Brief Report

Serum and Ascites Neutralizing Antibodies in Ovarian Cancer Patients Treated with Intraperitoneal Adenoviral Gene Therapy

AKSELI HEMMINKI,1,2 MINGHUI WANG,1 RENEE A. DESMOND,3 THERESA V. STRONG,4 RONALD D. ALVAREZ,5 and DAVID T. CURIEL1

OVERVIEW SUMMARY

Neutralizing antibodies (NAbs) can inhibit gene transfer by adenovirus in preclinical models, but this has not been confirmed in human trials, which have mostly utilized local delivery. Therefore, it has not been studied whether intraperitoneal administration of adenovirus to ovarian cancer patients results in induction of NAbs in ascites or serum. Also, it is not known how ascites and serum NAbs correlate, or if preexisting ascites NAbs block gene transfer in humans. Before treatment, 33% of patients had a NAb titer of >1000. Good correlation existed between serum and ascites NAb titers before \( p = 0.0003 \) and after \( p = 0.0008 \) treatment, and serum and ascites NAb titers were rapidly induced after treatment. Transgene expression was affected but not prevented by preexisting NAbs. Total anti-adenoviral antibodies did not correlate well with NAbs and the administered dose did not affect the level of NAb induction. Although serum and ascites NAbs correlated well, serum total protein concentration was over 4-fold higher, suggesting selective accumulation of proteins in ascites. These results imply that serum NAbs can be used as a surrogate for ovarian cancer-associated ascites NAbs. Also, although NAbs did not prevent gene transfer, it could be useful to attempt removal of ascites before intraperitoneal adenoviral therapy.

INTRODUCTION

Most ovarian cancer patients present with metastatic disease, for which treatment options remain inadequate and survival figures low (Kristensen and Trope, 1997). The majority of these patients exhibit intraperitoneal dissemination but lack of detectable metastases elsewhere. Unfortunately, this patient group also has the worst prognosis. Therefore, it is logical that cancer gene therapy by intraperitoneal administration be attempted (Alvarez et al., 2000a,b; Hasenburg et al., 2000; Buller et al., 2001; Muller et al., 2001).

Adenovirus serotype 5 has emerged as a popular agent for cancer gene therapy because of its unparalleled capacity for gene transfer to dividing and quiescent cells and the ease of high-titer production. Also, more than 700 cancer patients have been treated with excellent safety data and no mortality attributable to the agent (Hemminki and Alvarez, 2002). All ovarian cancer gene therapy trials published have relied on intraperitoneal administration, which has been well tolerated, and dose-limiting toxicity has usually not been encountered. A contributing factor could be the degree of compartmentalization offered by the peritoneal cavity, which may have helped reduce side effects while increasing access of the vector to target tissue.

Most trials published have been phase I trials, the main end point of which is not efficacy or clinical benefit. Nevertheless, in three studies, ascites cells were collected and the vector and expression of the transgene were detected (Alvarez et al., 2000a,b; Muller et al., 2001). In another study, expression of the transgene was detected in biopsy specimens (Buller et al., 2001). Thus, it seems likely that gene transfer to intraperitoneal ovarian cancer cells can be achieved, but it is unclear at this point if this is sufficient to alter the course of the disease.

Adenovirus is a strongly immunogenic virus, which could help in local and distant tumor eradication, if tumor cells can be effectively infected with subsequent recognition by the immune system. However, because of nearly universal exposure, circulating anti-adenovirus antibodies are common. Neutralizing antibodies (NAbs) form a subpopulation of the total anti-adenovirus antibody (TAb) pool, but this subgroup is most rel-
relevant for inhibiting adenoviral infection. Twenty-seven to 59% of the population have been reported NAb positive (Schulick \textit{et al.}, 1997; Chen \textit{et al.}, 2000; Khuri \textit{et al.}, 2000). Further, even if circulating NAb titers are low, reexposure can quickly induce high NAb titers. Obviously, this is potentially problematic for readministration of adenovirus-based agents.

