Selectivity of TAG-72-targeted Adenovirus Gene Transfer to Primary Ovarian Carcinoma Cells versus Autologous Mesothelial Cells in Vitro

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ABSTRACT

Efficient gene transfer by recombinant adenovirus (Ad) vectors depends on expression of CAR and αv integrin on target cells. Because Ad may also infect nearby nontarget cells expressing these receptors, such as peritoneal mesothelial cells after i.p. injection, we hypothesized that targeting Ad gene delivery to a receptor overexpressed on most ovarian carcinoma cells, such as TAG-72, would enhance the selectivity of Ad gene transfer when used in this context. A monoclonal antibody that has been investigated clinically for immunotherapy and immunodetection of ovarian carcinomas, namely CC49, was used to construct a bispecific conjugate with the Fab fragment of a neutralizing anti-knob mAb to target Ad binding via TAG-72. This conjugate facilitated TAG-72-specific, CAR-independent Ad reporter gene transfer to both ovarian cancer cell lines and primary ovarian cancer cells cultured from malignant ascites fluid. Fab-CC49 was very selective for tumor cells, augmenting Ad gene transfer to primary ovarian cancer cells 4- to 9-fold relative to untargeted Ad, while also decreasing gene transfer to autologous cultured mesothelial cells 4- to 9-fold. These data suggest that targeting Ad via TAG-72 may improve the selectivity of Ad gene transfer for ovarian tumors 8- to 252-fold on i.p. vector injection. These results also define the requirements for a candidate target receptor in the rational design of a targeted Ad vector for ultimate clinical utility, one that selectively infects tumor cells and spares normal cells on i.p. injection. Such a vector may increase gene transfer and decrease the toxicity of Ad vectors, which would improve the therapeutic index of cytotoxic gene therapy for ovarian cancer in clinical trials.

INTRODUCTION

Ovarian cancer is the most common cause of gynecological cancer death in women with an estimated 23,000 new cases and 14,000 deaths in 2000 (1). The lifetime risk of developing ovarian cancer is estimated at 1:70. Because of a paucity of symptoms early in the course of the disease process, most cases of ovarian cancer are diagnosed in late stages of the disease, which is largely responsible for the high mortality rate among these patients. Advanced cases are usually treated with initial surgical debulking followed by chemotherapeutic agents such as paclitaxel and platinum-based agents, resulting in an 80% overall response rate. However, at 5 years, the overall survival is only 15–20% mainly because of the high rate of recurrent, drug-resistant disease. Second-line systemic chemotherapeutic agents that have been used to date have generally proven ineffective (2).

Therapeutic modalities other than systemic chemotherapy are currently being investigated for advanced-stage, treatment-refractory ovarian cancer (3–6). Of the vectors currently being investigated in clinical trials, Ad3 is among the most promising. The potential to address changes at the molecular level makes gene therapy a particularly promising treatment alternative, because tumors arise because of the sequential accumulation of genetic alteration in genes controlling the balance between cellular proliferation and cell death (7). A number of different strategies have already been devised for genetic manipulation of tumors (5). These approaches modulate various stages of tumorigenesis, making gene therapy a much more flexible option compared with chemotherapy and radiation therapy. Additionally, the combination of different genetic approaches has the potential to address the molecular heterogeneity inherent in treatment-refractory tumors.

Although gene therapy represents a potentially attractive treatment alternative for ovarian cancer, its efficacy is currently limited by the relative inefficiency of existing vectors to effect gene transfer in vivo (4, 5). Of the vectors currently being investigated in clinical trials, Ad3 is among the most promising. Ad can efficiently infect a wide variety of tissues both in vitro

3 The abbreviations used are: Ad, adenovirus; CAR, coxsackie-adenovirus receptor; MFI, mean fluorescence intensity; MOI, multiplicity of infection; pfu, plaque-forming unit(s); RLU, relative light units; RT, relative transduction; mAb, monoclonal antibody; FBS, fetal bovine serum; UAB, University of Alabama at Birmingham; ATCC, American Type Culture Collection; R-MFI, relative MFI; PE, phycoerythrin;
and in vivo (8). This finding has recently been attributed to the rather ubiquitous expression pattern of the receptors necessary for Ad entry into cells, namely the CAR and the α5 integrins (9–13). Moreover, the efficiency of Ad gene transfer has been shown to correlate directly with CAR levels on target cells in vitro (14–18). A corollary to this fact is that certain target cells may be refractory to Ad gene transfer because of their lack of CAR expression. Whereas the role of CAR expression on the efficiency of Ad gene transfer has yet to be established in vivo, it is likely that ubiquitous CAR expression on nontarget tissues may permit extensive ectopic gene transfer and that low CAR expression on target tissues may decrease Ad gene transfer efficiency, both ultimately limiting the clinical efficacy of Ad-based gene therapies.

Recent technological advances have permitted the generation of targeted Ad vectors capable of binding alternative cellular receptors, effecting gene transfer in a CAR-independent fashion (19). We and others have demonstrated this concept by generating Ad vectors targeted via several model receptors, including folate receptor (20), heparan sulfate (21), basic fibroblast growth factor receptor (22), and the epidermal growth factor receptor (14, 23). Several of these model receptors were chosen for the initial generation of a targeted Ad vector based on results obtained from studies of mAb-based cancer immunotherapy. Their appeal lies in the fact that they are overexpressed on certain individual tumor types relative to their surrounding normal tissues. However, none of these markers are selectively expressed solely on tumors.

