Clinical Studies

Oncolytic Adenovirus Coding for Granulocyte Macrophage Colony-Stimulating Factor Induces Antitumoral Immunity in Cancer Patients

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Abstract
Granulocyte macrophage colony-stimulating factor (GMCSF) can mediate antitumor effects by recruiting natural killer cells and by induction of tumor-specific cytotoxic T-cells through antigen-presenting cells. Oncolytic tumor cell–killing can produce a potent costimulatory danger signal and release of tumor epitopes for antigen-presenting cell sampling. Therefore, an oncolytic adenovirus coding for GMCSF was engineered and shown to induce tumor-specific immunity in an immunocompetent syngeneic hamster model. Subsequently, 20 patients with advanced solid tumors refractory to standard therapies were treated with Ad5-D24-GMCSF. Of the 16 radiologically evaluable patients, 2 had complete responses, 1 had a minor response, and 5 had disease stabilization. Responses were frequently seen in injected and noninjected tumors. Treatment was well tolerated and resulted in the induction of both tumor-specific and virus-specific immunity as measured by ELISPOT and pentamer analysis. This is the first time that oncolytic virus–mediated antitumor immunity has been shown in humans. Ad5-D24-GMCSF is promising for further clinical testing. Cancer Res; 70(11); 4297–309. ©2010 AACR.

Introduction
New approaches are needed for the treatment of metastatic solid tumors. One strategy is oncolytic viruses, which selectively replicate in and kill tumor cells (1–3). Replication leads to effective local amplification and the virus could also enter the blood-stream for transduction of the same tumor or metastases. Oncolytic replication is an immunogenic phenomenon, and recent preclinical and clinical evidence suggests that antitumor efficacy is partially mediated by the immune response (4, 5). Adenoviruses are among the most advanced cancer gene therapy platforms and their safety and efficacy has been validated in several randomized studies (6–9). With regard to oncolytic adenoviruses, a few dozen trials have been performed and although there is anecdotal evidence of activity in most trials, overall single-agent efficacy has been nonimpressive. This is partially due to the attenuated nature of the few agents tested thus far. However, even such early generation viruses seem quite effective when combined with standard therapy (10). In the only randomized study performed thus far, combining oncolytic adenovirus to chemotherapy doubled the response rate (8).

Adenoviruses are quite immunogenic (11), and recent preclinical data suggests that this might be a key aspect of their antitumor activity (4). In part because of the lack of suitable animal models, the combination of oncolytic adenoviruses and immunotherapy has not been explored in detail. However, it is known that the replication-deficient adenovirus is a useful vaccination agent due to its unparalleled capacity for gene delivery, high titer production, and immunogenicity (12). Nevertheless, because the antitumor immune response induced by adenovirus replication per se is usually not sufficient for clinical responses, arming oncolytic viruses with immunostimulatory molecules is attractive.

Although regularly present at tumors, the frequency and activity of CD8+ T cells is often too low to mount an effective response. This might be caused partly by the immunosuppressive environment of tumors (13). However, preclinical data suggests that by increasing costimulatory "danger" signals, immunosuppressive mechanisms could be overridden (4, 13,
Oncolytic replication provides strong danger signals and lysis of tumor cells releases tumor-associated antigens for sampling (15). Nevertheless, this may be insufficient in the absence of specific activation of antigen-presenting cells (13).

Granulocyte macrophage colony-stimulating factor (GMCSF) is among the most potent inducers of antitumor immunity (16). It acts through several mechanisms, including direct recruitment of natural killer cells and antigen-presenting cells such as dendritic cells (17, 18). GMCSF could also specifically activate dendritic cells at the tumor site to increase their expression of costimulatory molecules to enhance cross-priming and T-cell activation rather than cross-tolerance. Although these effects are well studied in preclinical systems, and there is emerging data from cancer vaccine trials, the immunologic aspects of oncolytic viruses in cancer patients are not well understood. For example, given the dogma of tumor immunosuppression, it is unknown if oncolytic viruses could induce virus- or tumor-specific T-cell responses.

Here, we generated an oncolytic adenovirus armed with GMCSF. Following preclinical testing, 20 patients with advanced and treatment-refractory solid tumors were treated. This is the first time that antitumoral immunity has been shown to result from oncolytic virus treatment of human cancer patients.

Materials and Methods

Adenoviruses

Ad5-D24-GMCSF was generated and amplified using standard adenovirus preparation techniques (19). Briefly, human GMCSF (Invitrogen) was amplified with specific primers featuring insertion of specific restriction sites Sall/MunI. GMCSF was then subcloned into pTHSN and subsequently recombined with an Ad5 rescue plasmid to generate pAd5-D24-GMCSF. This plasmid was linearized with Pael and transfected into A549 cells for amplification and rescue.

All phases of the cloning were confirmed with PCR and multiple restriction digestions. The shuttle plasmid pTHSN-GMCSF was sequenced. The absence of wild-type E1 was confirmed with PCR. The E1 region, transgene, and fiber were checked in the final virus with sequencing and PCR. To this end, viral DNA was extracted and PCR and sequencing was performed to analyze the integrity of the fiber and GMCSF cDNA. All phases of the virus production were done on A549 cells to avoid risk of wild-type recombination. GMCSF is under the E3 promoter, which results in replication-associated transgene expression starting ∼8 hours after infection. E3 is intact except for deletion of 6.7K/gp19K.