Most patients with peritoneally disseminated ovarian cancer have malignant ascites, which usually quickly recurs on drainage (Kristensen and Trope, 1997). Although the formation of malignant ascites is not well understood, most investigators agree that the main factors are increased permeability of afferent vessels of the peritoneal lining, and reduced uptake by the efferent vessels (Nagy \textit{et al.}, 1993; Parsons \textit{et al.}, 1996). The result is net accumulation of fluids and other constituents of blood, including proteins. A preliminary report suggested that malignant murine ascites may contain up to 85% of the protein of serum (Nagy \textit{et al.}, 1993). Therefore, it seems possible that also NAbs would be present in malignant ascites. Indeed, it has been shown that human malignant ascites contains anti-adenovirus antibodies, mostly of the IgG class (Blackwell \textit{et al.}, 2000; Stallwood \textit{et al.}, 2000). Further, it has been shown that many of these antibodies are neutralizing and capable of blocking transduction with adenovirus (Blackwell \textit{et al.}, 2000; Stallwood \textit{et al.}, 2000; Hemminki \textit{et al.}, 2001).

Presently, it is unknown how well NAb levels in malignant ascites correlate with NAb levels in blood. Also, NAb induction after intraperitoneal adenoviral gene therapy has not been studied. These aspects could have relevance with regard to efficacy and scheduling of readministration. Finally, it has not been studied whether preexisting NAbs present in ascites have an effect on transgene expression in the context of administration of adenovirus to humans.

**MATERIALS AND METHODS**

**Patient samples**

Fifteen women with recurrent ovarian cancer were enrolled in a phase I trial, the purpose of which was to investigate the safety of intraperitoneal administration of an adenovirus encoding an intracellular antibody against ErbB2 (Alvarez \textit{et al.}, 2000a). Dose escalation was performed from $10^{11}$ to $10^{9}$ plaque-forming units (PFU), and a single intraperitoneal injection through a Tenckhoff catheter was performed in each case. Patient demographics, safety, toxicity, and gene transfer have been reported previously (Alvarez \textit{et al.}, 2000a). Malignant ascites and serum samples were collected from the patients on day 0 (immediately before treatment), day 2 (second day after treatment), and day 56 after treatment. On occasion, the ascites samples had to be aspirated with a small volume of saline. In many cases, ascites samples were also collected on day 14 for analysis of transgene expression. All patients did not have sam-
### Table 1. Neutralizing and Total Antibodies in Patients Receiving Intraperitoneal Adenovirus

<table>
<thead>
<tr>
<th>Patient&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Dose (PFU)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ascites NAb&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Serum NAb</th>
<th>Serum TAb&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Ascites NAb</th>
<th>Serum NAb</th>
<th>Serum TAb</th>
<th>Ascites NAb</th>
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<th>Serum TAb</th>
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<td>127</td>
<td>16,383</td>
<td>1.830</td>
<td>1,023</td>
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<tr>
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<td>0.380</td>
<td>1,023</td>
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<td></td>
</tr>
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<td>0.558</td>
<td>1</td>
<td>1</td>
<td>0.610</td>
<td>16,383</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>$3.3 \times 10^9$</td>
<td>31</td>
<td>127</td>
<td>0.650</td>
<td>63</td>
<td>127</td>
<td>0.530</td>
<td>1,023</td>
<td>&gt;31,768</td>
<td>0.880</td>
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<td>1.314</td>
<td>63</td>
<td>511</td>
<td>1.174</td>
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<td>31</td>
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</table>

<sup>a</sup>Patients are grouped by dose level; lettering is random for blinding analyses.

<sup>b</sup>PFU, Plaque-forming units.

<sup>c</sup>NAb, Neutralizing antibody titer.

