A Multicenter Phase I Gene Therapy Clinical Trial Involving Intraperitoneal Administration of E1A-Lipid Complex in Patients with Recurrent Epithelial Ovarian Cancer Overexpressing HER-2/neu Oncogene

Srinivasa Madhusudan,1 Ayala Tamir,8 Nicholas Bates,1 Elizabeth Flanagan,1 Martin E. Gore,3 Desmond P. J. Barton,4 Peter Harper,5 Michael Seckl,6 Hilary Thomas,7 Nicholas R. Lemoine,6 Mark Charnock,2 Nagy A. Habib,8 Robert Lechler,8 Joanna Nicholls,8 Massimo Pignatelli,8 and Trivadi S. Ganesan1

1Cancer Research UK Medical Oncology Unit, The Churchill Hospital, Oxford; 2Department of Gynaecology, John Radcliffe Hospital, Oxford; 3Royal Marsden Hospital, Sutton, Surrey; 4St. George’s Hospital, London; 5Guy’s Hospital, London; 6Charing Cross Hospital, London; 7Royal Surrey County Hospital, Guildford; and 8Hammersmith Hospital, London, United Kingdom

ABSTRACT

Purpose: HER-2/neu oncogene is overexpressed in 10–30% of epithelial ovarian cancers and is associated with a poor prognosis. The E1A gene product of adenovirus type 5 down-regulates HER-2/neu and causes tumor regression in animal models. In the current study, we sought to determine the toxicity and biological activity of E1A-lipid complex in ovarian cancer patients.

Experimental Design: A Phase I trial involving intraperitoneal (i.p.) administration of E1A-lipid complex was initiated in ovarian cancer patients to assess biological activity (E1A gene transfer/transcription/translation and HER-2/neu expression) and to determine the maximum tolerated dose. Successive cohorts received E1A-lipid complex at doses of 1.8, 3.6, and 7.2 mg DNA/m2, given as weekly i.p. infusions for 3 of 4 weeks (each cycle) up to a maximum of 2 months. Peritoneal fluid was sampled at baseline and twice monthly for cellularity, cytology, CA-125, and biological activity.

Results: Fifteen patients, with a median age of 57 years (range, 43–81) were recruited. Three (1.8 mg DNA/m2), 4 (3.6 mg DNA/m2), and 8 patients (7.2 mg DNA/m2) received i.p. E1A. A total of 91 infusions (range, 1–18) was administered. Abdominal pain was the dose-limiting toxicity, and the maximum-tolerated dose was 3.6 mg DNA/m2. E1A gene transfer and expression was observed in all of the patients and at all of the dose levels. HER-2/neu down-regulation could be demonstrated in the tumor cells of 2 patients (18%). There was no correlation between dose and biological activity.

Conclusions: I.P. EIA-lipid complex gene therapy is feasible and safe. Future studies, either alone or in combination with chemotherapy, particularly in patients with minimal residual disease, should be evaluated.

INTRODUCTION

Advanced ovarian cancer is the most common cause of gynecological cancer death and is the fifth leading cause of cancer death in women (1). Despite recent advances in surgery and chemotherapy, the overall survival of patients with Fédération Internationale des Gynécologues et Obstétristes stages III-IV remains poor. Hence, there is an urgent need for novel therapies to improve patient outcome.

Several predictive and prognostic factors have been identified over the past decade in an effort to optimize therapy for ovarian cancer patients. HER-2/neu proto-oncogene encodes a 185 kDa transmembrane receptor tyrosine kinase that belongs to the epidermal growth factor receptor family. Activation of HER-2/neu causes initiation of complex signaling pathways involved in the regulation of growth, differentiation, adhesion, migration, and apoptosis of cells (2). Overexpression of HER-2/neu enhances the tumorigenic and metastatic potential of cancer cells in experimental models (3, 4), and has been shown to be a rate-limiting factor for ovarian cancer growth in tumor xenografts (5). HER-2/neu overexpression has been observed in all of the 20 ovarian cancer cell lines derived from advanced tumors or tumor cells from malignant ascites or tumor samples from second surgery suggesting a selective survival advantage for HER-2/neu-overexpressing cells (6). Moreover, its expression has been associated with resistance to chemotherapy in several preclinical studies (7, 8). HER-2/neu is overexpressed in several human tumors including ovarian cancer and has been shown to be associated with a poor prognosis (9–14). The predictive value of HER-2/neu overexpression and chemoresistance has been demonstrated in breast cancer (15) and ovarian cancer patients (10, 16).

