A Phase I Clinical Trial of Single-Dose Intrapleural IFN-β Gene Transfer for Malignant Pleural Mesothelioma and Metastatic Pleural Effusions: High Rate of Antitumor Immune Responses

Daniel H. Sterman, Adi Recio, Richard G. Carroll, Colin T. Gillespie, Andrew Haas, Anil Vachani, Veena Kapoor, Jing Sun, Richard Hodinka, Jennifer L. Brown, Michael J. Corbley, Michael Parr, Daniel H. Sterman, Interventional Pulmonology Program, Abramson Family Cancer Research Institute, University of Pennsylvania Medical Center; Virology Laboratory, Children’s Hospital of Philadelphia; Department of Genitourinary Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas; Biogen Idec, Cambridge, Massachusetts; Laboratory of Molecular Biostatistics and Epidemiology, University of Pennsylvania, Philadelphia, Pennsylvania; Biogen Idec, Cambridge, Massachusetts; Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland; Department of Genitourinary Medical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

Received 2/16/07; revised 4/22/07; accepted 5/10/07.

Note: Supplementary data for this article are available at Clinical Cancer Research online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Daniel H. Sterman, Interventional Pulmonology Program, Pulmonary, Allergy and Critical Care Division, University of Pennsylvania Medical Center, 833 West Gates Building, 3400 Spruce Street, Philadelphia, PA 19104-4283. Phone: 215-614-0984; Fax: 215-662-3226; E-mail: sterman@mail.med.upenn.edu.

© 2007 American Association for Cancer Research. doi:10.1158/1078-0432.CCR-07-0403

Abstract

Purpose: This phase 1 dose escalation study evaluated the safety and feasibility of single-dose intrapleural IFN-β gene transfer using an adenoviral vector (Ad.IFN-β) in patients with malignant pleural mesothelioma (MPM) and metastatic pleural effusions (MPE).

Experimental Design: Ad.IFN-β was administered through an indwelling pleural catheter in doses ranging from 9 × 10^11 to 3 × 10^12 viral particles (vp) in two cohorts of patients with MPM (7 patients) and MPE (3 patients). Subjects were evaluated for (a) toxicity, (b) gene transfer, (c) humoral, cellular, and cytokine-mediated immune responses, and (d) tumor responses via 18-fluorodeoxyglucose-positron emission tomography scans and chest computed tomography scans.

Results: Intrapleural Ad.IFN-β was generally well tolerated with transient lymphopenia as the most common side effect. The maximally tolerated dose achieved was 9 × 10^11 vp secondary to idiosyncratic dose-limiting toxicities (hypoxia and liver function abnormalities) in two patients treated at 3 × 10^12 vp. The presence of the vector did not elicit a marked cellular infiltrate in the pleural space. Intrapleural levels of cytokines were highly variable at baseline and after response to gene transfer. Gene transfer was documented in 7 of the 10 patients by demonstration of IFN-β message or protein. Antitumor immune responses were elicited in 7 of the 10 patients and included the detection of cytotoxic T cells (1 patient), activation of circulating natural killer cells (2 patients), and humoral responses to known (Simian virus 40 large T antigen and mesothelin) and unknown tumor antigens (7 patients). Four of 10 patients showed meaningful clinical responses defined as disease stability and/or regression on 18-fluorodeoxyglucose-positron emission tomography and computed tomography scans at day 60 after vector infusion.

Conclusions: Intrapleural instillation of Ad.IFN-β is a potentially useful approach for the generation of antitumor immune responses in MPM and MPE patients and should be investigated further for overall clinical efficacy.

Malignant pleural mesothelioma (MPM) is a refractory neoplasm. Except for a few patients who benefit from aggressive multimodality approaches, the majority of patients die from the disease within 8 to 14 months of diagnosis (1, 2). Metastatic pleural effusions (MPE) also portend a poor prognosis and are typically treated solely with palliative measures.

Given this current lack of effective therapies, our group has focused on the development of adenoviral vectors for the treatment of intrapleural malignancies. We hypothesized that MPM and MPE would be particularly attractive targets for gene transfer studies given preclinical data showing evidence of effective adenoviral gene therapy in peritoneal and pleural models of tumor (3–5).

Our initial clinical trials used intrapleural delivery of adenoviral vectors expressing the suicide gene, herpes simplex thymidine kinase (Ad.HSVtk), into patients with MPE followed by 2 weeks of i.v. ganciclovir (6–8). Toxicity was minimal, no maximally tolerated dose was reached, and post-gene transfer...
pleural biopsies revealed dose-related transgene expression at the higher doses, but only in the superficial tumor layers. Interestingly, two patients with stage 1 MPM had significant tumor responses, as documented by serial 18-fluorodeoxyglucose-positron emission tomography (18FDG-PET) and chest computed tomography (CT) scans, associated with very long survivals (>8 years; ref. 9).

Given the facts that the Ad.HSVtk gene transfer transduced only a few tumor cells and that the observed clinical responses were delayed and persistent, we reasoned that our successful antitumor responses were not primarily due to massive tumor cell killing by the suicide gene, as we had first postulated, but rather due to ‘secondary’ antitumor immune responses induced by the Ad.HSVtk/GCV treatment. This idea was supported by data in mice showing that cell death induced by HSVtk generates a strong Th-1 type antitumor immune response (10, 11). In addition, it is well established that adenovirus induces strong activation of the innate and acquired immune system (12, 13). We thus decided to develop a new vector that would (a) continue to take advantage of adenoviral-induced inflammation, (b) directly induce cell death (like Ad.HSVtk/GCV), and (c) enhance antitumor immune responses by secretion of an immunostimulatory cytokine.

Several published clinical trials have shown clinical responses to intrapleural infusion of IFN-β, IFN-α, and IFN-γ in patients with MPM or MPE (14–18). Type 1 IFNs (such as IFN-α and IFN-β) are known to inhibit tumor cell growth and stimulate the immune system (19). IFNs have immunoregulatory effects on antibody production, natural killer (NK) and T-cell activation, macrophage function, delayed-type hypersensitivity, and MHC antigen expression, as well as antiproliferative effects and antiangiogenic properties (20–24). Finally, IFN-γ gene transfer in animal models of various malignancies (both xenografts and autologous tumors) has shown impressive antitumor effects (25–29).

