Previously, we demonstrated that the capsid modified vectors, via ablation of both CAR and integrin binding, dramatically reduced gene transfer to normal tissues after IP injection (44). Here, we tested the effect of adenovirus capsid modifications on gene transfer to ovarian tumor nodules and normal tissues in vivo. Vectors with wild-type tropism (AdGFP) and vectors without native tropism but containing RGD-4C in the fiber knob (AdGFP.PB*F*RGD) were compared. All contained the same marker gene expression cassette in which the green fluorescent protein (GFP) coding sequence is placed under the direction of the CMV promoter. Tumor nodules were established in the ip space of nude mice by injecting Caov3 or Igr-ov1 cell lines. Following single ip injection of wild-type vector, AdGFP, expression was detected in the tumor nodules, peritoneal wall, liver (Fig. 2), mesentery, lung, kidney, spleen and diaphragm (data not shown). Importantly, the AdGFP.PB*F*RGD vector transduced tumor nodules but reduced GFP activity was detected in normal tissue. To determine the levels of reporter gene expression, vectors encoding the luciferase reporter gene were administered to Caov3 tumor-bearing mice and assayed for luciferase activity in the tumor and abdominal organs (Fig. 3). As above, a vector with wild-type tropism (AdL) was compared with the vector that lacked native receptor binding but contained RGD-4C in the fiber knob (AdL.PB*F*RGD). The modified
vector showed significantly reduced transductions of liver (P<0.05), kidney and peritoneum (P<0.005). Levels of gene expression were similar in the target tumor. When the transduction was normalized to the level of tumor transduction, the AdL.PB*F*RGD vector gave reductions relative to AdL of 57%, 78% and 78% for liver, kidney and peritoneum respectively. These data indicated that the RGD-4C vector achieved selective gene delivery to tumor nodules in vivo by retaining the interaction with tumor cells while reducing the transduction to some normal abdominal organs.

As a consequence of these promising improvements in the selectivity for ip administration we focused all additional analysis on vectors containing both ablations in the native adenovirus receptor interactions and the RGD-4C targeting ligand.

**Table I. Comparison of CMV, RSV, E2F, MUC-1 and hTERT promoter activity in established human ovarian cancer cell lines.**

<table>
<thead>
<tr>
<th>Promoters</th>
<th>CAOV3</th>
<th>IGR-OV1</th>
<th>PA-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV*</td>
<td>24000±2200</td>
<td>25600±1000</td>
<td>1100±30</td>
</tr>
<tr>
<td>RSV</td>
<td>40±5</td>
<td>500±80</td>
<td>100±3</td>
</tr>
<tr>
<td>E2F</td>
<td>60±1</td>
<td>270±10</td>
<td>80±4</td>
</tr>
<tr>
<td>MUC-1</td>
<td>80±10</td>
<td>150±10</td>
<td>0±0</td>
</tr>
<tr>
<td>hTERT</td>
<td>9±7</td>
<td>300±10</td>
<td>10±1</td>
</tr>
</tbody>
</table>

The ovarian cancer cell lines, PA-1, Caov3, and Igr-ov1 were cultured in 48-well plates (1x10^5 cells/well). The cells were infected with RGD targeted vectors with hTNFα under the control of the indicated promoters at 1x10^9 pu/well. Following 24-h incubation, the hTNFα expression was determined. Results shown are mean values (pg/ml) of duplicate infections a standard deviation. All the promoters appear to be positive in all the ovarian cancer cell lines. Of note, CMV exhibited significantly higher activity than other promoters (p<0.05). PA-1 lacks MUC-1 promoter activity. *p<0.05 versus all other promoters (one-way ANOVA).

**Table II. Evaluation of the tolerable dose of RGD-targeted adenovirus vectors that drive TNF protein under the control of a viral or tumor-specific promoter.**

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Dose</th>
<th>Mortality (%)</th>
<th>Body weight loss (%)</th>
<th>Tolerable dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFB</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>AdCMV.TNF.PB<em>F</em>RGD</td>
<td>3.00E+10</td>
<td>0</td>
<td>12.7±1.3</td>
<td>3.00E+10</td>
</tr>
<tr>
<td></td>
<td>6.00E+10</td>
<td>0</td>
<td>17.2±3.1</td>
<td></td>
</tr>
<tr>
<td>AdRSV.TNF.PB<em>F</em>RGD</td>
<td>6.00E+10</td>
<td>0</td>
<td>0</td>
<td>1.20E+11</td>
</tr>
<tr>
<td></td>
<td>1.20E+11</td>
<td>0</td>
<td>8.4±1.6</td>
<td></td>
</tr>
<tr>
<td>AdE2F.TNF.PB<em>F</em>RGD</td>
<td>6.00E+10</td>
<td>0</td>
<td>0</td>
<td>1.20E+11</td>
</tr>
<tr>
<td></td>
<td>1.20E+11</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AdMUC1.TNF.PB<em>F</em>RGD</td>
<td>6.00E+10</td>
<td>0</td>
<td>0</td>
<td>1.20E+11</td>
</tr>
<tr>
<td></td>
<td>1.20E+11</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AdhTERT.TNF.PB<em>F</em>RGD</td>
<td>6.00E+10</td>
<td>0</td>
<td>0</td>
<td>1.20E+11</td>
</tr>
<tr>
<td></td>
<td>1.20E+11</td>
<td>0</td>
<td>3.6±2.3</td>
<td></td>
</tr>
</tbody>
</table>