The biodistribution of adenovirus is determined by the capsid and Ad5-based viruses have been studied extensively previously (20). Toxicity studies with oncolytic Ad5-based adenoviruses have been performed previously in mice, hamsters, and cotton rats (20). Ad5-D24-E3 and Ad5Luc1 have been published previously (19).

Cell lines

Hamster pancreatic carcinoma–derived cell line HaP-T1 was kindly provided by Dr. Ruben Hernandez-Alcocera (Pamplona, Spain). The kidney hamster–derived cell line HaK was courtesy of Prof. William S.M. Wold (St. Louis, MO). These cell lines were maintained in 10% DMEM with 1% pen/strep and 1% L-glutamine. Human transformed embryonal kidney cell line 293 was purchased from Microbix, and human lung adenocarcinoma cell line A549 and TF1 were from the American Type Culture Collection.

Animals

All animal protocols were reviewed and approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Syrian hamsters (Mesocricetus auratus) were obtained from Taconic at 4 to 5 weeks of age and quarantined for at least 1 week prior to the study. Nude mice were purchased from Taconic. The health status of the animals was frequently monitored and as soon as any sign of pain or distress was evident, they were killed.

Patients

Nineteen patients with advanced metastatic tumors refractory to conventional therapies were treated with a single round of Ad5-D24-GMCSF (Table 1). Inclusion criteria were solid tumors refractory to conventional therapies, WHO performance score 3 or less, and no major organ function deficiencies. Exclusion criteria were organ transplant, HIV, severe cardiovascular, and metabolic or pulmonary disease (e.g., symptomatic coronary heart disease, uncontrolled blood pressure). Written informed consent was obtained and the study was completed according to Good Clinical Practice guidelines and the Declaration of Helsinki. This compassionate use scheme was evaluated by the Medicolegal Department of the Finnish Ministry of Social Affairs and Health and the Gene Technology Board.

Treatment protocol

Patients received a single round of treatment with Ad5-D24-GMCSF on day 0. Virus administration was performed by ultrasound-guided intratumoral or intracavitary (ovarian cancer, mesothelioma) injection, and one fifth of the dose was given i.v. The starting dose of 8 × 109 viral particles (VP) was chosen based on safety results published by others (1, 5–9, 21). Subsequently, the dose was escalated to 1 × 1010, 3.6 × 1010, 1 × 1011, 2 × 1011, 2.5 × 1011, 3 × 1011, and 4 × 1011 (Table 2). Patients were monitored for 24 hours in the hospital and for the following 4 weeks as outpatients. Follow-up for survival was performed for a maximum of 18 months. Physical assessment and medical history were done at each visit. Side effects were recorded according to CTCEA 3.0. Because many cancer patients have symptoms due to disease, preexisting symptoms were not scored if they did not become worse. However, if the symptom became more severe, e.g., pretreatment grade 1 changed to grade 2 after treatment, it was scored as grade 2.

Tumor size was assessed by contrast-enhanced computer tomography scanning typically performed at 6 weeks. Maximum tumor diameters were obtained and in some liver metastatic cases, Hounsfield units (density estimate) could also be counted (22). Response Evaluation Criteria in Solid Tumors (RECIST) were applied to overall disease, including...
injected and noninjected lesions. These criteria include complete response, partial response (>30% reduction in the sum of tumor diameters), stable disease (i.e., no response/progression), and progressive disease (>20% increase; refs. 23, 24). Serum tumor markers were also evaluated when elevated at baseline. The same percentages were used except that 12% to 29% reductions are indicated as minor responses.

A panel of three different proinflammatory cytokines (interleukin-6, interleukin-8, and tumor necrosis factor-α) were analyzed by BD Cytometric Bead Array Human Soluble Protein Flex Set (Becton Dickinson) according to the instructions of the manufacturer. In addition, subjective symptoms were collected by the treating physicians. For all patients, serum levels of GMCSF were also measured.

**Neutralizing antibody titer**

293 cells were seeded at $1 \times 10^4$ cells/well on 96-well plates and cultured overnight. Next day, cells were washed with DMEM without FCS. Serum samples were incubated at 56°C for 90 minutes to inactivate complement, and a 4-fold dilution series (1:1 to 1:16,384) was prepared in serum-free DMEM. Ad5lac1 (20) was mixed with serum dilutions and incubated at room temperature for 30 minutes. Thereafter, cells in triplicates were infected with 100 VP/cell in 50 µL of mix, and