We therefore conducted preclinical studies using an adenoviral vector expressing mouse IFN-β (Ad.muIFN-β). Our preclinical data showed that (a) Ad.muIFN-β had dramatic therapeutic efficacy in syngeneic animal models of MPM and lung cancer, (b) i.p. and i.t. injections of Ad.muIFN-β showed significant antitumor activity both in the injected tumor site and in distant tumors, and (c) in these models, this effect was due, in large part, to the generation of CTLs directed against tumor antigens and activation of NK cells (30–32).

Based on these data, as well as the availability of clinical grade Ad.humanIFN-β from BiogenIdec, we conducted a single-center dose escalation phase 1 clinical trial of single-dose intrapleural infusion of Ad.huIFN-β in patients with MPM/MPE. The goals of the trial were as follows: (a) determine safety and toxicities of a single intrapleural infusion of Ad.huIFN-β; (b) establish the maximally tolerated dose of a single intrapleural infusion of Ad.huIFN-β; (c) evaluate induced antitumor and antitumor immune responses; and (d) assess antitumor activity of Ad.huIFN-β before and after vector instillation using chest CT and whole-body 18FDG-PET scanning. Materials and Methods

Preclinical and regulatory issues

After conducting a formal animal toxicology study with intrapleural delivery of Ad.muIFN-β (and Ad.huIFN-β) under Food and Drug Administration Good Laboratory Practices guidelines, the study was approved by all appropriate human study and biosafety committees at Penn and the Recombinant DNA Advisory Committee of the NIH’s Office of Biotechnology Activities. We obtained a physician-sponsored investigational new drug from the Food and Drug Administration (BB-IND 10603).

Vector

We used Ad.huIFN-β virus (BG00001), developed at BiogenIdec. The vector is a good manufacturing practices grade, E1/E3-deleted replication-incompetent adenovirus with an insertion of the human IFN-β gene in the E1 region of the adenoviral genome. The transgene was driven by a human cytomegalovirus promoter. Food and Drug Administration approval for this vector was obtained by BiogenIdec and cross-referenced in our investigational new drug proposal.

Patients

Patients were eligible for these studies based on the following: (a) a pathologically confirmed diagnosis of MPM or MPE; (b) an Eastern Cooperative Oncology Group performance status of 0, 1, or 2; and (c) an accessible pleural space for instillation of vector. Exclusion criteria included prior surgical resection, successful pleurodesis, recent chemotherapy or radiotherapy, inadequate pulmonary function, or the presence of significant cardiac/hepatic/renal disease. Patients 102 and 103 signed the consent form but were not dosed because of interval disease progression.

Protocol summary

Eligible patients underwent insertion of a tunneled intrapleural catheter (Pleurx, Cardinal Health) under local anesthesia and had a single-blood volume leukapheresis for harvesting peripheral blood mononuclear cells to be used for immunoassessment. On study day 1, patients were admitted to the Penn General Clinical Research Center, hydrated i.v., and premedicated with acetaminophen. All obtainable pleural fluid was drained from the chest through the Pleurx catheter. Subsequently, a single dose of Ad.huIFN-β (BG00001), diluted in 50 cc of sterile normal saline, was instilled via the catheter into the pleural space. The catheter was then flushed and capped to maximize vector delivery to intrapleural tumor. Patients were monitored in the General Clinical Research Center and discharged to home after 72 h with the pleural catheter capped. Patients were followed closely as out-patients for the next 6 months. Approximately 4 weeks after vector instillation, the pleural catheter was removed, unless still needed for control of symptomatic malignant pleural effusion. Chest CT scans and dual-time point 18FDG-PET scans were done at baseline and 2 months after vector instillation, as well as at 6 months, if clinically indicated.

Sample collection and generation of cell lines from patient samples

Peripheral blood mononuclear cells were purified by Ficoll density gradient centrifugation and were viably cryopreserved as bulk peripheral blood mononuclear cells. Cells contained in malignant pleural effusions (or 20 mL pleural lavages, if no pleural fluid was present) were purified by density gradient centrifugation using either Ficoll or Percoll. The cells were generally frozen in bulk, but if needed, were fractionated into specific subsets using either magnetic beads or the MoFlo cell sorter (DakoCytomation).

Radiographic response assessment

We used the ‘modified Response Evaluation Criteria in Solid Tumors (RECIST)’ schema for the evaluation of tumor response in the special case of mesothelioma (2, 33). These criteria apply equally well to evaluation of MPE. In addition, we evaluated response using 18FDG-PET, which is increasingly being used to assess mesothelioma tumor activity before and after therapy, with the potential for earlier and more accurate determinations of treatment efficacy (34).
Measurement of viral shedding

Samples of whole blood, pleural fluid, and swabs from the pleural catheter insertion site were obtained at baseline and various times after gene instillation and sent to the Children’s Hospital of Philadelphia Virology Laboratory for analysis. Viral DNA was detected by PCR. Specimens were also cultured for wild-type adenovirus on A549 cells and replication-deficient virus (vector) on 293 cells. Details of these procedures can be found in Supplementary Data.

Assessment of gene transfer

Transgene and endogenous IFN-β mRNA assays. PCR primers were designed to target an area of the transcript within the 3′ untranslated region where the endogenous and vector-produced IFN-β mRNA differed. Glyceraldehyde-3-phosphate dehydrogenase served as a control for RNA isolation and stability. Studies with primer pairs designed as controls for DNA contamination of the RNA prep showed no contamination (data not shown). Control studies are described in Supplementary data.

Cells that had been isolated from the pleural effusions of patients 104, 108, 110, 111, and 112 were thawed and 1 × 10⁴ total cells were used for RNA preparations. Patients 101, 106, and 107 had pleural tumor, but no effusion; therefore, no cells were available for RNA analysis. Samples from time points taken before dose, 24 h after vector treatment, 48 h, and 7 days after vector treatment (when cells were available) were analyzed for glyceraldehyde-3-phosphate dehydrogenase, endogenous, and transgene RNA transcripts.

IFN-β intracellular staining

IFN-β intracellular staining was done from two patients’ pleural fluid cytospins (patients 108 and 110) using a primary anti-IFN-β antibody (Chemicon). See Supplementary Data for details.