<sup>d</sup>TAb, Total antibody titer (OD at 405 nm).
Preexisting neutralizing antibodies (NAbs) and correlation of preexisting NAbs with transgene expression. Five of 15 (33%) patients (columns C, D, E, H, and K) had >1000 ascites or serum NAb titer before treatment (A and B). Ascites (C and E) and serum (D and F) NAb titers and transgene expression levels were natural log transformed and plotted against each other (triangles). Spearman correlation coefficients \( r \) are shown with associated \( p \) values. The squares and solid lines represent the predicted values obtained by linear regression. A downward slope indicates inverse, albeit not statistically significant, correlation of transgene expression with baseline NAb titer.
amples available at all time points, but all available samples were analyzed. Cells were separated by centrifugation and the supernatant containing the soluble proteins was stored at –80°C. Total protein concentration was determined as described (Hemminki et al., 2001).

Analysis of antibody titers

For determination of the NAb titer, quadruplicates of SKOV3.ip1 cells (from J. Price, M.D. Anderson Cancer Center, Houston, TX) were plated into 96-well plates (10,000/well) and allowed to grow overnight before infection. A 2-fold dilution series of serum or ascites was prepared in OptiMEM (Media Preparation Shared Facility, University of Alabama at Birmingham [UAB]), in a normalized volume. Luciferase-expressing, nonreplicating Ad5Luc1 at 100 PFU/cell (Kanerva et al., 2002) was mixed with each dilution for 30 min. Then, using a multipipette, the mix was added to cells and infection was allowed to proceed for 2 hr. Infection medium was then replaced with growth medium. Luciferase expression was measured 48 hr later with cell culture lysis reagent and the luciferase assay system (Promega, Madison, WI), utilizing an Orion microplate luminometer (Berthold, Pforzheim, Germany) with Culturplate-96 (Research Parkway, Meriden, CT) according to the manufacturer’s suggestions. The raw data were measured for 5 sec/well. Analysis of total antibodies was performed by enzyme-linked immunosorbent assay (ELISA) and partial data have been reported (Alvarez et al., 2000a). Briefly, 96-well plates were coated with various doses of adenovirus and blocked with 1% bovine serum albumin. After washing, dilutions of serum were added, total anti-adenovirus antibodies were detected with goat anti-human IgG conjugated to alkaline phosphatase, and absorbance was detected at 405 nm.

Statistical analysis

Relationships between ascites NAb titers and transgene expression were examined by Spearman correlation coefficients after natural log transformations of both variables. Linear regression was performed to obtain the predicted transgene expression values. Separate comparisons were performed for serum and ascites NAbs and for each day. For testing the effect of dose on induction of NAbs, the NAb titer on day 56 was divided by the NAb titer on day 0, and the ratio was correlated to the administered dose as described above. Likewise, Spearman correlation coefficients and natural log transformation were utilized for comparison of total and NAb titer. SAS version 8.2 (SAS Institute, Cary NC) was used for all calculations and modeling.

RESULTS AND DISCUSSION

Serum and ascites samples were collected from patients with disseminated ovarian cancer and enrolled in an adenovirus gene therapy trial (Alvarez et al., 2000a). We developed a novel approach for rapid, reliable, and automatable NAb titer determination from large numbers of samples. Quadruplicates of serial dilutions were incubated with an adenovirus encoding luciferase followed by automated analysis of transgene expression in a multiwell luminometer. The smallest dilution factor that still allowed more than 50% transgene expression was set as the NAb titer (Fig. 1). In most cases, the NAb titer increased after administration of adenovirus, with dramatic induction seen by day 56 (Fig. 2). These findings are in accord with previous reports that have reported high levels of NAb induction in cancer patients after intrapleural, intravenous, or intratumoral ade-noviral gene therapy (Gahery-Segard et al., 1997, 1998; Molnar-Kimber et al., 1998; Harvey et al., 1999; Trask et al., 2000; DeWeese et al., 2001; Nemunaitis et al., 2001). However, this is the first report in which induction of NAbs has been studied after intraperitoneal administration.

One of the patients evaluable on day 56 did not display induction of high NAb titers (patient 1). Her titer increased only to 31 (Table 1). It has been suggested that advanced cancer may cause severe immune suppression and lack of antibody responses (Pollock and Roth, 1989). This may have caused the lack of NAb response in this patient.