**Table 1. Patients at baseline**

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Oncologic therapies prior to Ad5/3-D24-GMCSF</th>
<th>WHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3</td>
<td>30</td>
<td>M</td>
<td>Hepatocellular cancer</td>
<td>Surgery, 5-fluorouracil, folic acid, cisplatin, epirubicin, gemcitabine, capecitabine, oxaliplatin, IFNs, vinorelbine, erlotinib, raltitrexed</td>
<td>1</td>
</tr>
<tr>
<td>C3</td>
<td>57</td>
<td>F</td>
<td>Jejunum cancer</td>
<td>Surgery, irinotecan, 5-fluorouracil, leucovorin, oxaliplatin, bevacizumab, capecitabine, radiation</td>
<td>1</td>
</tr>
<tr>
<td>R8</td>
<td>60</td>
<td>F</td>
<td>Breast cancer</td>
<td>5-fluorouracil, cyclophosphamide, epirubicin, tamoxifen, docetaxel, capecitabine, paclitaxel, gemcitabine, vinorelbine, carboplatin, radiation</td>
<td>2</td>
</tr>
<tr>
<td>O12</td>
<td>28</td>
<td>F</td>
<td>Ovarian cancer</td>
<td>Surgery, docetaxel, carboplatin, paclitaxel, cisplatin, gemcitabine, doxorubicin, etoposide</td>
<td>1</td>
</tr>
<tr>
<td>O14</td>
<td>57</td>
<td>F</td>
<td>Ovarian cancer</td>
<td>Surgery, 5-fluorouracil, cyclophosphamide, epirubicin, docetaxel, paclitaxel, carboplatin, bevacizumab, erlotinib, topotecan</td>
<td>1</td>
</tr>
<tr>
<td>G15</td>
<td>71</td>
<td>M</td>
<td>Gastric cancer</td>
<td>Epirubicin, oxaliplatin, capecitabine, docetaxel</td>
<td>1</td>
</tr>
<tr>
<td>K18</td>
<td>62</td>
<td>M</td>
<td>Non–small cell lung cancer</td>
<td>Radiation, cisplatin, docetaxel, carboplatin, paclitaxel, gemcitabine, erlotinib</td>
<td>2</td>
</tr>
<tr>
<td>T19</td>
<td>55</td>
<td>F</td>
<td>Medullar thyroid cancer</td>
<td>Surgery, radiation (including $^{131}$I), dacarbazine, somatostatin analogues, IFNs</td>
<td>2</td>
</tr>
<tr>
<td>M32</td>
<td>62</td>
<td>M</td>
<td>Mesothelioma</td>
<td>Cisplatin, erlotinib, vinorelbine</td>
<td>1-2</td>
</tr>
<tr>
<td>X49</td>
<td>60</td>
<td>F</td>
<td>Cervical cancer</td>
<td>Surgery, radiation, cisplatin, paclitaxel, carboplatin, topotecan, bevacizumab</td>
<td>2</td>
</tr>
<tr>
<td>M50</td>
<td>60</td>
<td>M</td>
<td>Mesothelioma</td>
<td>Radiation, cisplatin, erlotinib</td>
<td>1</td>
</tr>
<tr>
<td>IS2</td>
<td>58</td>
<td>F</td>
<td>Melanoma</td>
<td>Surgery, dacarbazine, vincristine, lomustine, bleomycin, IFNs, iplimumab, radiation</td>
<td>2</td>
</tr>
<tr>
<td>C58</td>
<td>54</td>
<td>M</td>
<td>Colon cancer</td>
<td>Bevacizumab, 5-fluorouracil, leucovorin, oxaliplatin, irinotecan, capectabine</td>
<td>2</td>
</tr>
<tr>
<td>R73</td>
<td>58</td>
<td>F</td>
<td>Breast cancer</td>
<td>Surgery, cyclophosphamide, epirubicin, 5-fluorouracil, vinorelbine, tamoxifen, anastrozole, letrozole, docetaxel</td>
<td>1</td>
</tr>
<tr>
<td>I78</td>
<td>60</td>
<td>F</td>
<td>Choroidal melanoma</td>
<td>Surgery, dacarbazine, IFNs, temozolomide, cisplatin, doxorubicin</td>
<td>0</td>
</tr>
<tr>
<td>O88</td>
<td>59</td>
<td>F</td>
<td>Ovarian cancer</td>
<td>Paclitaxel, carboplatin, doxorubicin, gemcitabine, topotecan, vinorelbine, docetaxel, etoposide</td>
<td>3</td>
</tr>
<tr>
<td>U89</td>
<td>60</td>
<td>M</td>
<td>Renal cancer</td>
<td>Surgery, IFNs, sunitinib, sorafenib, radiation</td>
<td>1</td>
</tr>
<tr>
<td>S100</td>
<td>63</td>
<td>F</td>
<td>Leiomyosarcoma</td>
<td>Surgery, radiation, doxorubicin, ifosfamide, docetaxel, gemcitabine, trabectedin, oxaliplatin</td>
<td>2</td>
</tr>
<tr>
<td>S108</td>
<td>42</td>
<td>M</td>
<td>Synovial sarcoma</td>
<td>Surgery, doxorubicin, ifosfamide, doxorubicin, docetaxel, paclitaxel, topotecan, bevacizumab, radiation</td>
<td>2</td>
</tr>
<tr>
<td>O9</td>
<td>61</td>
<td>F</td>
<td>Ovarian cancer</td>
<td>Paclitaxel, gemcitabine, carboplatin, doxorubicin, topotecan, Ad5/3-Cox2L-D24 $2 \times 10^{10}$ VP as first viral treatment</td>
<td>1</td>
</tr>
</tbody>
</table>

**NOTE:** Description of patient characteristics at baseline: age, sex, primary tumor, WHO performance score, and summary of previous therapies.
Table 2. Summary of treatment doses, antibody titer, viral load in serum and treatment response