Cytokine assays

Commercial cytokine ELISA assays were used to measure the levels of IFN-β, IFN-α, IFN-γ, interleukin (IL)-6, transforming growth factor-β (total and heat activated), and vascular endothelial growth factor in serial dilutions of pleural fluid and serum at various time points. Additional cytokines (IL-1β, IL-10, Macrophage Chemotactic Protein-1 [MCP-1/CCL2], IL-8, and Regulated Upon Activation, Normal T-cell Expressed and Secreted [RANTES/CCL5]) were measured using a cytokine bead assay from Luminex according to the manufacturer’s instructions.

Cellular analysis of pleural fluids

Pleural fluid mononuclear cells were isolated from patient samples by Ficoll centrifugation. Cells were labeled with fluorochrome-conjugated monoclonal antibodies as described previously (35) and four-color flow cytometric analysis was done using a Becton Dickinson FACSCanto flow cytometer (BD Biosciences). All labeled antibodies were from BD Biosciences.

Immunoblots

To detect humoral responses against tumor antigens, immunoblotting against purified proteins and extracts from mesothelioma, lung cancer, and ovarian cancer cell lines was done. Purified WT-1 protein was purchased from Santa Cruz Biotechnology. Purified Simian virus 40 (SV40) large T-antigen (Tag) protein was purchased from Chimerix. Purified mesothelin was provided by Drs. M. Ho and I. Pastan (National Cancer Institute, Bethesda, MD). Cells lines derived from patient samples were grown in culture. Extracts from cells or purified proteins were prepared and immunoblotted with patient serum as described previously (9). See Supplementary Data for details. Patient serum samples (diluted at 1:1,500) from time points before treatment and 6 weeks to 6 months after treatment were used and bound human antibody was visualized.

Anti-mesothelin antibody ELISA

Quantitative estimates of levels of anti-mesothelin antibodies were obtained using a modified ELISA on 1:100 dilutions of serum samples at various time points before and after vector instillation, as described by Ho et al. (36).

Cellular antitumor immune responses

Cellular immune responses were measured by transfecting total tumor RNA or control RNA into autologous dendritic cells. Autologous T cells were stimulated in vitro and used for CTL activity against chromium 53–labeled autologous tumor cells or HLA-matched allogeneic tumor cell lines as described previously (37).

Adenovirus serotype 5 neutralizing antibody levels

Serum neutralizing antibodies specific for adenovirus were evaluated as described previously (4, 9). See Supplementary Data for details.

Statistical analysis

We used a standard 3+3 design to determine the maximally tolerated dose, with an implicit 50% chance of further dose escalation after

<table>
<thead>
<tr>
<th>ID</th>
<th>G</th>
<th>Age</th>
<th>Primary (stage at entry)</th>
<th>Dose level</th>
<th>Dosing</th>
<th>Day 60 CT response (RECIST/modified RECIST)</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>M</td>
<td>80</td>
<td>Mesothelioma (IA)</td>
<td>1</td>
<td>Tolerated</td>
<td>PD</td>
</tr>
<tr>
<td>104</td>
<td>F</td>
<td>57</td>
<td>Lung (IIIB)</td>
<td>1</td>
<td>Tolerated</td>
<td>PD</td>
</tr>
<tr>
<td>105</td>
<td>F</td>
<td>47</td>
<td>Ovarian (IV)</td>
<td>1</td>
<td>Tolerated</td>
<td>SD</td>
</tr>
<tr>
<td>106</td>
<td>M</td>
<td>72</td>
<td>Mesothelioma (IIA)</td>
<td>2</td>
<td>DLT: hypoxia</td>
<td>SD</td>
</tr>
<tr>
<td>107</td>
<td>M</td>
<td>64</td>
<td>Mesothelioma (IIIB)</td>
<td>2</td>
<td>Tolerated</td>
<td>PD</td>
</tr>
<tr>
<td>108</td>
<td>M</td>
<td>66</td>
<td>Mesothelioma (IV)</td>
<td>2</td>
<td>Tolerated</td>
<td>PD</td>
</tr>
<tr>
<td>109</td>
<td>F</td>
<td>40</td>
<td>Mesothelioma (IIIA)</td>
<td>2</td>
<td>DLT: elevated transaminases</td>
<td>PD</td>
</tr>
<tr>
<td>110</td>
<td>M</td>
<td>78</td>
<td>Mesothelioma (IIIA)</td>
<td>1</td>
<td>Tolerated</td>
<td>SD</td>
</tr>
<tr>
<td>111</td>
<td>M</td>
<td>76</td>
<td>Mesothelioma (IV)</td>
<td>1</td>
<td>Tolerated</td>
<td>PD</td>
</tr>
<tr>
<td>112*</td>
<td>M</td>
<td>61</td>
<td>Mesothelioma (IIIA)</td>
<td>1</td>
<td>Tolerated (×2)</td>
<td>SD (status post dose 1)</td>
</tr>
</tbody>
</table>

Abbreviations: G, gender; SD, stable disease; PD, progressive disease; CR, complete response; PR, partial response; DLT, dose-limiting toxicity; mAb, monoclonal antibody; VP16, etoposide.

*Patient 112 received a second vector dose at dose level 1 under an Institutional Review Board and Food and Drug Administration–approved protocol amendment 4 mo after initial dosing.
achievement of a toxicity rate of 30%. Immunologic responses (cytokine levels, lymphocyte response to tumor antigens, levels of antibodies in tumor lysates, and fractions of lymphocytes measured by flow cytometry) and overall tumor response rates were secondary end points. We analyzed these variables using standard parametric and nonparametric approaches, as applicable.

Results

Patients
Ten patients (patients 101 and 104-112) underwent intrapleural infusion of a single dose of Ad.huIFN-β at two dose levels after pretreatment leukapheresis. Seven patients had mesothelioma; two patients had metastatic non–small cell lung cancer; and the other patient had metastatic ovarian cancer. Patient details are shown in Table 1. The trial began in August 2003 and the last patient was dosed in July, 2005.