The ideal candidate tumor marker must satisfy two criteria. First, it should be selectively expressed solely on tumor cells, not surrounding normal cells. Second, it should be expressed at high levels on virtually all of the tumors of a given type. A potentially ideal tumor marker for ovarian carcinoma has recently been identified through studies with radioimmunotherapy, the pancarcinoma antigen tumor-associated glycoprotein 72 (TAG-72). At our institution (UAB), a Phase I/II trial of therapy, the pancarcinoma antigen tumor-associated glycoprotein 72 (TAG-72) appears to be a unique ovarian cancer marker in that it is: (a) expressed on virtually every ovarian carcinoma; and (b) selectively expressed on tumor cells, not surrounding normal peritoneal cells (26, 27). These unique features suggested to us that TAG-72 could be exploited for purposes other than radioimmunotherapy, in particular the rational design of a targeted vector to improve the selectivity of ovarian cancer gene therapy.

We investigated the potential utility of a TAG-72-targeted Ad vector for ovarian cancer gene delivery using a bispecific antibody conjugate consisting of CC49 and the Fab fragment of a neutralizing anti-Ad5 kn6 mAb (Fab-CC49). Results of studies using an Ad vector-encoding luciferase (AdCMVLuc) demonstrated that Fab-CC49 enhanced AdCMVLuc gene transfer 9- to 57-fold relative to untargeted AdCMVLuc with two of five established human ovarian carcinoma cell lines and 2- to 28-fold with three samples of primary human ovarian carcinoma cells derived from malignant ascites. Importantly, Fab-CC49 also decreased AdCMVLuc gene transfer to autologous primary human mesothelial cells 4- to 9-fold relative to untargeted AdCMVLuc. Thus, a genetically modified, TAG-72-targeted Ad vector may prove superior to Ad vectors currently being investigated in clinical trials for selective gene transfer to recurrent ovarian cancers after i.p. delivery.

**MATeRIALS AND METHODS**

**Tumor Cells.** Human ovarian (CAOV-4, OVCAR-3, OV-1063, PA-1) and colon (LS174T, WiDr) tumor cells were obtained from the ATCC (Manassas, VA). CAOV-4 cells were cultured in L-15 medium (Mediatech, Herndon, VA) containing 20% FBS (Summit Biotechnology, Fort Collins, CO). OVCAR-3 and OV-1063 cells, and LS174T and WiDr cells, were cultured in RPMI 1640 containing 20% and 10% FBS, respectively. PA-1 cells were cultured in Earle’s-MEM with 10% FBS, SKOV3.ip1 human ovarian carcinoma cells were obtained from Janet Price (M. D. Anderson Cancer Center, Houston, TX) and cultured in DMEM containing 10% FBS. D54 MG human glioma cells were kindly provided by Darrell Bigner (Duke University, Durham, NC) and were cultured in DMEM/F12 containing 7% FBS. All of the media formulations contained 2 mM glutamine. All of the cells were cultured at 37°C in a 5% CO2 atmosphere and passaged less than 12 times during the course of these experiments.

Primary human ovarian carcinoma cells were established in culture from fresh malignant ascites fluid obtained during surgery at the University of Alabama Hospital during 1999. Permission to obtain these specimens was reviewed and approved by the Institutional Review Board for Human Experimentation at UAB. Cellular material from the ascites was obtained by centrifugation as described previously (28) and cultured in RPMI 1640 containing 10% FBS and 2 mM glutamine without prior immunoaffinity separation. Although these early cultures were heterogeneous, tumor cells vastly outnumbered mesothelial cells as confirmed by phase contrast microscopy as described previously (28). After two or three passages, only mesothelial cells continued to grow, and they appeared by phase-contrast microscopy as fibroblastic, polygonal cells with no evidence of any remaining ovarian tumor cells.

**Viruses and mAbs.** AdCMVLuc and AdCMVGFP first-generation E1-, E3-deleted vectors expressing firefly luciferase and green fluorescent protein, respectively, driven by the cytomegalovirus (CMV) immediate early promoter, were obtained from Robert Gerard (University of Leuven, Leuven, Belgium) and Corey Goldman (Cleveland Clinic, Cleveland, OH), respectively. Viruses were propagated and plaque titered on the permissive human embryonic kidney cell line 293 (Microbix, Hamilton, Ontario) and purified twice by centrifugation on CsCl gradients. Virus aliquots were maintained at −80°C until use. The particle:pfu ratio of different virus preparations was found to be important in determining the level of Fab-conjugate-targeted Ad gene transfer (14). To maintain a constant particle: pfu ratio (indicated in parentheses), all of the gene transfer experiments were conducted with a single lot of either

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GFP, green fluorescent protein; Ti, targeting index; RGD, arginine-glycine-glycine-asparagine.
AdCMVLuc (8:1) or AdCMVGFP (27:1) as well as with single lot of Fab-CC49.

The anti-CAR mAb RmcB (a generous gift from Robert W. Finberg, Harvard Medical School, Boston, MA) has been previously described (29). Murine mAb LM609 to α5β1 integrin and P1F6 to αvβ3 integrin were both purchased from Chemicon (Temecula, CA). The neutralizing murine mAb ID6.14 specific for the COOH-terminal, receptor-binding knob domain of Ad serotype 5 fiber has been described previously (20). The anti-TAG-72 mAb CC49 (a generous gift from Jeffrey Schлом, National Cancer Institute, Bethesda, MD) has been described previously (30).