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Dose (VP)</th>
<th>Primary Tumor</th>
<th>Neutralizing antibody titer</th>
<th>Virus load in serum</th>
<th>Response</th>
<th>RECIST*</th>
<th>Density/other</th>
<th>Marker</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Week posttreatment</td>
<td>Days posttreatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0  1  2  3  4</td>
<td>0  1  2  3–7  8–12 21–40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>8 × 10^9</td>
<td>Jejunum cancer</td>
<td>0  1,024</td>
<td>16,834</td>
<td>0  0  &lt;500  &lt;500  0  0  0</td>
<td>MR 120</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>1 × 10^{10}</td>
<td>HCC</td>
<td>0  16,384</td>
<td>4,096</td>
<td>0  0  &lt;4,896  0  0  0  0  0</td>
<td>SD (+5.2%)</td>
<td>548†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O12</td>
<td>3.6 × 10^{10}</td>
<td>Ovarian cancer</td>
<td>0  16,384</td>
<td>16,384</td>
<td>0  0  &lt;500  0  0  0  0  0</td>
<td>SD (+7.7%)</td>
<td>106</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O14</td>
<td>1 × 10^{10}</td>
<td>Ovarian cancer</td>
<td>64  64</td>
<td>16,384</td>
<td>0  0  0  &lt;500  0  0  0  0</td>
<td>CR (&lt;100%)</td>
<td>528†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G15</td>
<td>1 × 10^{10}</td>
<td>Gastric cancer</td>
<td>1,024 16,384</td>
<td>16,384</td>
<td>0  0  565  &lt;500  0  0  0  0</td>
<td>–4.6%</td>
<td>308†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K18</td>
<td>2 × 10^{10}</td>
<td>NSCLC</td>
<td>16,384 16,384</td>
<td>16,384</td>
<td>0  &lt;500  0  0  0  856  0  0</td>
<td>PD (+15%)</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T19</td>
<td>2 × 10^{10}</td>
<td>Thyroid cancer</td>
<td>0  16,384</td>
<td>0  0  0  765  &lt;500  0  0  0</td>
<td>SD (&lt;8.9%)</td>
<td>490†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U89</td>
<td>2 × 10^{10}</td>
<td>Renal cancer</td>
<td>64  16,384</td>
<td>0  0  0  0  0  0  0  0</td>
<td>PD (&lt;13%)</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S100</td>
<td>2 × 10^{10}</td>
<td>Leiomysarcoma</td>
<td>0  0  16,384</td>
<td>16,384</td>
<td>0  &lt;500  &lt;500  0  0  0  0  0</td>
<td>PD (+39%)</td>
<td>121</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S108</td>
<td>2 × 10^{10}</td>
<td>Synovial sarcoma</td>
<td>0  0  256</td>
<td>0  0  0  0  0  0  0  0</td>
<td>PD (&lt;59%)</td>
<td>74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M50</td>
<td>2.5 × 10^{11}</td>
<td>Mesothelioma</td>
<td>256  16,384</td>
<td>16,384</td>
<td>0  0  &lt;500  &lt;500  0  0  0  0</td>
<td>CR (&lt;100%)</td>
<td>403†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R8</td>
<td>3 × 10^{11}</td>
<td>Breast cancer</td>
<td>64  16,384</td>
<td>16,384</td>
<td>0  &lt;500  &lt;500  0  0  0  0  0</td>
<td>PR (&lt;44%)</td>
<td>112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M32</td>
<td>3 × 10^{11}</td>
<td>Mesothelioma</td>
<td>0  0  256 16,384</td>
<td>16,384</td>
<td>0  0  0  0  0  0  0  0</td>
<td>PD&lt;sup&gt;3&lt;/sup&gt;</td>
<td>125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X49</td>
<td>3 × 10^{11}</td>
<td>Cervical cancer</td>
<td>16  4,096 1,024</td>
<td>0  4,290 1,211</td>
<td>1,024  0  0  0  0  0  0  0</td>
<td>PD (+55%)</td>
<td>92</td>
<td>–27%</td>
<td>112</td>
</tr>
<tr>
<td>I52</td>
<td>3 × 10^{11}</td>
<td>Melanoma</td>
<td>0  256 16,384</td>
<td>256  0  0  0  0  0  0  0</td>
<td>PD (+25%)</td>
<td>129</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I78</td>
<td>3 × 10^{11}</td>
<td>Choroidal</td>
<td>0  0  64</td>
<td>16,384</td>
<td>16,384 0  0  0  0  0  0  0</td>
<td>PD&lt;sup&gt;3&lt;/sup&gt;</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C58</td>
<td>4 × 10^{11}</td>
<td>Colon cancer</td>
<td>256  16,384</td>
<td>16,384</td>
<td>0  0  1,978  4,236 0  0  0  0</td>
<td>PD (+37%)</td>
<td>118</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R73</td>
<td>4 × 10^{11}</td>
<td>Breast cancer</td>
<td>0  256 1,024</td>
<td>1,024 0  0  0  0  0  0  0  0</td>
<td>SD (&lt;3.6%)</td>
<td>245†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O88</td>
<td>4 × 10^{11}</td>
<td>Ovarian cancer</td>
<td>0  1,024</td>
<td>0  0  0  0  0  0  0  0</td>
<td>PD (+37%)</td>
<td>118</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O9&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2 × 10^{11}</td>
<td>Ovarian cancer</td>
<td>16,384 16,384</td>
<td>0  0  2,133&lt;sup&gt;5&lt;/sup&gt; 0  0  0  0  0</td>
<td>MR (&lt;20%)</td>
<td>142</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: CR indicates complete disappearance of measurable tumors as defined by RECIST criteria. SD indicates stable disease, whereas PD is progressive disease (more than 20% increase in sum of tumor diameters). With regard to tumor markers, CR indicates reduction of the marker to within normal limits. MR indicates a 12% to 29% decrease in marker, whereas PR is a more than 30% decrease. Best response indicated. SD indicates stabilization of the tumor marker (no progression or response). Decrease in tumor density has been suggested to correlate with antitumor efficacy. Abbreviations: HCC, hepatocellular carcinoma; NSCLC, non–small cell lung cancers.