Safety and toxicities
The first three patients in our dose 1 cohort [receiving $9 \times 10^{11}$ viral particles (vp) of Ad.huIFN-β] tolerated dosing with grade 1 to 2 toxicities. Transient lymphopenia was seen in two of three patients. Other toxicities included chest pain, coryza, fever, anemia, and elevated liver enzymes (Table 1).

Four MPM patients were enrolled at dose level 2 ($3 \times 10^{12}$ vp). Two had adverse events. The first patient in this cohort (patient 106) experienced an episode of transient hypoxia (grade 3) 11 h after dosing but rapidly recovered to baseline. The etiology of this response is not clear. This patient did have a history of chronic compensated congestive heart failure, well controlled on medical therapy, before enrollment in our clinical trial. We had held his diuretics immediately on development of his hypoxia to rapid resolution of his dyspnea and decrease in oxygen requirements, highly suggestive of a transitory congestive heart failure exacerbation. On the other hand, patient 106 was one of the patients with an undetectable baseline level of anti-adenoviral neutralizing antibodies (see below). The low anti-adenoviral neutralizing antibody levels could have contributed to the finding of detectable virus in the blood (see below) and to high levels of intrapleural macrophage transduction by adenovirus with resultant increases in release of inflammatory cytokines. Consistent with this hypothesis, patient 106 had the highest level of serum IL-6 (a peak of 854 pg/mL at 12 h after vector delivery) of any of the patients. This level dropped to undetectable levels 24 h after delivery. The rapid increase in serum IL-6 levels showed by patient 106 is likely reflective of this immediate macrophage activation. In any case, this patient has now completed 32 months of follow-up without any further study-related complications.

The fourth patient treated at dose level 2 (patient 109) developed grade 3 elevations in serum transaminases (alanine aminotransferase peaked at 435 units/L and her AST at 231 units/L) 3 weeks after vector instillation without clinical evidence of hepatic dysfunction. These transaminase elevations returned to baseline levels over a period of several weeks. This patient had a distant history of Hodgkin’s lymphoma of the chest and abdomen treated with external beam radiation therapy, including partial irradiation of the liver that may have predisposed her to some liver toxicity.

These events were classified as dose-limiting toxicities (although were not severe enough to be categorized as serious adverse events), so per protocol, three additional patients were dosed at dose level 1. Patient 112 also received a second dose of vector ($9 \times 10^{11}$ vp) 4 months after his first dose under an Institutional Review Board and Food and Drug Administration–approved protocol amendment. None of these three patients experienced any dose-related serious adverse events nor showed evidence of dose-limiting toxicities. Our maximally tolerated dose was thus $9 \times 10^{11}$ vp.

Table 1. Patient summary (Cont’d)

<table>
<thead>
<tr>
<th>Day 60 metabolic response (18FDG-PET)</th>
<th>Month 6 CT response (RECIST/modified RECIST)</th>
<th>Survival after dosing (mo)</th>
<th>Additional therapy</th>
<th>Status/cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td>SD</td>
<td>34</td>
<td>Chemotherapy (pemetrexed, gemcitabine)</td>
<td>Deceased/Progression</td>
</tr>
<tr>
<td>PD</td>
<td>PD</td>
<td>8</td>
<td>Radiation therapy, gefitinib</td>
<td>Deceased/progression</td>
</tr>
<tr>
<td>PD</td>
<td>PD</td>
<td>&gt;35</td>
<td>Chemotherapy (bevacizumab + carboplatin, cyclophosphamide/IFN-α); debulking surgery (abdominal)</td>
<td>Alive</td>
</tr>
<tr>
<td>SD</td>
<td>SD</td>
<td>&gt;32</td>
<td>Chemotherapy (pemetrexed + cisplatin)</td>
<td>Alive</td>
</tr>
<tr>
<td>SD</td>
<td>SD</td>
<td>23</td>
<td>Chemotherapy (pemetrexed/cisplatin; navelbine + carboplatin; erlotinib; carboplatin + VP-16; adriamycin; vinblastin/gemcitabine; Taxotere); radiation therapy</td>
<td>Deceased/progression</td>
</tr>
<tr>
<td>PD</td>
<td>PD</td>
<td>6</td>
<td>Chemotherapy (pemetrexed cisplatin)</td>
<td>Deceased/progression</td>
</tr>
<tr>
<td>PD</td>
<td>N/A</td>
<td>4</td>
<td>Chemotherapy (pemetrexed)</td>
<td>Deceased/progression</td>
</tr>
<tr>
<td>PR</td>
<td>PD</td>
<td>&gt;26</td>
<td>Chemotherapy (pemetrexed/cisplatin, gemcitabine, navelbine); radiofrequency ablation</td>
<td>Alive</td>
</tr>
<tr>
<td>PD/SD</td>
<td>PR (on erlotinib)</td>
<td>17</td>
<td>Erlotinib CP-870,893 antibody (anti-CD40 mAb); chemotherapy (vinorelbine, Alimta)</td>
<td>Deceased/progression</td>
</tr>
<tr>
<td>PD/SD (6 mo/s/p dose 1, day 60 s/p dose 2)</td>
<td></td>
<td>&gt;21</td>
<td></td>
<td>Alive</td>
</tr>
</tbody>
</table>
Viral shedding

Chest wall swabs, pleural fluid, and serum were analyzed on day 0 (pretreatment) and days 2, 3, 4, 7, 14, 21, and 28 by addition to cell monolayers with subsequent analysis for cytopathic effects. Samples were cultured on A549 cells to detect replication-competent adenovirus and on 293 cells (which express adenoviral E1 protein) to detect replication-defective vector (Supplementary Table S1). No samples were positive for replication-competent adenovirus. Pleural fluid cultures showing replication-defective vector were positive in five of the patients (for up to 7 days in three patients) but were negative in all patients by 14 days. Only one patient (patient 106) had a positive culture for vector in serum (and only on day 1; Table 2).

Specimens were also analyzed for vector-specific DNA sequences by PCR. Four patients (patients 104, 106, 107, and 110) had positive serum adenoviral PCRs for up to 4 days after vector instillation. Pleural fluid samples (or flushes, if needed) were analyzed at days 2, 3, 4, 7, and 14 for all 10 patients. Thereafter, pleural fluid was analyzed in the patients where Pleurex catheter fluid was obtainable (see Table 2). We identified adenoviral vector DNA for 10 days in pleural fluid in all of the patients. PCR was positive in 6 of 8 patients at day 28, in 3 of 5 patients at day 42, and in none of the 4 evaluable patients by day 56.