Indirect Immunofluorescence. Receptor expression was analyzed by indirect immunofluorescence as described previously (14). Briefly, subconfluent cultures were harvested with versene and resuspended at 1–2 × 10⁶ cells/ml in PBS containing 0.1% BSA and 0.1% sodium azide. Cells were then incubated with primary mAbs to CAR (RmcB), αvβ3, and α5β3 integrin (LM609 and P1F6, respectively), or TAG-72 (CC49). After washing with buffer, cells were stained with an Alexa 488-conjugated goat-antimouse IgG secondary antibody (Molecular Probes, Eugene, OR). Cells (10⁴ per sample) were then analyzed on a Becton Dickinson FACSVantage or FACSscan at the UAB Rheumatology or UAB Comprehensive Cancer Center FACS Core Facilities, respectively. Percent positive was calculated by setting at 1% the percentage of cells incubated in the absence of primary mAb (mock) as positive. R-MFI was calculated as the ratio of the MFI of the sample of interest to the MFI for the corresponding mock sample for each individual cell line.

Receptor expression on AdCMVGFP-infected cells was performed as above with the exception that PE-conjugated secondary antibodies were used (Molecular Probes). Concurrent GFP and receptor expression was determined by two-color flow cytometry. Scatter plot data were analyzed by linear regression to determine the correlation between GFP (X axis) and receptor (Y axis) expression on infected cells.

AdCMVLuc Targeting. To facilitate targeted Ad gene transfer to TAG-72, a Fab-mAb conjugate was generated using CC49 (Fab-CC49), and the conjugate was characterized as described previously (14). Established or primary human tumor cells were plated at 1.2 × 10⁴ cells/well in 24-well dishes and were allowed to adhere overnight at 37°C. AdCMVLuc (MOI, 100 pfu/cell) was incubated for 30 min at room temperature with various concentrations of Fab-CC49 to determine the optimum conjugate:virus ratio. OVCAR-3 or SKOV3.ip1 cells were pre-incubated for 1 h at 4°C in the presence or absence of 20 μg/ml recombinant Ad5 knob diluted in Opti-MEM (Life Technologies, Gaithersburg, MD) and were then infected with virus complexed with or without Fab-CC49 for 1 h at 37°C. Twenty-four h postinfection, cells were harvested and analyzed for luciferase expression as described previously (14). Subsequent experiments were performed using a single, optimized conjugate:virus ratio. In these experiments, cells were preincubated with OPTI-Mem alone (negative control), 20 μg/ml of Ad5 knob, or 50 μg/ml of CC49 to determine the receptor specificity of gene transfer by Fab-CC49-targeted AdCMVLuc versus untargeted AdCMVLuc. These data were statistically analyzed using a two-factor ANOVA on the logarithm of RLU values, as described previously (14). Differences were determined at the 0.05 level of significance.

AdCMVGFP Targeting. Established or primary human ovarian carcinoma cells were plated in 6-well dishes and were allowed to adhere overnight at 37°C. AdCMVGFP was incubated with the optimum concentration of Fab-CC49, and serial dilutions were made to infect cells at various MOI. Cells were infected with conjugated or unconjugated virus for 1 h at 37°C. Twenty-four h postinfection, cells were harvested with versene and were analyzed for GFP expression by flow cytometry as described above for indirect immunofluorescence.

RESULTS

Expression of CAR, αv Integrins, and TAG-72 on Established Human Tumor Cells. The expression of the Ad receptors CAR, αvβ3, and α5β3 integrins, and the candidate target receptor for the generation of an ovarian cancer-targeted Ad vector, TAG-72, were examined by indirect immunofluorescence using a panel of human tumor cell lines (Table 1). The human glioblastoma multiforme cell line D54 MG was included because it is known that these cells express high levels of CAR and αvβ3 integrin, and are, hence, readily infected with recombinant Ad vectors (14). The human colon cancer cell line LS174T was included in the analysis because it is known to express high levels of the target receptor TAG-72 (31, 32).

Finally, an additional human colon cancer cell line, WiDR, and five human ovarian cancer cell lines were analyzed, including

### Table 1: Indirect immunofluorescence analysis of CAR, αv Integrins, and TAG-72 expression on human cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Histology</th>
<th>CAR % R-MFI</th>
<th>αvβ3 % R-MFI</th>
<th>αvβ5 % R-MFI</th>
<th>TAG-72 % R-MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>D54MG</td>
<td>Glioblastoma multiforme (43)</td>
<td>94.6</td>
<td>9.3</td>
<td>58.4</td>
<td>4.8</td>
</tr>
<tr>
<td>LS174T</td>
<td>Colon adenocarcinoma (44)</td>
<td>73.6</td>
<td>5.8</td>
<td>15.2</td>
<td>2.6</td>
</tr>
<tr>
<td>WiDR</td>
<td>Colon adenocarcinoma (45)</td>
<td>75.8</td>
<td>4.8</td>
<td>30.2</td>
<td>3.5</td>
</tr>
<tr>
<td>SKOV3.ip1</td>
<td>Ovarian carcinoma (46)</td>
<td>60.1</td>
<td>6.0</td>
<td>51.4</td>
<td>5.2</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>Ovarian carcinoma (47)</td>
<td>50.3</td>
<td>5.8</td>
<td>33.5</td>
<td>5.1</td>
</tr>
<tr>
<td>PA-1</td>
<td>Ovarian teratocarcinoma (48)</td>
<td>99.2</td>
<td>8.0</td>
<td>21.7</td>
<td>2.4</td>
</tr>
<tr>
<td>OV-1063</td>
<td>Ovarian carcinoma (49)</td>
<td>41.3</td>
<td>2.9</td>
<td>28.8</td>
<td>2.4</td>
</tr>
<tr>
<td>CAOV-4</td>
<td>Ovarian carcinoma (50)</td>
<td>7.7</td>
<td>0.9</td>
<td>44.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

aData presented as the percentage of cells gated positive (%) and the R-MFI from a representative of two to five separate experiments.

bOV-1063 cells have recently been shown by the ATCC to contain a Y chromosome (ATCC web page http://www.atcc.org/phage/probe.html).
CAOV-4, OV-1063, PA-1, SKOV3.ip1, and the known TAG-72-positive cell line OVCAR-3 (33).