Generally, the serum and ascites NAb titers correlated well with each other (day 0: r = 0.86, p = 0.0003; day 2: r = 0.95, p = 0.0008; day 56: r = 0.53, p = 0.36) (Fig. 2). This suggests that malignant ascites contains most of the NAbs present in serum, with relatively dynamic equilibrium maintained between the two fluid compartments, considering the good correlation seen already 2 days after treatment. Good correlation of serum and ascites NAbs could be clinically relevant when considering a patient with ascites for treatment by adenovirus-mediated therapy—it is often easier to test serum than ascites. Also, this finding suggests that if the preexisting NAb titer is high, it is probably useful to attempt to remove as much ascites as possible before treatment.

Total anti-adenovirus antibodies (TAbS) have been used as a measure of anti-adenovirus immunity. We compared the NAb titers with the serum TAb titers (Table 1). On day 0, there was no correlation (TAbS vs. ascites NAbs: r = –0.02, p = 0.96; TAbS vs. serum NAbs: r = –0.26, p = 0.38). After administration of the agent, some evidence of correlation was seen (day 2—TAbS vs. ascites NAbs: r = 0.48, p = 0.19; TAbS vs. serum NAbs: r = 0.61, p = 0.14; day 56—TAbS vs. ascites NAbs: r = 0.46, p = 0.43; TAbS vs. serum NAbs: r = 0.26, p = 0.61), but results were not statistically significant. Although the number of patients is too small to make definite conclusions, these results suggest that NAbs do not completely correlate with TAbS. Theoretically, NAbs are more relevant for gene transfer as they can functionally inhibit transduction and are thus the preferred measurement.

Conceivably, the administered dose could affect the level of NAb induction (titer on day 56/titer on day 0) (Molnar-Kimber et al., 1998). However, in accord with another report (Harvey et al., 1999), we did not see any evidence of this (ascites NAb induction vs. dose: r = –0.05, p = 0.91; serum NAb induction vs. dose: r = 0.18, p = 0.70) (Fig. 3). Instead, 88% of evaluable patients demonstrated high NAb levels on day 56 (Fig. 2). The patient whose NAb titers were not induced received the second highest dose (3.3 × 1010 PFU).

To investigate the correlation of NAb titer with the protein content of ascites and serum, total protein concentration was determined (Table 2). It was somewhat surprising that, on average, serum contained more than 4-fold more protein than as-
<table>
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<tr>
<th>Patient</th>
<th>Ascites</th>
<th>Serum</th>
<th>Serum/ascites ratio</th>
<th>Ascites</th>
<th>Serum</th>
<th>Serum/ascites ratio</th>
<th>Ascites</th>
<th>Serum</th>
<th>Serum/ascites ratio</th>
<th>Mean of ratios</th>
</tr>
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<tbody>
<tr>
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<td></td>
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<td></td>
<td></td>
<td>1.100</td>
<td></td>
<td></td>
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<tr>
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<td>1.245</td>
<td>4.405</td>
<td>3.538</td>
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cites (mean of serum:ascites total protein concentrations: day 0, 4.517; day 2, 4.125; day 56, 4.281). Although this result may be affected by the fact that occasionally the ascites sample was aspirated with saline, it seems unlikely that this would be the sole cause of the findings. Saline was used only in a proportion of the cases, and the highest protein concentration found in the ascites samples was 1.430 mg/ml, while the lowest concentration in the serum was 4.000 mg/ml. It has been suggested in an animal study that malignant ascites may contain up to 85% of the protein of plasma, whereas nonmalignant peritoneal fluid contains only 25% (Nagy et al., 1993). Our results suggest that in ovarian cancer patients, the total protein content of ascites is clearly lower than that of serum, and therefore the NAb titer is unrelated to total protein concentration. If confirmed, this finding could have relevance for investigations into the pathophysiology of malignant ascites formation. The difference between serum and ascites protein concentration suggests that ascites protein accumulation is selective in a currently unidentified manner.