Antiviral immune responses

Serum anti-adenoviral neutralizing antibody titers were measured before and after gene transfer. As shown in Table 3, baseline titers of anti-adenoviral neutralizing antibody ranged from <1:10 to 1:750. Only three patients had baseline titers >1:100. All patients increased their neutralizing antibody titers after gene transfer. Four patients had weak antibody responses to vector instillation (defined as an increase of titer by <10-fold). Six patients had more than a 10-fold increase in titer (average of a 45-fold increase). Patient 112 received two doses of vector. The first dose led to an increase from 1:50 to 1:3,200. The second dose of vector led to a doubling of neutralizing antibody titers from 1:3,200 to 1:6,400 (Table 3).

Gene transfer

Gene transfer was assessed using pleural fluid obtained through the tunneled pleural catheter (Table 4). In patients with no accessible pleural fluid (patients 106, 107, and 109), we collected pleural lavages.

Pleural fluid samples were tested for the presence of adenoviral DNA by PCR (Table 2). No patient had detectable adenoviral DNA in pretreatment samples. All 10 patients had detectable adenoviral DNA after gene transfer.

We measured IFN-β protein levels in the before and after vector instillation pleural fluid and serum samples. These pleural measurements should be considered semiquantitative because the volume of pleural fluid clearly affected the final concentration, and, in three of the patients, saline lavages were done to obtain samples. As summarized in Table 4, detectable levels of IFN-β in pleural fluid (ranging from 200 pg/mL to 160 ng/mL) were found in all but two patients. The time course is shown in Fig. 1A. IFN-β was detectable for up to 3 days in most patients. Serum levels of IFN-β were undetectable in most patients (<0.2 ng/mL) and quite low in those who had detectable levels (Table 4).

To further document gene transfer, we assessed endogenous and vector-produced IFN-β mRNA using reverse transcription-PCR in the five samples in which enough cellular material was available (Fig. 1B). Transgene-specific RNA was detected in all five patients (patients 104, 108, 110, 111, and 112). Like the protein measurements, vector mRNA expression was relatively transient, usually detectable for only 48 h, although in patient 111, mRNA was seen at 7 days. Only two patients had detectable message for endogenous IFN-β; patient 104 had endogenous message on day 7 and patient 111 at 24 h.

Finally, to further confirm successful intrapleural IFN-β gene transfer and to show the actual transduction of tumor cells, we did IFN-β staining on pleural fluid cytospins obtained before and 48 h after BG00001 vector instillation in two patients where sufficient numbers of tumor cells were present. Positively staining cells were seen in both patients. Results are shown for patient 110 (Fig. 1C). Whereas tumor cells obtained from pre-vector transfer pleural fluid showed no positive staining, the majority of tumor cells visualized from the sample 48 h after gene transfer showed clear evidence of strong perinuclear IFN-β staining.

Inflammatory and cytokine responses

The patterns of inflammatory pleural leukocyte cell response were quite variable among patients. The average “fold increase” in total leukocytes (comparing preinstillation with the peak count in the next 4 days) was 3.3-fold (± 0.7-fold SE) with any increases due to monocytes and lymphocytes (not neutrophils). We did not observe consistent alterations in the percentage or absolute count of pleural T cells, regulatory T cells, NK cells, or dendritic cells. No consistent change in the activation of T cells or dendritic cells was observed.

<table>
<thead>
<tr>
<th>Table 2. Viral shedding table</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultures</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Pleural fluid Serum</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

NOTE: Chest wall swabs, pleural fluid, and serum from all 10 patients were analyzed on day 1 (pretreatment) and days 2, 3, 4, 7, 14, 21, and 28. Samples were cultured in 293 cells to detect vector. PCR was done to detect vector DNA sequences.
Levels of pleural cytokines (including IL-6, IL-1β, IL-10, vascular endothelial growth factor, MCP-1, IL-8, RANTES, transforming growth factor-β1 and transforming growth factor-β2, and IFN-γ) were highly variable among the patients, both at baseline and after response to gene transfer, perhaps reflecting the dilutional issues mentioned above.

Levels of serum cytokines were also monitored with special focus on IL-6, which can be elevated in mesothelioma (38) and has been reported as a marker of systemic inflammatory response after adenoviral instillation (39, 40). Serum IL-6 levels were detectable in four patients, and in these patients, the peaks were only ~250 pg/mL. Only one other cytokine, MCP-1, was consistently detected in serum after gene transfer, which averaged 72 ± 17 pg/mL at baseline with peaks averaging 943 ± 407 pg/mL, primarily day 1 after vector instillation.

Antitumor immunologic responses

**Innate immune responses.** Because IFN-β has the ability to activate NK cells (20, 31), we examined the activation state of circulating NK cells at an early time point (3 days) after vector instillation. Using flow cytometry, we first identified NK cells by the CD56+/CD3- cell surface phenotype among lymphocytes. Five of the nine patients had <1% circulating NK cells before and after vector administration and were not analyzed further. In the remaining four patients, the percentages of NK cells among total peripheral blood lymphocytes were 3% (patient 107), 3.6% (patient 101), 10.2% (patient 106), and 10.4% (patient 108). These percentages did not change after gene transfer. We evaluated the activation state of NK cells in these patients by determining the percentage of NK cells expressing the activation marker CD69 before and after gene transfer (41). In two of these patients, the percentage of CD69+ activated cells did not change. However, as shown in Fig. 1D, the percentage of CD69+ activated NK cells went up remarkably in patient 106 (14.1-98.2%) and patient 107 (4.1-78.3%). Thus, in two of four evaluable patients, a single Ad.IFN-β intrapleural infusion led to the activation of circulating NK cells (see Supplementary Table S1).

**Humoral responses to known MPM tumor antigens.** Humoral responses to three defined mesothelioma-associated antigens were evaluated by immunoblotting purified proteins using patient sera from before and after gene transfer. Most patients had low-level baseline reactivity to purified Wilms’ tumor antigen-1 (WT-1 protein), but none showed clear increases after treatment. Most patients also had some baseline reactivity against purified SV40 large Tag (SV40 Tag protein) with no increases after gene transfer. However, one patient (patient 107) developed a significant increase in antibody response to SV40 Tag after gene transfer (Fig. 2A; see Supplementary Table S1).