Although LS174T and WiDR colon cancer cell lines expressed high levels of CAR, relative to the known CAR-positive human glioma cell line D54 MG, only one (PA-1) of the five ovarian cancer cell lines expressed comparably high levels of CAR, with MFI relative to negative control samples (R-MFI) of 9.3 and 8.0, respectively. At the other end of the spectrum, both OV-1063 and CAOV-4 cells displayed low levels of CAR (R-MFI, 2.9 and 0.9, respectively). All of the cell lines tested, with the exception of PA-1 and CAOV-4 cells, expressed high levels of $\alpha_v\beta_3$ integrin (R-MFI, >3.9). $\alpha_v\beta_3$ integrin expression was variable, with R-MFI ranging from 2.4 with OV-1063 and CAOV-4 cells to 5.2 with SKOV3.ip1 cells. These results are consistent with previous reports describing $\alpha_v\beta_3$ integrin expression on cells of epithelial origin (34, 35). All of the cell lines tested expressed TAG-72, with SKOV3.ip1 and CAOV-4 cells displaying the lowest level of expression (R-MFI, 1.8 and 2.0, respectively). In contrast, WiDR, OVCAR-3, and PA-1 cells expressed very high levels of TAG-72, with R-MFI values of 28.2, 21.6, and 48.7, respectively.

**Optimization of Fab-CC49-targeted AdCMVLuc Gene Transfer to Human Ovarian Carcinoma Cell Lines.** Both OVCAR-3 and SKOV3.ip1 cells were infected by AdCMVLuc via a fiber (CAR)-dependent pathway, because preincubating cells with recombinant Ad5 knob blocked >99% of luciferase gene transfer to both of these cell lines (Fig. 1). Preincubating AdCMVLuc with increasing concentrations of Fab-CC49 increased the level of TAG-72-targeted AdCMVLuc gene transfer and decreased the level of CAR-dependent AdCMVLuc gene transfer with both cell lines, as determined with cells pretreated in the presence and absence, respectively, of a neutralizing amount of recombinant Ad5 knob. The optimum Fab-CC49:AdCMVLuc ratios for OVCAR-3 and SKOV3.ip1 cells were 0.3 ng and 10 ng of Fab-CC49 per $1 \times 10^6$ pfu AdCMVLuc, respectively. Increasing the conjugate:virus ratio further had a minimal effect on AdCMVLuc gene transfer with both of the cell lines. The conjugate:virus ratio of 10 ng of Fab-CC49 per $1 \times 10^6$ pfu of AdCMVLuc or of AdCMVGFP was, thus, chosen for all of the subsequent targeting experiments to facilitate CAR-independent, TAG-72-specific gene transfer.

**TAG-72-targeted AdCMVLuc Gene Delivery to Established Human Carcinoma Cells.** The relative level and specificity of TAG-72-targeted AdCMVLuc gene transfer compared with AdCMVLuc gene transfer to the various human cancer cell lines was determined in vitro by analyzing luciferase activity in cell lysates at 24 h postinfection. As shown in Table 2, D54 MG human glioma cells were readily infected by AdCMVLuc and, thus, served as a reference for Ad transducibility, as described previously (14). Both LS174T and WiDR colon carcinoma cells displayed high levels of luciferase gene transfer, similar to levels found with D54 MG cells. These results indicated that these cells too were readily infectable by AdCMVLuc, presumably because of their high expression of CAR and $\alpha_v\beta_3$ integrins (Table 1). Of the human ovarian carcinoma cells analyzed, OVCAR-3 cells were the most AdCMVLuc-transducible, displaying approximately 30% of the luciferase activity found with D54 MG cells. AdCMVLuc gene transfer was lowest with OV-1063 cells, two orders of magnitude lower than with D54 MG cells, which indicated that these cells are relatively refractory to AdCMVLuc infection. The remaining human ovarian cancer cell lines, SKOV3.ip1, PA-1, and CAOV-4 cells, were efficiently infected by AdCMVLuc, although levels were one order of magnitude lower than with OVCAR-3 cells. Importantly, AdCMVLuc gene transfer to all of the eight cell lines was fiber-dependent, because preincubation with saturating concentrations of Ad5 knob significantly inhibited luciferase expression in all of the eight cell lines tested ($P < 0.05$).

The magnitude of luciferase gene transfer of TAG-72-targeted AdCMVLuc (virus preincubated in the presence of Fab-CC49 at the optimum conjugate:virus ratio) was then compared with untargeted AdCMVLuc and expressed as a ratio defined as the targeting index (Ti, Table 2). Fab-CC49-targeted AdCMVLuc significantly enhanced luciferase gene transfer to only two cell lines ($P < 0.05$), namely OVCAR-3 (9.0-fold) and CAOV-4 (57.0-fold) ovarian cells. Targeted AdCMVLuc gene transfer to all of the other cell lines was fiber-dependent, because preincubation with saturating concentrations of Ad5 knob significantly inhibited luciferase expression in all of the eight cell lines tested ($P < 0.05$).