<sup>1</sup>Percentages indicate actual overall change in total tumor diameter.
<sup>2</sup>Patients still alive at the time of follow-up cutoff.
<sup>3</sup>Percentage not measurable.
<sup>4</sup>Thinning of carcinomatosis (not measurable with RECIST).
<sup>5</sup>Second treatment with oncolytic virus. Patient was treated with Ad5/3-Cox2L-D24(40) 9 weeks earlier (PD).
<sup>6</sup>Refer to Ad5-D24-GMCSF specifically.
100 μL of growth medium with 10% FCS was added 1 hour later. Twenty-four hours postinfection, cells were lysed and luciferase activity was measured with Luciferase Assay System (Promega) using TopCount luminometer (Perkin-Elmer). To evaluate the effect of neutralizing antibodies in the serum of patients treated with Ad5-D24-GMCSF, luciferase readings were plotted relative to gene transfer achieved with Ad5Luc1 alone. The neutralizing antibody titer was determined as the lowest degree of dilution that blocked gene transfer by >80%.

**Determination of virus in serum samples**

Total DNA was extracted by adding 3 μg of carrier DNA (polydeoxyadenylic acid; Roche) to 400 μL of serum and using the QIAamp DNA mini kit. Extracted DNA was eluted in 60 μL of nuclease-free water and DNA concentration was measured by spectrophotometry. PCR amplification was based on primers and probe targeting the E1A region flanking the 24 bp deletion (forward primer, 5′-TCCGTTTCTATGCAAAACCT-3′; reverse primer, 5′-TCCCTCCGGTAGAATGACAAAGA-3′; and probe onco 5′-FAM-TGATCGATCCACCCAGTGA-3′MGBNFQ). In addition, a probe complementary to a sequence included in the 24 bp region targeted for deletion was used to test the samples for the presence of wild-type adenovirus infection (probe wt 5′VIC-TACCTGCCACGAGCT-3′MGBNFQ).

The real-time PCR conditions for each 25 μL reaction were as follows: 2× LightCycler480 Probes Master Mix (Roche), 800 nmol/L of each forward and reverse primer, 200 nmol/L of each probe, and 250 ng of extracted DNA. PCR reactions were carried out in a LightCycler (Roche) under the following cycling conditions: 10 minutes at 95°C, 50 cycles of 10 seconds at 95°C, 30 seconds at 62°C, 20 seconds at 72°C, and 10 minutes at 40°C. All samples were tested in duplicate. TaqMan exogenous internal positive control reagents (Applied Biosystems) were used in the same PCR runs to test each sample for the presence of PCR inhibitors. A regression standard curve was generated using DNA extracted from serial dilutions of Ad5-D24-GMCSF (1 × 10^6—10 VP/mL) in normal human serum. The limit of detection and limit of quantification for the assay were 500 VP/mL of serum. Positive samples were confirmed by real-time PCR using LightCycler480 SYBR Green I Master mix (Roche) and primers specific for adenovirus and GMCSF sequences (forward primer, 5′-AAAACCCACCTCCTCTTACTG-3′; and reverse primer, 5′-TCATT-CATCTCAGCAGCAGT-3′).

**ELISPOT and flow cytometry analysis**

Peripheral blood mononuclear cells (PBMC) were isolated by Percoll gradient according to standard protocols. Cells were immediately frozen in CTL-CryoABC serum-free media (Cellular Technology, Ltd.) for further ELISPOT and flow cytometry analyses. ELISPOT was performed on a Becton Dickinson instrument (FACSCalibur). The following antibodies were used: human CD3 PerCP-labeled (Becton Dickinson), human CD8 clone LTB FITC-labeled (Promimmune), and for survivin, Pro5 Pentamer A*0201-MLMGEFLKL PE-labeled (Promimmune). For adenoviruses, a custom-made PE-labeled tetramer was ordered from Glycotope A*0201-GLYRBSML. Monoclonal antibody W6/36 (Mabtech) was used to selectively block MHC class I presentation.

**Statistical analysis**

Statistical tests were performed with SPSS 15.0 and GraphPad Prism version 4.00 for Mac (GraphPad Software). Mann-Whitney test was used in the animal experiments. t tests were used to assess significance in the in vitro assays.

**Results**

**In vitro and in vivo characterization of Ad5-D24-GMCSF**

Ad5-D24-GMCSF was generated by replacing viral gp19k and 6.7k with GMCSF (Supplementary Fig. S1A). This strategy couples transgene expression with viral replication to ensure high expression of the transgene at the tumor site (25). The virus replicates in a tumor-selective manner, thus resulting in low systemic expression of GMCSF. The tumor specificity of the virus was achieved with a 24 bp deletion, which abrogates the retinoblastoma (Rb)-binding site of E1A. Previous reports have shown that this results in selective virus replication in cells with p16-Rb pathway defects, including most if not all human cancers (19).

Ad5-D24-GMCSF had oncolytic potency in all tested cell lines, and in most cases, we observed no significant differences between Ad5-D24-E3 and Ad5-D24-GMCSF (Supplementary Fig. S2A–C). Therefore, replacement of gp19k and 6.7k with GMCSF did not seem to reduce the oncolytic potency of the virus significantly.