### Table 3. Adenovirus neutralizing antibody titers

<table>
<thead>
<tr>
<th>Patient</th>
<th>After Rx</th>
<th>6 Wk after Rx</th>
<th>4-6 Mo after Rx</th>
<th>Fold response: before to peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>50</td>
<td>80</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>104</td>
<td>&lt;10</td>
<td>800</td>
<td>NA</td>
<td>&gt;80</td>
</tr>
<tr>
<td>105</td>
<td>&lt;10</td>
<td>750</td>
<td>250</td>
<td>&gt;75</td>
</tr>
<tr>
<td>106</td>
<td>&lt;10</td>
<td>70</td>
<td>80</td>
<td>&gt;8</td>
</tr>
<tr>
<td>107</td>
<td>250</td>
<td>7,500</td>
<td>3,500</td>
<td>30</td>
</tr>
<tr>
<td>108</td>
<td>600</td>
<td>9,000</td>
<td>7,000</td>
<td>15</td>
</tr>
<tr>
<td>109</td>
<td>750</td>
<td>&gt;10,000</td>
<td>NA</td>
<td>&gt;13</td>
</tr>
<tr>
<td>110</td>
<td>&lt;10</td>
<td>60</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>111</td>
<td>10</td>
<td>90</td>
<td>60</td>
<td>9</td>
</tr>
<tr>
<td>112 (first dose)</td>
<td>50</td>
<td>2,800</td>
<td>3,200</td>
<td>64</td>
</tr>
<tr>
<td>112 (second dose)</td>
<td>3,200</td>
<td>6,800</td>
<td>NA</td>
<td>2-fold</td>
</tr>
</tbody>
</table>

**NOTE:** Varying dilutions of serum were mixed with a fixed dose of Ad.LacZ and then plated onto human mesothelioma cells. After 48 h, cells were stained for β-galactosidase expression and the amount of blue color was quantified on a spectrophotometer. The dilution, where staining was inhibited by 50%, was defined as the neutralizing antibody titer and expressed as 1/titer.

**Abbreviation:** NA, serum not available.

### Table 4. Summary of pleural gene transfer

<table>
<thead>
<tr>
<th>ID</th>
<th>Primary</th>
<th>Dose level</th>
<th>Type of sample</th>
<th>Adenoviral DNA (+PCR)</th>
<th>Peak IFN-β level (ng/mL)</th>
<th>Transgene mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>Mesothelioma</td>
<td>1</td>
<td>Effusion</td>
<td>Positive until week 4</td>
<td>Nondetectable</td>
<td>Not tested, insufficient no. cells</td>
</tr>
<tr>
<td>104</td>
<td>Lung</td>
<td>1</td>
<td>Effusion</td>
<td>Positive until week 6</td>
<td>59</td>
<td>Positive for 24 h</td>
</tr>
<tr>
<td>105</td>
<td>Ovarian</td>
<td>1</td>
<td>Effusion</td>
<td>Positive until week 6</td>
<td>12</td>
<td>Not tested, insufficient no. cells</td>
</tr>
<tr>
<td>106</td>
<td>Mesothelioma</td>
<td>2</td>
<td>Pleural lavage</td>
<td>Positive until week 1</td>
<td>Nondetectable</td>
<td>Not tested, insufficient no. cells</td>
</tr>
<tr>
<td>107</td>
<td>Mesothelioma</td>
<td>2</td>
<td>Pleural lavage</td>
<td>Positive until week 4</td>
<td>123</td>
<td>Not tested, insufficient no. cells</td>
</tr>
<tr>
<td>108</td>
<td>Mesothelioma</td>
<td>2</td>
<td>Effusion</td>
<td>Positive until week 4</td>
<td>8</td>
<td>Positive for 48 h</td>
</tr>
<tr>
<td>109</td>
<td>Mesothelioma</td>
<td>2</td>
<td>Pleural lavage</td>
<td>Positive until week 3</td>
<td>30</td>
<td>Not tested, insufficient no. cells</td>
</tr>
<tr>
<td>110</td>
<td>Mesothelioma</td>
<td>1</td>
<td>Effusion</td>
<td>Positive until week 3</td>
<td>160</td>
<td>Positive for 72 h</td>
</tr>
<tr>
<td>111</td>
<td>Lung</td>
<td>1</td>
<td>Effusion</td>
<td>Positive until week 6</td>
<td>27</td>
<td>Positive for 7 h</td>
</tr>
<tr>
<td>112</td>
<td>Mesothelioma</td>
<td>1</td>
<td>Effusion</td>
<td>Positive until week 4</td>
<td>0.2</td>
<td>Positive for 48 h</td>
</tr>
<tr>
<td>112</td>
<td>Dose 2</td>
<td>1</td>
<td>Effusion</td>
<td>Positive until week 4</td>
<td>Not detectable</td>
<td>Not tested, insufficient no. cells</td>
</tr>
</tbody>
</table>
Patient sera were also tested for reactivity against purified mesothelin. We observed a low baseline level of staining in almost all patients. However, clear increases in antibody reactivity were seen in patients 106 and 112 (Fig. 2B and C). We also evaluated anti-mesothelin antibody reactivity using a quantitative ELISA assay (36). As shown in Fig. 3, patients 101, 104, 111, and 112 had significant baseline levels of anti-mesothelin antibodies. Similar to the immunoblotting results, patient 106 had a marked increase in levels after therapy and patient 112 had a marked increase in his baseline levels after his second treatment.

Humoral responses to tumor antigens on cell line extracts. We also analyzed serum in immunoblotting to identify new or
increased intensity bands on extracts of mesothelioma, lung cancer, or ovarian cancer cell lines. The sera from eight patients recognized new antigens in the posttreatment samples (Supplementary Table S1). Figure 2D to F shows examples of strong new bands or clear increases in intensity of staining in the postgene transfer serum.