The expected pattern of significant CC49 inhibition and lack of significant inhibition by
Ad5 knob was seen with five of eight cell lines, which indicated that Fab-CC49-targeted AdCMVLuc facilitated CAR-independent, TAG-72-specific gene transfer with the cells. Results with the remaining three cell lines, LS174T, PA-1, and OV-1063, were unexpected. Fab-CC49-targeted AdCMVLuc gene transfer to PA-1 and OV-1063 cells was significantly inhibited by Ad5 knob and not by CC49. These results suggested that Fab-CC49-targeted AdCMVLuc failed to facilitate CAR-independent, TAG-72-specific gene transfer to these cell lines. Results with PA-1 and OV-1063 cells could be explained by the observation that targeted AdCMVLuc gene transfer to both of these cell lines was relatively low, and, although the levels of blocking observed were statistically significant, the level of luciferase expression in these samples approached the lower limit of detection of this assay. The pattern of Ad5 knob and CC49 mAb inhibition obtained with LS174T cells, however, could not be readily explained.

To more stringently analyze the specificity of Fab-CC49-targeted Ad gene transfer, we used two-color flow cytometry to detect concurrent CAR and TAG-72 receptor expression and reporter gene transfer from an Ad vector encoding green fluorescent protein (AdCMVGFP) with OVCAR-3 cells (Fig. 2). Cells were infected at increasing MOI with either AdCMVGFP or Fab-CC49-targeted AdCMVGFP, were harvested 24 h postinfection, and were analyzed for CAR and TAG-72 expression by indirect immunofluorescence using a R-PE-conjugated secondary antibody. As shown in Fig. 2A, virtually all of the OVCAR-3 cells were transduced by AdCMVGFP at a MOI of 100 pfu/cell. This MOI was thus chosen for the two-color flow cytometry analysis. To determine the correlation between AdCMVGFP expression and target receptor expression, a linear regression analysis was performed on the logarithm of fluorescence from GFP (X/axis) and the logarithm of fluorescence from PE (Y axis). Compared with control cells incubated without a primary antibody (Fig. 2C, slope, 0.10; r², 0.11), GFP expression in cells infected with AdCMVGFP strongly correlated with CAR expression (Fig. 2E, slope, 0.27; r², 0.52), but not with TAG-72 expression (Fig. 2F, slope, 0.08; r², 0.07). In contrast, compared with negative control cells incubated without a primary antibody (Fig. 2D, slope, 0.18; r², 0.16), Fab-CC49-targeted AdCMVGFP-mediated expression of GFP correlated with TAG-72 expression (Fig. 2H, slope, 0.25; r², 0.40), but not with CAR expression (Fig. 2G, slope, 0.12; r², 0.08). This pattern of correlation between GFP and receptor expression in OVCAR-3 cells infected with either AdCMVGFP or Fab-CC49-targeted AdCMVGFP was also evident at multiple MOI, ranging from 10 to 100 pfu/cell (data not shown).

Expression of CAR, α5 integrins, and TAG-72 on Primary Ovarian Carcinoma and Mesothelial Cells Cultured from Malignant Ascitic Fluid. Relative to OVCAR-3 cells, CAR expression on primary ovarian carcinoma cells was variable, with OVP0217 cells demonstrating moderate levels of CAR expression relative to OVCAR-3, and both OVP0222 and OVP0305 cells expressing low levels of CAR (Fig. 3). All of the samples of primary ovarian carcinoma cells expressed high levels of α5β3 integrin and TAG-72, but two of three expressed low levels of α3β1 integrin. In contrast, cultured mesothelial cells expressed high levels of CAR and α5β3 integrin and moderate levels of α3β1 integrin (Fig. 3). TAG-72 expression was undetectable on both mesothelial cell cultures, consistent with previous reports demonstrating the lack of TAG-72 expression on normal peritoneum (36). On the basis of these results, we hypothesized that targeting Ad-mediated gene transfer via TAG-72 may be enhanced relative to untargeted Ad with cultured primary human ovarian carcinoma cells and may be decreased with cultured human mesothelial cells.

Gene Transfer to Primary Ovarian Carcinoma and Mesothelial Cells Cultured from Malignant Ascites Fluid. Results of studies analyzing Fab-CC49-targeted versus untargeted AdCMVLuc gene transfer at a single MOI of 100 pfu/cell confirmed this hypothesis. AdCMVLuc gene transfer to autologous primary ovarian carcinoma cells was two to three orders of magnitude lower than with OVCAR-3 cells, whereas both samples of mesothelial cells demonstrated one order of magnitude higher levels of luciferase gene transfer relative to OVCAR-3 cells (Table 3). Furthermore, like OVCAR-3 cells, AdCMVLuc gene transfer to both primary ovarian tumor cells and mesothelial cells was fiber-dependent, as Ad5 knob inhibited gene transfer >96% with all of the samples tested. These results were consistent with the results from the receptor anal-