Naïve Balb/c mice (n=5) were given a single ip injection of vector at the indicated dose. Body weight loss at day two and mortality were noted. Body weight loss is listed as the mean percentage of loss ± the standard deviation. Tolerable dose was selected as the highest dose that caused no mortality and <20% weight loss. FFB, final formulation buffer; NA, not applicable.

**RGD-4C Ad vectors that provide selective expression of TNFα.** We have previously reported that vectors containing deletions in the wild-type receptor interactions can exit the peritoneal cavity as evidenced by trafficking to the liver and other organs (44). Similarly, the RGD-4C targeted vector is detected in samples of spleen and lung following administration into the peritoneal space (Fig. 3). Consequently, systemic exposure to vectors cannot be ruled out. This indicates that other mechanisms to provide tumor selectivity may be desirable. We tested different promoters driving the expression of TNFα as possible strategies to increase the selectivity of gene product delivery. We created vectors that contain the CMV, RSV, E2F, hTERT, and MUC-1 promoters configured to drive expression of TNFα. We selected these promoters as they have been associated with rapidly proliferating cells (E2F), immortalized cells (hTERT) and with upregulation in adenocarcinoma (MUC-1). Other elements of the genome of the adenoviral vector genome remain identical between the vectors including the E3 and E4 deletion.

We assessed the expression of TNFα using established human ovarian cancer cell lines (PA-1, Caov3, and Igr-ov1). The results demonstrate that among the five promoters examined, the CMV promoter is the strongest for TNFα expression in all the ovarian cancer cells (Table I). The tumor selective promoters, MUC-1, hTERT and E2F induce lower expression comparable to the RSV promoter. For the PA-1 tumor cell line no TNFα expression was detectable following the infection of the MUC-1 driven vector. This is consistent with the reported adenocarcinoma selectivity of the MUC-1 promoter.
promoter as PA-1 is derived from a teratocarcinoma showing no MUC-1 expression (39).

**Efficacy of RGD-4C Ad vectors.** To conduct efficacy testing, we first established a dose that could be safely administered, causing <20% body weight loss and no evident morbidity (Table II). The maximum dose of CMV-driven vector was 3x10^10 pu/20 g body weight (BW). All the other vectors caused no significant body weight loss up to 1.2x10^11 pu/20 g BW.

Efficacy testing was conducted in two intraperitoneal tumor models of human ovarian cancer (Caov3 and Igr-ov1) in athymic nude mice. We initiated treatment under conditions where established tumor nodules were known to be present. We first screened all vector constructs for activity using the Caov3 cell line. Treatment was initiated five days after tumor cell inoculation, and a total of three injections were administered over seven days. The animals' behavior and body weights were monitored daily for any sign of morbidity. Thirty days following treatment and in a manner where the investigator was blinded to the treatment group, tumors were harvested and weighed. Fig. 4 illustrates the size of tumor nodules following treatment with CMV, RSV, MUC-1, E2F and hTERT. Vectors containing the CMV, RSV and MUC-1 promoters showed significant reductions (p<0.05) in tumor burden compared to mice treated with control media. E2F and hTERT showed no statistically significant anti-tumor effect. The size of tumor nodules in animals that received vector with MUC-1 or RSV promoter was significantly reduced. Six out of ten mice were tumor free following treatment with vector that contains hTNFα under the control of MUC-1.

Similar dosing scheme as used above, three times 1.2x10^11 pu/20 g over one week. Both MUC-1 and RSV vectors displayed reduced tumor burden compared to control mice (Fig. 5).

**RGD-4C Ad vectors can reduce systemic exposure to TNFα.** We measured the level of TNFα protein in the serum and intraperitoneal fluid lavage of non-tumor bearing mice following a single ip injection at the tolerable dose (Fig. 6). Vectors containing the CMV and RSV promoters resulted in TNFα in both serum and ip fluid. TNFα induced by the CMV promoter was transient with a peak level at 24 h after administration and decreased to non-detectable by 4 days. Expression from the RSV promoter was lower than CMV at 24 h, but unlike CMV, detectable expression persisted for at
least 7 days. Low levels of TNFα were detected only at 24 h in ip lavage fluid following administration of vector containing the MUC-1 promoter. Using this vector TNFα levels were not detected in serum at any time point. Body weights of the animals were monitored as a measurement of TNFα-associated toxicity. A significant drop in body weight of >8% was noticed at day 1 after CMV, or RSV vector application (p<0.05). Importantly, body weight loss was <3% with MUC-1. For CMV and RSV containing vectors, the observed elevations in serum or ip TNF levels correlate well with body weight loss. These results suggest that the MUC-1 promoter accounts for the reduced expression of TNFα in non-target tissues compared to the non-regulated viral promoters and that this may improve tolerability.