**Cellular immune responses.** The induction of antitumor CTLs following gene transfer was observed in only one of seven patients evaluated (patient 105). Details of this patient have been published recently (see Supplementary Table S1; ref. 37).

**Clinical responses**

Four of 10 patients showed meaningful clinical responses defined as disease stability or regression noted on 18F-DG-PET and CT scans at day 60 after vector infusion (Table 1).

In our first cohort of patients, one MPM patient (patient 101) had progression of disease at his 60-day follow-up and subsequently received palliative chemotherapy with good response, ultimately dying of progressive disease ~34 months after Ad.IFN-β instillation. The second patient, with metastatic lung cancer (patient 104), died with progressive disease 8 months after intrapleural administration of Ad.IFN-β. The third patient in this cohort (patient 105) had ovarian carcinoma and had received chemotherapy and surgical debulking with residual intra-abdominal disease and a large metastatic left pleural effusion. This patient had a complete metabolic response on follow-up 18F-DG-PET imaging that has been described in detail in a recent case report (37).

In our second cohort of patients (all with MPM), three patients had stable disease at 60 days. The first patient (patient 106), who has sarcomatoid histology, remained stable on CT (Fig. 4A) and positron emission tomography scan without additional therapies for ~18 months after his single dose of intrapleural Ad.IFN-β (9 × 10¹ⁱ vp) before developing disease.

![Fig. 2. Humoral immune responses. Antibody responses against tumor antigens were visualized on immunoblots using patient serum (diluted 1:1,500) before and after gene transfer. A, response of patient 107 to purified SV40 large TAg (SV40). Extracts from a mesothelioma cell line (REN) and purified SV40 protein were run on gels and immunoblotted with pre- and post-gene transfer serum (top). The blots were stripped and reprobed with a monoclonal antibody against SV40 to ensure equal loading. Posttreatment serum showed a marked increase in the reactivity against SV40 visualized by the band at ~ 80 KDa (arrow). B and C, responses of patients 106 and 112 to purified mesothelin. Purified mesothelin protein was run on gels and immunoblotted with pre- and post-gene transfer serum (top). The blots were stripped and reprobed with a monoclonal antibody against mesothelin to ensure equal loading. Posttreatment serum showed clear increases in the reactivity against mesothelin visualized by the band at ~70 KDa (arrows). D, response of patient 109 to an ovarian cancer cell extract. Extracts from an ovarian cell line (OV1) were run on a gel and immunoblotted with pre- and post-gene transfer serum. The posttreatment serum showed a marked increase in the reactivity against a band at ~55 KDa (arrow). E, response of patient 106 to a mesothelioma cell extract. Extracts from a mesothelioma cell line (M30) were run on a gel and immunoblotted with pre- and post-gene transfer serum. The posttreatment serum showed marked increases in at least four bands (arrows). F, response of patient 108 to an autologous mesothelioma cell extract. Extracts from a mesothelioma cell line generated from the patient’s pleural fluid were run on a gel and immunoblotted with pre- and post-gene transfer serum. The posttreatment serum showed increases in at least two bands (arrow).
progression. Patients 107 and 108 had stable disease at 60 days. Subsequently, however, both had evidence of progressive disease (at ~12 and 3 months after dosing, respectively) and initiated palliative chemotherapy. The final patient in this cohort (patient 109), who had an aggressive mesothelioma variant, developed evidence of disease progression at the 60 days of postdosing time point and died ~4 months after treatment.

In our last cohort of patients (three additional patients at dose level 1), one patient (patient 110) with sarcomatoid mesothelioma had stable anatomic disease by CT scan at 2-month follow-up with a near-complete metabolic tumor response on PET scan (Fig. 4B). At the 6-month evaluation, with no interval systemic or local therapy, patient 110 had stable anatomic disease on CT scan but probable metabolic progression on PET (data not shown). At 60 days, patient 111 (stage IIIB/IV non–small cell lung cancer) showed disease progression and was started on erlotinib (Tarceva, Genentech, Inc.). The last patient enrolled in this dose cohort (patient 112), with advanced MPM, had stable disease on chest CT scan at 60 days after Ad.IFN-β administration (per modified RECIST schema) and stable tumor metabolic activity on day 60 of 18FDG-PET scanning. Patient 112 received an additional dose (also at \(9 \times 10^{11} \text{ vp}\)) 4 months after initial dosing and had evidence of stable disease on chest CT and 18FDG-PET imaging on day 60 after repeat dosing.

**Discussion**

The most important findings of this study are the following: (a) administration of intrapleural Ad.IFN-β is feasible and well-tolerated with an maximally tolerated dose of \(9 \times 10^{11} \text{ vp}\); (b) intrapleural Ad.IFN-β administration results in gene transfer to tumor (and possibly other cells) manifested by the production of transgene-derived protein (IFN-β); (c) a single dose of vector is able to activate circulating NK cells and induce measurable antitumor humoral immune responses in most patients; and (d) a single dose of Ad.IFN-β gene transfer resulted in 18FDG-PET responses and prolonged disease stability in some patients.

**Gene transfer.** An important component of any gene transfer trial is to document transgene production. Because type I IFNs could potentially be produced as an innate immune response to adenoviral vector instillation (42), we looked for expression of the other type 1 IFN, IFN-α. No IFN-α was detected in any pleural fluid or pleural lavage sample from any of the 10 patients enrolled (data not shown). We also used multiple methods to confirm gene transfer including measuring protein levels, mRNA levels, and immunohistochemical staining. We found that levels of IFN-β peaked the day after gene transfer (Fig. 1A) and then rapidly declined (no detectable levels at day 7). The reason for this decline is not known for
certain. However, given that we were able to detect adenoviral DNA for many weeks after instillation of the vector (Table 2), as well as transgene mRNA in some patients for up to 7 days (Fig. 1B), promoter shutdown may have been involved. An important point, however, is that even these short periods of transgene expression seemed to be sufficient to induce antivector immune responses (see below).

**Neutralizing anti-adenovirus antibodies.** Our neutralizing antibody results were similar to our previous intrapleural Ad.HSVtk study (7). We saw no obvious correlation between baseline titers of antibodies and levels of pleural IFN-β. Interestingly, in patient 112, we observed a strong antitumor immune response to mesothelin after his second dose of Ad.IFN-β, at time when his baseline neutralizing antibody titers were 1:3,200, suggesting effective local gene delivery. Thus, the significance of these neutralizing antibodies on gene transfer in our trial is unknown. We hope to study this issue further by correlating gene transfer to neutralizing antibody titers in our current trial where patients are receiving two doses of vector at a 2-week interval.