### Table 2 Untargeted versus TAG-72-targeted Ad gene transfer to human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line (k)</th>
<th>No block (RLU × 10⁷)</th>
<th>Ad5 knob (RLU × 10⁷)</th>
<th>Ad5 knob (%) inhibition</th>
<th>CC49 mAb (RLU × 10⁷)</th>
<th>Ti²</th>
</tr>
</thead>
<tbody>
<tr>
<td>D54MG (1)</td>
<td>393 ± 92</td>
<td>96 ± 0.3</td>
<td>326 ± 27</td>
<td>70 ± 2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>LS174T (1)</td>
<td>291 ± 13</td>
<td>96 ± 0.2</td>
<td>177 ± 22</td>
<td>88 ± 24</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>WiDr (1)</td>
<td>321 ± 23</td>
<td>96 ± 0.6</td>
<td>252 ± 8.6</td>
<td>67 ± 3</td>
<td>0.8 ± 0.03d</td>
</tr>
<tr>
<td>SKOV3.ip1 (2)</td>
<td>40 ± 19</td>
<td>90 ± 2.8</td>
<td>36 ± 11</td>
<td>48 ± 33</td>
<td>0.9 ± 0.3d</td>
</tr>
<tr>
<td>OVCAR-3 (2)</td>
<td>111 ± 12</td>
<td>94 ± 5.0</td>
<td>1000 ± 496</td>
<td>57 ± 18</td>
<td>9.0 ± 4.5</td>
</tr>
<tr>
<td>PA-1 (2)</td>
<td>38 ± 10</td>
<td>71 ± 30</td>
<td>26 ± 13</td>
<td>13 ± 50</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>OV-1063 (2)</td>
<td>4.7 ± 2.1</td>
<td>83 ± 9.0</td>
<td>1.7 ± 0.9</td>
<td>14 ± 58e</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>CAOV-4 (1)</td>
<td>19 ± 6.7</td>
<td>92 ± 1.3</td>
<td>1080 ± 139</td>
<td>43 ± 5</td>
<td>57.0 ± 7.3</td>
</tr>
</tbody>
</table>

a Data determined from one or two representatives (k) of one to five separate experiments with each cell line.
b Ti defined as the ratio of luciferase gene transfer (RLU) of Fab-CC49 targeted AdCMVLuc versus untargeted AdCMVLuc.
c Luciferase gene transfer by Fab-CC49-targeted AdCMVLuc in the presence of either Ad5 knob or CC49 not statistically different from Fab-CC49-targeted AdCMVLuc gene transfer in the absence of block (P > 0.05).
d Luciferase gene transfer by Fab-CC49-targeted AdCMVLuc not statistically different from untargeted AdCMVLuc gene transfer (P > 0.05).
ysis demonstrating low CAR and α₅β₅ integrin expression on primary ovarian tumor cells but high levels of CAR and α₅β₅ integrin expression on mesothelial cells (Fig. 3).

Relative to untargeted AdCMVLuc, Fab-CC49-targeted AdCMVLuc enhanced the magnitude of luciferase gene transfer 1.92- to 27.92-fold to primary ovarian carcinoma cells at 100 pfu/cell (Table 3). As shown in Fig. 4A, relative to untargeted AdCMVGFP, targeting AdCMVGFP via TAG-72 also augmented gene transfer efficiency to all of the three samples of primary ovarian carcinoma cells over a range of MOI. Furthermore, the level of GFP gene transfer of both Fab-CC49-targeted AdCMVGFP and untargeted AdCMVGFP was directly proportional to the amount of input virus (Fig. 4B), because the relationship of the logarithm of MOI with the logarithm of the MFI was linear for all of the three primary samples ($r^2 > 0.95$) as well as for OVCAR-3 cells ($r^2 > 0.88$, data not shown). A comparison of the magnitude of GFP expression from TAG-72-targeted AdCMVGFP and untargeted AdCMVGFP (Ti) confirmed the results obtained with AdCMVLuc (Table 3). With this virus, the Ti ranged from 0.8 to 11.4 for the three primary ovarian carcinoma samples at a MOI of 100 pfu/cell. However, the Ti of AdCMVGFP gene transfer was not independent of MOI with any of the cell lines (Fig. 3C, $r^2 < 0.5$). The maximum enhancement of gene transfer with Fab-CC49 targeting AdCMVGFP was obtained at 10–100 MOI. Because of acute viral toxicity, Ti was decreased with MOI $> 200$ as we have previously observed (14). These results demonstrate the rationale for using a MOI of 100 pfu/cell for Ti analyses (Tables 2 and 3).

In contrast to results with primary ovarian carcinoma cells,
TAG-72 targeting decreased luciferase gene transfer expression 4.0- to 8.8-fold to cultured mesothelial cells (Table 3). Specificity determination by blocking with Ad5 knob or CC49 demonstrated CAR independent, TAG-72-specific Fab-CC49-targeted AdCMVLuc gene transfer to two of three primary ovarian carcinoma samples (OVP0217 and OVP0222). However, CC49 failed to block Fab-CC49-targeted AdCMVLuc gene transfer to the remaining primary tumor (OVP0305) and the two mesothelial cell cultures, perhaps because of the relatively low level of TAG-72 expression on these cells.

DISCUSSION

The high rate of recurrence of ovarian cancer after initial cytoreductive surgery and front-line chemotherapy has necessitated the exploration of additional therapeutic options. Gene therapy is a rational therapeutic modality in this clinical setting because it maintains promise in eradicating the chemotherapy-resistant, recurrent disease that invariably arises in this patient population (2). Phase I gene therapy trials using Ad vectors are ongoing for such patients. Whereas Ads are among the most promising vectors available, they are currently limited by the low gene transfer efficiency seen clinically on i.p. injection in patients with ovarian cancer. Several recently published reports have suggested that this phenomenon may be attributable in part to low level expression on target cells of the receptors necessary for Ad entry, particularly the Ad fiber-binding protein CAR (14–18). To overcome this limitation, tropism-modified Ad vectors may be used because they mediate CAR-independent, target receptor-specific gene transfer. A number of such vectors have been described, generated either by genetic incorporation of targeting ligands into Ad capsid proteins or through the use of bispecific antibody conjugates. The target receptors used for these vectors have included RGD-binding integrins (37), heparan sulfate proteoglycans (21), basic fibroblast growth factor receptor (22), epidermal growth factor receptor (14, 23), and the pancarcinoma antigen EpCAM (38). Although each of these target receptors is overexpressed on neoplastic tissues, the lack of tumor specificity of these markers may limit the selectivity of the corresponding targeted Ad vector for neoplastic tissue.