Neutralizing antibodies can block infection with adenovirus, but the effect is dose dependent. Preclinical studies with ovarian cancer substrates have suggested that NAs in malignant ascites can block or reduce gene transfer to ovarian cancer cells in vitro (Blackwell et al., 2000; Stallwood et al., 2000; Hemminki et al., 2001) and in animal models (Hemminki et al., 2002). However, these aspects have not been investigated in humans. Thus, we studied the correlation between preexisting NAs and gene transfer. Preexisting NAb titers were variable, and 5 of 15 patients (C, D, E, H, and K) had titers of >1000 on day 0 (Fig. 3). An inverse but not statistically significant correlation was seen between preexisting ascites NAb titer and transgene expression (day 2: \( r = -0.34, p = 0.37 \); day 14: \( r = -0.63, p = 0.25 \) (Fig. 3). The same was true when serum NAs were compared with transgene expression (day 2: \( r = -0.39, p = 0.29 \); day 14: \( r = -0.70, p = 0.18 \)). In general, where evaluable, transgene expression levels were similar on days 2 and 14 (Alvarez et al., 2000a). Allowing for modest sample size, these results suggest that the preexisting NAb titer may reduce transgene expression, but perhaps not prevent it. This is in accord with other trials in which local administration was performed (Ganly et al., 2000; Nemunaitis et al., 2000; DeWeese et al., 2001; Kirn, 2001). Therefore, although there is little doubt that NAs affect adenoviral gene transfer, and may even block gene transfer in vitro, the current clinical evidence suggests that high NAb titers do not prevent gene transfer when local administration is utilized (Ganly et al., 2000; Nemunaitis et al., 2000; DeWeese et al., 2001; Kirn, 2001). Saturating the neutralizing capacity of plasma due to the high concentrations of virus achieved by local administration may have contributed to this.

Intravascular administration of adenovirus has been endeavors in a few trials, and gene transfer in general has been low. Nevertheless, the available anecdotal evidence suggests that high NAb titers do not prevent gene delivery (Kirn, 2001; Nemunaitis et al., 2001; Reid et al., 2001). However, as demonstrated in an experimental model of vascular delivery (Chen et al., 2000), it seems intuitive that high NAb titers would affect the level of transduction. Interestingly, the same study also suggested that high NAb titers reduce toxicity. To understand the relationship between these variables in humans, it is crucial to perform clinical trials in a manner allowing comprehensive analysis of gene transfer and immune responses. In case further studies show that high NAb titers can indeed significantly affect clinical gene transfer, ways to overcome neutralization have been suggested. Modification of the adenovirus capsid has been shown to allow avoidance of preexisting NAs (Blackwell et al., 2000; Hemminki et al., 2001, 2002; Kanerva et al., 2002). Alternatively, using differential polyethylene glycosylation could allow reduced neutralization (Croyle et al., 2001). Further, an adenoviral capsid protein column, capable of removing anti-adenoviral antibodies from plasma, has been developed (Chen et al., 2000).

In conclusion, we have measured the serum and ascites NAb titers of ovarian cancer patients enrolled in an adenovirus gene therapy trial. The preexisting NAb titer was variable and a third of the patients had a titer >1000. High preexisting NAb titers did not significantly affect gene transfer, although some evidence of lower efficacy was seen. In 88% of patients, treatment resulted in induction of high NAb titers by day 56, although induction was already often seen after 2 days. Dose did not affect the level of induction. Serum and ascites NAs correlated well with each other, and NAs seem more reliable than TAs for predicting gene transfer. This is the first study of the association of NAs and intraperitoneal gene therapy. The results suggest that preexisting NAs are not likely to prevent the success of intraperitoneal adenovirus gene therapy, but, because of the rapid induction of high intraperitoneal NAb titers even in patients with advanced cancer, early instead of delayed readministration may be advisable. Finally, removal of ascites, perhaps followed by lavage, could minimize interference by NAs and therefore increase the efficacy of adenovirus-based intraperitoneal gene therapy.

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SERUM AND ASCITES NEUTRALIZING ANTIBODIES


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