**Pleural immune responses.** The presence of an indwelling pleural catheter gave us the opportunity to obtain samples of pleural fluid at multiple time points, thus allowing us to define the response to gene transfer. Key observations in this regard were that the vector did not induce a reproducible cellular influx into the pleural fluid and that intrapleural levels of cytokines (IL-6, IL-1, IL-10, vascular endothelial growth factor, MCP-1, IL-8, RANTES, and IFNγ) were highly variable at baseline and after response to gene transfer. It should be kept in mind, however, that unlike serum measurements, the variable amount of pleural fluid production by each patient likely influenced the final concentration of cytokines. Thus, the concentrations of measured substances should be considered only “semiquantitative.”

**Antitumor immune responses.** Given our underlying hypothesis for the potential efficacy of Ad.IFN-β, one of the most important results of this trial was to show antitumor immune responses (summarized in Supplementary Table S1). Three types of immune responses were identified. First, we were able to show activation of circulating NK cells in two of four evaluable patients (Fig. 1D). This is consistent with known effects of IFN-β (20, 31) and suggests that although serum levels of IFN-β were low, systemic activation of these important immune cells was achievable. Activation of NK cells may thus serve as a useful biomarker of gene transfer in future trials.

Second, CTL responses, assessed by transfecting autologous dendritic cells with tumor RNA, were evaluated in seven patients. In two of these patients, we were able to test responses to autologous tumor cells. A clear CTL response was only seen in one patient (37).

Third, we also examined antitumor humoral responses. In contrast to the CTL data, there was evidence of increased antibody levels against mesothelioma antigens in 7 of the 10 patients. In these patients (summarized in Supplementary Table S1), we most frequently detected increased antibodies in the posttreatment samples that recognized tumor antigens contained in extracts of mesothelioma, lung cancer, or ovarian cancer cell lines (thus the identity of these antigens is unknown; see Fig. 2D-F). For the mesothelioma cases, we were also able to test for antibody responses against specific tumor-associated antigens, including WT-1 (43), mesothelin (44, 45), and the putative mesothelioma carcinoxin SV40 large Tag (46, 47). Using purified Tag protein, we found that one of the seven MPM patients (patient 107) developed a very strong humoral response after gene transfer to this tumor antigen (Fig. 2A). We doubt that this had any therapeutic effect, but rather serves an indication of immune response against a tumor antigen. Mesothelin is a 40-kDa glycoprotein located on the surface of normal mesothelial cells and is also overexpressed in mesotheliomas, as well as cancers of the ovary, pancreas, stomach, lung, and endometrium (44). Thomas et al. (45) have found that mesothelin was a dominant tumor antigen in their immunotherapy trial using an allogeneic pancreatic cancer vaccine. We therefore evaluated anti-mesothelin antibody reactivity using immunoblotting (Fig. 2B and C) and a quantitative ELISA assay (Fig. 3). Four patients had significant baseline levels of anti-mesothelin antibodies and two patients (patients 106 and 110) had clear increases in levels after therapy.

These data indicate that intrapleural Ad.IFN-β led to clearly detectable antitumor immune responses (primarily humoral in nature) in the majority of cases (7 of 10 patients) and are an important proof of principle for our cytokine gene therapy approach. We think that these responses reflect an especially robust response because, unlike most cancer vaccines that are administered in multiple doses over long periods, our trial used only one instillation of vector.

**Clinical responses.** Defining “meaningful” clinical responses in a phase I clinical trial is challenging, especially in immunologic/biological trials where the best clinical end point remains unclear. Data from other immunotherapy trials, our first gene therapy trial, and our current study (in which we think we generated cytostatic or slow cytotoxic effects) suggest that the best primary end points will be time to treatment failure (progression), progression-free survival, and/or overall survival. In addition, there is growing evidence that decreased radiochemical uptake on follow-up 18FDG-PET scans done early after treatment may be an excellent predictor of overall clinical response (34). One interesting, but as yet unresolved, challenge will be to try to identify which immunologic biomarkers might predict response to therapy. As we increase our patient accrual, important correlations may become apparent (e.g., antibody responses to mesothelin or SV40 may be predictive of clinical responses). These relationships may help guide us in designing our future trials.

Given these considerations, we believe that we have seen encouraging, clinically meaningful radiographic responses in this trial, including four patients who showed stabilization of their disease on CT scan at the 2-month post-vector time point. Additionally, two of these patients had evidence of stable and two had markedly diminished tumor metabolic activity on day 60 follow-up of 18FDG-PET scans (Table 1). Four of the 10 patients were still alive, 21 to 35 months after gene transfer.

**Summary and future directions.** In summary, we have shown that introduction of Ad.IFN-β into tumors can generate antitumor immune responses at a high rate. Some preliminary indication of clinical responses (PET responses and prolonged disease stability) suggests that this is a potentially useful approach for the treatment of patients with malignant effusions and mesothelioma and should be investigated further. Accordingly, we have begun a new phase 1 trial in which patients are being given two intrapleural doses of Ad.IFN-β separated by
References


Article on Peptide Epitopes from the Wilms’ Tumor 1 Oncoprotein

In the article on peptide epitopes from the WT1 oncoprotein stimulating CD4⁺ and CD8⁺ T cells that recognize and kill human malignant mesothelioma tumor cells, beginning on page 4547 of the August 1, 2007, issue of Clinical Cancer Research, the following sentence was omitted from the end of the article:

The authors acknowledge Dr. Bo Dupont and Ms. Alice Yeh of the Immunology Program, Sloan-Kettering Institute, for performing the HLA genomic typing for this study.
A Phase I Clinical Trial of Single-Dose Intrapleural IFN-β Gene Transfer for Malignant Pleural Mesothelioma and Metastatic Pleural Effusions: High Rate of Antitumor Immune Responses


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/13/15/4456

Cited articles
This article cites 48 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/13/15/4456.full.html#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
/content/13/15/4456.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.