In this investigation, we demonstrate that the pancarcinoma antigen TAG-72 may represent an ideal marker for the generation of a targeted Ad vector with selectivity for ovarian cancer.

Unpublished observations.

Fig. 3 Cell surface expression of CAR (black bars), \(\alpha_\beta_1\) integrin (white bars), \(\alpha_\beta_5\) integrin (dotted bars), and TAG-72 (hatched bars) via indirect immunofluorescence on primary human ovarian carcinoma (A, B) and autologous mesothelial (C, D) cells derived from malignant ascites. Data from a representative of two separate experiments are presented as the percentage of cells gated positive (A, C) and MFI (B, D) relative to mock cells (gray bars).
in i.p. gene therapy applications. TAG-72 has been shown to be selectively expressed at high levels on virtually all of the epithelial ovarian malignancies but not on surrounding normal mesothelial cells lining the peritoneal cavity (26, 27, 36, 39).

Several mAbs to this receptor have been investigated for the diagnosis and immunotherapy of ovarian cancer (24–27, 36, 39). One such mAb, CC49, was selected for its high binding affinity and favorably high tumor:normal tissue ratio relative to other TAG-72-specific mAbs to construct a bispecific antibody conjugate (Fab-CC49) to facilitate targeted Ad gene transfer to TAG-72 (30).

Results with ovarian cancer cell lines showed that the level of luciferase reporter gene transfer from AdCMVLuc (Table 2) correlated well with the levels of CAR expression and αv integrins on these cells (Table 1). As previously shown with cell lines derived from bladder (15), glioma (14), lung (18), and melanoma tumors (16), the lack of CAR expression on some carcinoma cell lines (CAOV-4) and αv integrins on others (PA-1, CAOV-4) could readily explain the low level of AdCMVLuc gene transfer seen with these cells.

However, the lack of CAR expression on target cells could be overcome by targeting Ad via TAG-72 using Fab-CC49. To compare the level of TAG-72-targeted Ad gene transfer to that of untargeted Ad, the Ti was calculated as the ratio of TAG-72-targeted AdCMVLuc-mediated luciferase expression to the luciferase expression of untargeted AdCMVLuc. Gene transfer was enhanced with two of five human ovarian carcinoma cell lines by TAG-72 targeting, with Ti for OVCAR-3 and CAOV-4 being 9.0 and 57, respectively (Table 3). The target receptor specificity and CAR-independence of TAG-72-targeted Ad gene transfer was confirmed by two separate methods: (a) inhibition of TAG-72-targeted AdCMVLuc gene transfer by CC49 mAb and lack of inhibition of Ad5 knob inhibition (Table 2) as described previously (14); and (b) a two-color flow cytometry assay to directly correlate untargeted and TAG-72-targeted AdCMVGFP-mediated GFP expression with concurrent cell surface CAR and TAG-72 receptor expression determined by indirect immunofluorescence with a R-PE conjugated secondary antibody (Fig. 3).

At the optimal conjugate:virus ratio, TAG-72-targeted AdCMVLuc gene transfer facilitated target receptor-specific, CAR-independent gene transfer to five of eight TAG-72-positive cell lines (Table 2). The remaining three cell lines, PA-1 and OV-1063 ovarian cells and LS174T colon carcinoma cells, all expressed TAG-72. The low level of TAG-72-targeted AdCMVLuc gene transfer to these cells could possibly be attributable to their low expression of αv integrins, which may be necessary for the internalization of the TAG-72-targeted vector (40).

Similar results, demonstrating the importance of additional factors in determining the level of targeted Ad gene transfer, have been previously reported (14). The expected CC49 and Ad5 knob inhibition pattern of TAG-72-targeted AdCMVLuc gene transfer was also not seen with LS174T cells, but these results could not be readily explained based on the LS174T profile of target receptor expression. Two-color flow cytometry demonstrated that GFP expression mediated by untargeted AdCMVGFP infection of OVCAR-3 cells at a MOI of 10–100 directly correlated with CAR expression but not with TAG-72 expression on these cells (Fig. 3 and data not shown). Conversely, Fab-CC49-targeted AdCMVGFP-mediated GFP expression directly correlated with TAG-72 expression but not with CAR expression.

On the basis of the results with cell lines, a similar analysis was performed using patient-matched primary ovarian carcinoma cells and autologous mesothelial cells cultured from malignant ascitic fluid. These experiments were performed with fresh ascites samples without separation of tumor cells from mesothelial cells before culture to more closely approximate the situation likely to be encountered clinically with a TAG-72-targeted Ad. Morphological confirmation of the absence of tumor cells, which die after 2–3 passages in culture, was performed by phase-contrast microscopy as described previously (28) to ensure that the mesothelial cells were homogeneous and that subsequent studies using these cells would be valid as a
This profile of Ad receptor expression correlated well with the high levels of AdCMVLuc gene transfer seen with these cells, which were more than one order of magnitude higher than that with the highly AdCMVLuc transducible ovarian cancer cell line OVCA-2 (RT values of 19.4 and 14.3 for 021 and 0305 mesothelial cells, respectively; Table 3). In contrast, targeting AdCMVLuc gene transfer via TAG-72 decreased the level of luciferase gene expression with both mesothelial samples compared with untargeted AdCMVLuc (Ti, 0.11 and 0.25), which was perhaps attributable to the relatively low TAG-72 expression levels on these cells (Fig. 3).