Discussion

Our results indicate that the improved design of adenovirus vectors for ovarian cancer treatment is possible. The results extend preclinical and clinical evidence that delivery of TNFα using adenovirus vectors may have anti-tumor activity. As a secreted molecule, TNFα can be expected to supply a strong bystander effect. We believe that this bystander effect of TNFα is necessary for the antitumor activity observed in this study of ovarian cancer. This view is supported by our results using targeted vectors containing the marker gene GFP, clearly indicating heterogeneity of transduction within tumors. The results in the present study support the conclusion that delivery of TNFα using adenovirus vectors causes only anti-tumor effects. There are reports that indicate that TNFα can play a tumor promotion role (45-47). However, we have not seen any evidence of increased tumor burden following delivery of any TNF expressing vector at any dose level.

In this study we extend our previous results and those of other groups showing that alteration in the virus capsid can produce improvements in selective transduction of target cells (43,48,49). The RGD-4C ligand used in this study has been reported to bind to αvβ3 and αvβ5 integrins (48,49). The frequency of αvβ3 and αvβ5 integrins in ovarian cancers has been estimated to be 62% and 65% respectively (28,50). Our results showing efficient transduction of ovarian cancer cell lines by RGD-4C vectors are consistent with these findings. We have previously reported that receptor-ablated vectors show reduced transduction of non-target tissue of the peritoneal space (44). In the present study similar results were obtained with vectors having both native receptor ablation and containing the RGD-4C ligand in the fiber knob. The RGD-4C adenovectors show clear advantages for intraperitoneal delivery over adenovector with wild-type receptor binding. Of particular interest was the limited delivery of RGD-4C targeted vector to the peritoneal wall since intraperitoneal administration will clearly bring vectors in contact with the thin surface membrane composed of mesothelial cells. A study of human biopsy samples of the mesothelial membrane (51) shows that α3 subunits were not detected in the mesothelial layer that surrounds the peritoneal space. Some integrins (α2, α3 and α6) were expressed while others (α1, α4, α5 and α6) were not observed (51). We interpret this study to indicate that our vectors containing the RGD-4C ligand may have limited targets on the normal lining of the peritoneal cavity in humans. We recognize that other studies of cultured peritoneal mesothelial cells and ascites-derived cells show αvβ3 and αvβ5 expression (29,52). This has led others to predict that the RGD-4C adenovirus vectors will have limited application in the peritoneal cavity.

Our most encouraging results were obtained using the MUC-1 (DF3) promoter. The MUC-1 promoter may have particular advantages for ovarian cancer application as it can be upregulated in this disease (33,34). Tai et al (33) also studied a replication-deficient AdDF3-Bgal vector in which the B-gal gene was placed under the control of the MUC-1 promoter and showed selective B-gal expression in the ip tumor and non-specific B-gal expression in the mesothelial cells of the peritoneum following ip administration. A similar finding was also demonstrated using non-replicating adenovirus with the MUC-1 promoter and transduction of MCF-7 cells (53). Our results show limited efficacy when TNFα expression was placed under direction of the E2F or telomerase promoters. For other applications and delivery methods these promoters may have important advantages. Other investigators have demonstrated tumor-selective gene expression using cell-type or tissue-type specific promoters, DF3/MUC-1 (33), E2F (36), hTERT (37), Cox-2 (38,40) and SLPI (54).

Our results convince us that the combination of native receptor binding ablation, RGD-4C targeting, and tumor selective expression of TNFα provides a means to overcome many of the limitations of the adenovirus vectors previously tested in clinical trials for ovarian cancer. Ongoing studies in our laboratory are examining the relative contribution of capsid targeting and expression control to allow selective delivery of TNF to tumors in the intraperitoneal space. Other investigators are also exploring strategies to improve the activity and selectivity of adenovirus vectors for ovarian cancer application. Preclinical investigations have been initiated with RGD containing replication-defective and replication-selective adenoviruses (38,52,54-56). It is hoped that a new generation of highly selective adenovirus vectors can provide better treatment for patients with ovarian cancer.

Acknowledgements

We acknowledge the expertise of Dawn Hritz in cell culture and in vitro studies, Randy Osborn and Leslie West for animal studies and Kenji Kawagishi for in vivo luciferase biodistribution study. Grant support: National Cancer Institute SBIR # 1R43CA107658-01 (C. Richter King).

References