GMCSF secretion in vitro was seen when the virus replicated (Supplementary Fig. S3A), but not when UV-inactivated virus was used (data not shown). To show the functionality of virus-derived GMCSF, we cultured the TF1 cell line (26), whose growth is strictly dependent on fully functional human GMCSF, with either the commercial GMCSF or with supernatant from cells infected with Ad5-D24-GMCSF. Although cells cultured without GMCSF started to die at day 3, the presence of GMCSF or supernatant allowed cells to grow (Supplementary Fig. S3B). GMCSF expression was also observed in tumor-bearing mice (Supplementary Fig. S3C).

In accord with the in vitro results, we did not see any significant difference between Ad5-D24-E3 and Ad5-D24-GMCSF in vivo, suggesting that the expression of the transgene does not impair the efficacy of the virus (Supplementary Fig. S3D). Human GMCSF has no biological activity in mice and thus it did not increase the efficacy of the virus.

To characterize the potency of Ad5-D24-GMCSF in an immuno-incompetent environment, we grew syngeneic tumors in Syrian hamsters, which have been reported to be semi-permissive for human adenovirus (27). Interestingly, previous data also
suggests the activity of human GMCSF in hamsters (28, 29). Tumors were eradicated in animals treated with both Ad5-D24-E3 and Ad5-D24-GMCSF within 16 days (Fig. 1A).

Fifteen days after tumor eradication, hamsters were challenged with the same cell line (HapT1) or a different syngeneic cell line (HaK) without further virus treatment. Interestingly, all animals in which HapT1 tumors had been eradicated with Ad5-D24-GMCSF rejected HapT1 but none rejected HaK tumors, suggesting that GMCSF expression from the virus had enhanced tumor-specific immunity (Fig. 1B and C). In animals in which HapT1 tumors had been eradicated with Ad5-D24-E3, we observed delayed HapT1 tumor growth, suggesting that oncolytic adenovirus replication per se could induce a degree of immunity, but not enough to protect from tumor challenge (Fig. 1B).

Safety of Ad5-D24-GMCSF in cancer patients
Treatments were generally well tolerated up to $4 \times 10^{11}$ VP, and no grade 4 to 5 side effects were observed (Supplementary Table S1). Most patients experienced grade 1 to 2 flu-like symptoms, including fever (14 of 19), chills (5 of 19), fatigue (7 of 19), and injection site pain (8 of 19). Patient O88 experienced temporary worsening of intestinal function (grade 3 ileus and pain). Intestinal problems are quite common in ovarian cancer patients with peritoneal metastatic disease and this patient had similar symptoms prior to virus administration.

As the levels of certain cytokines (e.g., interleukin-6) have been suggested to predict adenovirus-mediated toxicity (30, 31), it may be of importance that only minor elevations were seen (Supplementary Fig. S4; Supplementary Table S2).

Figure 1. Oncolytic adenoviruses eradicate pancreatic tumors in Syrian hamsters eliciting a tumor-specific immune response. A, 15 Syrian hamsters were inoculated s.c. on both flanks with $7 \times 10^6$ HapT1 cells. Animals were treated with $1 \times 10^9$ VP on days 0, 2, and 4, growth media was used as control. Syrian hamsters have been reported to be semi-permissive for replication of human adenovirus and activity of human GMCSF. B, animals previously treated with Ad5-D24-E3 or Ad5-D24-GMCSF were rechallenged with the same tumor (HapT1) and tumor growth was measured over time. C, animals with HapT1 tumors previously treated with Ad5-D24-E3 or Ad5-D24-GMCSF were rechallenged with a different tumor (HaK) and tumor growth was measured over time. For statistics, Mann-Whitney test was performed with GraphPad Prism version 4.00 for Mac (GraphPad Software, http://www.graphpad.com/; *, $P < 0.05$).
Therefore, the doses used might have been well within the therapeutic window for this virus.

**Presence of Ad5-D24-GMCSF and GMCSF in serum**

Because injected virus is rapidly cleared, the extended presence of virus genomes in serum has been suggested to indicate virus replication (32–35). All patients were negative for Ad5-D24-GMCSF before treatment. Two days or more after treatment, virus was detected in the blood of 15 of 18 cases (Table 2).

Systemic levels of GMCSF were low, which was in accord with the absence of significant effects on total WBC counts, suggesting effective restriction of GMCSF production to the tumor (Supplementary Fig. S4).

**Neutralizing antibodies**

Neutralizing antibody titers were positive in 8 of 19 cases (42%) at baseline (Table 2). Titers increased within 2 weeks in most patients regardless of viral dose. As suggested by others, no clear correlation was seen between neutralizing antibody titers and viral dose, antitumor activity, or toxicity (8, 32, 34, 36, 37). However, with regard to virus replication and antibody titers, it is interesting that the antibody titer of X49 increased to only 4,096 in 1 week and decreased thereafter, and she had high and persistent amounts of virus in her blood. The patient (I78) who showed the highest serum virus load had no antibodies at baseline, and at 4 weeks, had a titer of only 64. These two cases would seem to suggest that some cancer patients are indeed immunosuppressed to some degree which compromised their antibody production, leading to enhanced virus replication. In contrast, patient C58 also featured high serum virus load, but had antibodies at baseline with an increase to maximum in 1 week.