Unlike the mesothelial cells, primary ovarian carcinoma cells displayed moderate-to-low levels of both CAR and $\alpha_\text{v}\beta_3$ integrins, and high levels of $\alpha_\text{v}\beta_5$ integrins (Fig. 3). AdCMV-Luc-mediated luciferase expression was two to three orders of magnitude lower in these cells than in OVCA-2 cells (Table 3), results that correlated well with the levels of Ad expression on these cells (Fig. 3). However, because all three of the samples of primary ovarian cancer cells displayed high TAG-72 expression, the Ti of TAG-72-targeted AdCMVLuc at 100 pfu/cell was high for all three (Ti, 1.92–27.92). Moreover, equivalent results were obtained using AdCMVGFP (Fig. 4), which also showed that the Ti was not independent of input MOI at <200 pfu/cell (Fig. 4C).

Because mesothelial cells were derived from the same patient samples as the primary ovarian cancer cells, a relative level of selectivity (selectivity index) could be calculated for the TAG-72-targeted AdCMVLuc vector. For the 0217 and 0305 samples, the selectivity index was determined to be 252- and 8-fold, respectively. These data suggest that targeting Ad via TAG-72 may potentially increase the tumor selectivity of Ad gene transfer 1–2.5 orders of magnitude relative to CAR-dependent untargeted Ad vectors.

The results in this report have significant implications for the potential clinical utility of Ad vectors for i.p. gene therapy applications. High-level expression of CAR and $\alpha_\text{v}$ integrins on mesothelial cells lining the peritoneal cavity may significantly limit the bioavailability of untargeted Ad vectors for carcinoma cells. In essence, the peritoneal lining may serve as an “Ad sink,” and, in addition to limiting gene transfer to the target cancer cells, it may potentially increase Ad vector toxicity caused by ectopic transgene expression in nontarget mesothelial cells. Successful targeting to a receptor not found on mesothelium and found in abundance on tumor tissues, such as TAG-72, may thus improve the toxicity of Ad vectors. Furthermore, considering the vast surface area covered by “normal peritoneum” versus minimal residual tumor after cytoreductive surgery and adjunctive chemotherapy, an Ad vector with an extremely high selectivity index, on the order of three to four orders of magnitude, may be necessary for ultimate clinical efficacy. Additional limitations of untargeted Ad vector efficacy may be the low level of CAR expression on tumor cells, as shown in this report and others (14). With a selectivity index of one to three orders of magnitude and the capacity to achieve CAR-independent gene transfer, a TAG-72-targeted Ad may overcome these limitations.

We have previously shown that gene delivery may be enhanced by the genetic incorporation of targeting moieties into the capsid proteins of Ad vectors (34, 35, 37, 41). Specifically,
the addition of a RGD motif in the HI loop of the Ad5 fiber has been shown to enhance gene transfer to both established and primary ovarian carcinoma cells (35). Whereas this vector may significantly increase the level of Ad gene transfer in the peritoneum, a RGD-modified Ad vector may not be selective for ovarian cancer cells because of the ubiquitous expression of RGD-binding integrins on most cell types. However, a genetically modified Ad vector containing a TAG-72-specific peptide epitope in the HI loop of the fiber protein may be more efficacious and more selective in the clinical setting. Notably, TAG-72-specific peptides have been generated using hexapeptide and decapetide phage libraries (42). We are currently exploring the use of such peptides to develop a HI-loop modified TAG-72-specific Ad vector.

Other antigens selectively expressed on ovarian tumors may also be investigated in a similar manner for the design of targeted Ad vectors. Although expressed in a large proportion (65–100%) of ovarian tumors (26, 27, 36, 39), some ovarian tumors or subpopulations of cells within a single tumor may not express TAG-72. Thus, the heterogeneity of cell surface receptor expression may limit the universal applicability of a TAG-72-targeted Ad vector for ovarian cancer. To overcome this potential limitation, targeted Ad vectors may be generated to alternate ovarian cancer-specific targets. Studies currently ongoing in our laboratory that compare TAG-72-targeted Ad with Ad targeted to other ovarian tumor antigens using patient-matched primary ovarian and mesothelial cell cultures will be important in addressing the potential clinical utility of these vectors. In addition, the combination of targeted vectors in a “cocktail” may increase the proportion of tumor cells susceptible to Ad gene transfer.

In summary, the selective targeting of Ad vectors to the TAG-72 oncofetal antigen on tumor cells in the ovarian carcinoma i.p. gene therapy setting may increase the magnitude of Ad gene delivery to target tumor cells, render CAR-negative, Ad-refractory tumor cells susceptible to Ad gene transfer, and potentially decrease vector toxicity by exclusion of normal tissues from Ad gene transfer. Future gene therapy trials using TAG-72-targeted Ad vectors should evaluate these parameters. Additional ovarian carcinoma-specific cell surface receptors should be investigated for selective Ad vector targeting to improve current gene therapy for consolidation regimens or second-line treatment.

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Selectivity of TAG-72-targeted Adenovirus Gene Transfer to Primary Ovarian Carcinoma Cells versus Autologous Mesothelial Cells in Vitro

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