**Efficacy of Ad5-D24-GMCSF**

Fifteen patients were evaluated for radiologic responses (Table 2; Fig. 2). Two patients (13%) had complete eradication of all measurable disease (Fig. 2). In addition, five patients (33%) had stabilization of disease progressing before treatment. Therefore, the clinical benefit rate was 47% according to RECIST criteria. The duration of disease stabilization was 98, 106, 490, 398, and 245 days for M3, O12, T19, M50, and R73, respectively. Disease stabilization is a surrogate endpoint and thus further work is required to evaluate if this translated into actual patient benefits (38).

Previous reports suggest that a decrease in tumor density might correlate with antitumor efficacy (22). This was measurable in two patients, and in both cases, the density decreased (by 4.6% and 27%; Table 2). In addition, a nonmeasurable decrease of the intraperitoneal tumor was detected in one patient (O88). There was no detectable difference in response between injected and noninjected tumors. In fact, noninjected tumors sometimes decreased more clearly than injected lesions (Fig. 2A, patient M50). RECIST criteria takes into account both injected and noninjected tumors and therefore response or stabilization of disease previously progressing suggests a systemic antitumor effect (23, 24). Interestingly, about one third of the treated patients seemed to gain long-term survival (Fig. 2B).

**Second treatment with Ad5-D24-GMCSF resulted in rapid tumor reduction**

Encouraged by the results in the 19 patients who received Ad5-D24-GMCSF as their first oncolytic virus treatment, we treated one additional patient who had received another oncolytic virus (39) 9 weeks earlier. Subsequent to Ad5-D24-GMCSF treatment, she had a 52% reduction in tumor volume (20% decrease according to RECIST) in only 17 days (Fig. 2A, patient O9). After 24 hours, there was 2,133 VP/mL of Ad5-D24-GMCSF present in her serum (Table 2).

**Effect of Ad5-D24-GMCSF administration on WBC compartments**

GMCSF levels in the serum were monitored, but increase over baseline was observed in only three patients with a maximum level of 70 pg/mL, suggesting a restriction of GMCSF production in the tumor sites. No increases in WBC were seen (Supplementary Fig. S4). The complete phenotypic panel of circulating WBC was evaluated (Supplementary Table S3). Interestingly, 9 of 10 patients showed an increase in the total number of CD8+ T cells following treatment (Supplementary Fig. S5). Hence, we sought to investigate whether these cells were specific for adenovirus or if Ad5-D24-GMCSF could also stimulate a tumor-specific response.

**Adenovirus-specific T-cell response in cancer patients treated with Ad5-D24-GMCSF**

The tumor microenvironment has been thought to be immunosuppressive, which might work against induction of immunity at the tumor (15). However, because we saw an increase in total number of CD8+ T cells, and inspired by preclinical data (4, 13), we investigated adenovirus- and tumor-specific T-cell responses. For triggering T cell-specific immune responses, peptides need to be presented as MHC class I by antigen-presenting cells expressing the necessary costimulatory molecules. Interestingly, Ad5-specific ELISPOT suggested a strong activation of T-cell responses (Fig. 3A–D). To clarify the mechanism of the response, we blocked MHC class I presentation with a specific antibody. As expected, this resulted in a reduction of IFN-γ secretion by ELISPOT (Fig. 3A). Therefore, an immune response developed against adenoviruses replicating in tumors, suggesting that the tumor environment might not completely preclude the induction of an immune response.

**Tumor-specific immune response**

T cells were pulsed with a peptide mix from survivin, which is a classic tumor-associated epitope broadly expressed in a variety of different tumors (ref. 40; Fig. 4A–D). In all patients analyzed, we observed a posttreatment increase in T cells capable of recognizing survivin. When we pretreated the PBMCs with an antibody blocking MHC class I presentation, we observed a reduction of IFN-γ secretion. To deconvolute the peptide pool used in ELISPOT, we performed pentamer analyses in HLA-A2–positive patients.
Figure 2. Tumor responses and survival. A, computer tomography scan from patients treated with Ad5-D24-GMCSF. A noninjected tumor in mesothelioma patient M50 decreased by 88% (overall response SD), complete response in patient with ovarian cancer (O14), complete response in breast cancer patient (R8), and rapid response (52% reduction) in ovarian cancer patient (O9) previously treated with a different oncolytic adenovirus. B, overall survival of patients treated with Ad5-D24-GMCSF.
Figure 3. Ad5-D24-GMCSF treatment activates adenovirus-specific CTLs. Total PBMCs were isolated from treated patients at the indicated time points and pulsed with an adenovirus type 5 penton-derived peptide pool. IFNγ ELISPOT was performed. Each panel represents a different patient. W6/36 monoclonal antibody was used to selectively block MHC class I presentation.
Figure 4. Ad5-D24-GMCSF treatment activates tumor-specific CTLs. Total PBMCs were isolated from treated patients at the indicated time points and pulsed with survivin-derived peptide pools. IFNγ ELISPOT was performed. Each panel represents a different patient. The W6/36 monoclonal antibody was used to selectively block MHC class I presentation.
(Supplementary Fig. S6). PBMCs from patients before and after the treatment were stained with specific antibodies for CD3, CD8, and survivin and then analyzed by flow cytometry. We observed an increase of T cells specific for survivin, in correlation with the results seen in ELISPOT. There were more antiatedenovirus T-cells than antisurvivin T-cells, suggesting that the former is a stronger antigen. Thus, the majority of the overall induction of CD8+ cells was probably due to antiadenovirus T-cells (Supplementary Fig. S6). Nevertheless, the clear relative increase in antisurvivin cells suggests that Ad5-D24-GMCSF was able to also induce antisurvivin reactivity.

Although survivin is widely considered an ideal tumor-associated antigen (40, 41), some reports have also proposed expression by selected normal cells (42). Therefore, whereas induction of antisurvivin T-cells suggests tumor-specific immunity, it does not unequivocally prove it.

Discussion

Accumulating evidence indicates that a dynamic cross-talk between tumors and the immune system could regulate tumor growth and metastasis (15, 43). Viral oncolysis can be useful as a costimulatory danger signal for helping induce immunity instead of tolerance towards tumor antigens (4, 15). In addition, cell lysis provides an ample supply of antigens for sampling by antigen-presenting cells. Here, we armed a p16-Rb pathway–selective oncolytic adenovirus with GMCSF, which could recruit natural killer cells directly to the tumor. GMCSF could also recruit dendritic cells and stimulate them so that they can return to the lymph node for coordinating the cytotoxic T-cell attack on the tumor.

Recombinant GMCSF has been used in humans for both its mitogenic effect on leukocyte progenitors and for its immunostimulating properties. It has been used as a vaccine adjuvant and as systemic agent for general antitumor immune stimulation (44, 45). Therefore, its safety is well established. However, systemic use for immune stimulation was compromised by side effects related to systemic exposure, whereas efficacy may have been limited because of low local concentrations. Thus, the production of GMCSF locally at the tumor might yield safety and efficacy benefits.

Only gp16k and 6.7k were deleted from the virus genome, therefore retaining most of E3, which is useful for effective oncolysis (46). Also, the 24 bp deletion used to obtain tumor selectivity might be advantageous over the most widely clinically used virus with an E1B55k deletion (d1520/ONYX-015/H101), because it does not attenuate virus replication in tumor cells (46). It has in fact been proposed that the 24 bp constant region 2 deletion in E1A might actually increase the oncolytic potency of the virus (47). In contrast, replication in normal tissues is minimized (1, 46).

In vitro and in immunodeficient preclinical systems, Ad5-D24-GMCSF was found to be as potent as the same virus without the transgene. However, in the presence of T cells, only Ad5-D24-GMCSF treatment protected animals from subsequent challenge with the same tumor cells. Growth of a different cell line was not compromised by prior tumor eradication with Ad5-D24-GMCSF. These findings suggest that the expression of GMCSF by the virus (Ad5-D24-GMCSF) could induce tumor-specific immunity.

Ad5-D24-GMCSF was well tolerated in patients and signs of efficacy were seen in 63% of the patients, suggesting that Ad5-D24-GMCSF may have antitumor activity in humans. Also, one third of the patients seemed to receive a long-lasting benefit. These data are particularly intriguing considering that these were highly pretreated patients with advanced and often large tumors. In this patient population, a high rate of antitumor activity and long-term survival were unusual.

Despite relatively conservative dosing at doses 10 to 100 fold lower than reported safe in previous studies with other oncolytic adenoviruses (1, 5–9, 21), persistent virus shedding into the serum was seen in 15 of 18 patients. Systemic dissemination of virus from the tumor might allow the transduction of metastases which (together with the immune response) might help explain the antitumor efficacy seen in injected and noninjected lesions. In this study, we used a combination of intravenous and intratumoral injection. However, the optimal dosing schedule has not been studied and thus merits further investigation.

One of the key findings in this article is the emphatic cytotoxic T-cell response induced against adenovirus. As Ad5-D24-GMCSF replicates in a tumor-specific manner, this suggests that the tumor environment is capable of mounting an immune response against tumor epitopes. However, at present, we cannot exclude a low degree of adenoviral gene expression in normal tissues, which might also contribute to antiaadenoviral T-cell responses.

Most interestingly, Ad5-D24-GMCSF treatment resulted in an immune response against survivin, a classic tumor-associated epitope. This suggests that the immunologic tolerance typical of tumors could be broken with an approach combining oncolytic virus-mediated cell killing with GMCSF-mediated recruitment and activation of dendritic cell and natural killer cells.

Efficacy gains might be obtained by combining virus with standard treatments. For example, it has been reported that radiation and oncolytic adenovirus could be synergistic (1). The same is true for many chemotherapeutics (10, 48). Moreover, immune suppression could yield benefits by slowing antibody induction or modulating the immunologic tumor environment (49). Tumor load and the associated immunosuppressive tumor environment might act against the virus-mediated antitumor immune response. Therefore, earlier treatment might yield efficacy benefits. Furthermore, as we treated patients only once, it is possible that repeated treatment could increase both the antitumor immune response and the transduction of tumor masses for more effective oncolysis.

In summary, Ad5-D24-GMCSF seems safe for the treatment of human cancer. Further clinical studies are needed to study efficacy in larger numbers of patients and to optimize the treatment schedule. In particular, randomized trials would be needed to more reliably show the benefit of adding GMCSF to oncolytic adenovirus, and to assess the efficacy of the approach in general. Nevertheless, our data suggests that
the immune tolerance of the tumor environment can be broken for the induction of an antitumor T-cell response.

Disclosure of Potential Conflicts of Interest

A. Hemminki is a K. Albin Johansson Research Professor of the Foundation for the Finnish Cancer Institute. A. Hemminki is also a founder and a shareholder in Oncos Therapeutics, Inc. The other authors disclosed no potential conflicts of interest.

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## Oncolytic Adenovirus Coding for Granulocyte Macrophage Colony-Stimulating Factor Induces Antitumoral Immunity in Cancer Patients

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