The human adenovirus-5 E1A gene encodes two proteins of 289 and 243 amino acid residues. E1A is known to bind to...
transcriptional coactivators, corepressors, and cell cycle regulatory proteins involved in gene regulation and cell growth (17). This essentially reprograms the infected cells toward viral transcription and viral replication. Interestingly, E1A reverses the malignant phenotype and suppresses the growth of human tumor xenografts (18). This tumor-suppressing effect is achieved by several mechanisms including repression of HER-2/neu overexpression (19), induction of apoptosis (20), repression of protease gene expression (21), inhibition of angiogenesis (22), reduced anchorage independence (23), reversal of epithelial-mesenchymal transition, sensitization to anoikis (24), and sensitization to radiation-induced apoptosis (25). The malignant potential of HER-2/neu-overexpressing human ovarian cancer cell lines can be reduced by E1A in various animal models (26, 27). E1A also increases the chemosensitivity of SKOV-3 ovarian cancer cell line to multiple cytotoxic agents (28).

The novel cationic liposome prepared by combining 3β[N-(N′,N′-dimethylaminoethane)-carbamoyle] cholesterol with dioleoylphosphatidylethanolamine (29) has been used in several gene therapy protocols. Multiple administration of the cationic liposome is feasible, because it is easy to prepare, has a relatively low toxicity profile, and does not produce any immunological response despite repeated dosing. The above factors increase the DNA transfection efficiency of the cationic liposome substantially (30).

Ovarian cancer is largely confined to the abdominal cavity both at initial presentation and at relapse. This makes i.p. gene therapy an attractive treatment strategy, as high drug concentrations can be achieved locoregionally (compared with systemic therapy), thereby enhancing drug delivery and possibly drug efficacy (31).

Cationic liposome-based i.p. E1A gene therapy (E1A-lipid complex) caused significant suppression of tumor growth and improved survival in mice bearing i.p. tumors produced by i.p. inoculation of SKOV-3 ovarian cancer cells that overexpress HER-2/neu (32). Delivery of the E1A in an appropriate plasmid delivered using cationic liposomes was investigated preclinically, and no major toxicity was observed after delivering 40 times the proposed starting dose (1.8 mg/m² equivalent) in this trial (33). Furthermore, it has now been shown that a “mini-E1A” (without conserved domains CR2 and CR3) gene is still capable of repressing HER-2-mediated transformation (34).

On the basis of the above preclinical and clinical data, we initiated a multicenter Phase I study to demonstrate E1A gene transduction and HER-2/neu down-regulation in cancer cells of patients with ovarian cancer. Evaluation of the toxicity, tolerability, maximum tolerated dose, and reversibility of such toxicity after i.p. administration of E1A-lipid complex, determination of maximum biologically active dose, and evaluation of tumor response were the other objectives of the study.

MATERIALS AND METHODS

Patient Selection

This multicenter Phase I trial was conducted in compliance with the requirements of the declaration adopted by the world assemblies held at Helsinki, Tokyo, Venice, and Hong Kong (1989). Each participating center was required to obtain local research ethics committee approval before initiating the trial, in addition to approval from the Gene Therapy Advisory Committee (London, United Kingdom). All of the patients provided informed written consent. Patients were eligible to participate in the study if they had advanced, histologically confirmed epithelial ovarian cancer or peritoneal carcinomatosis (stage III or IV according to Tumor-Node-Metastasis classification) of ovarian origin relapsing after first-line chemotherapy. Patients had to have tumors that overexpress HER-2/neu (defined by immunohistochemical examination). Patients had to agree to undergo placement of an i.p. catheter (Tenckhoff catheter; Quinton Instruments, Seattle, WA). Patients without ascites had to undergo serial peritoneal lavages. Zubrod performance status of ≤3, serum creatinine <177 μmol/liter, serum bilirubin <34 μmol/liter, and a normal coagulation profile were necessary before entry.

Preparation of E1A-Lipid Complex

The cationic lipid carrier was prepared by mixing 3β[N-(N′,N′-dimethylaminoethane)-carbamoyle] cholesterol hydrochloride with dioleoylphosphatidylethanolamine in a 3:2 molar ratio (supplied as a lyophilized powder by Fournier Pharmaceuticals Ltd., Slough, United Kingdom) and stored at 2–8°C. The lipid carrier was reconstituted in sterile water to form a cationic lipid suspension before mixing. The original plasmid pE1A-K2 contains the unaltered active E1A gene insert under the control of its own promoter from adenovirus type 5 in a high copy pUC21 plasmid backbone (including a gene coding for kanamycin resistance). It was supplied as a buffered solution (3.36 mM Tris and 0.3 mM EDTA) by Fournier Pharmaceuticals Ltd. and stored at -20 ± 10°C. The E1A plasmid solution was thawed at 37°C, diluted in 5% dextrose, and mixed with cationic lipid suspension in a 1:1 volume ratio to yield a final concentration of 250 μg DNA:250 nmol lipid for administration to the patient.

Treatment Plan

Laparoscopic visualization of the peritoneal cavity was performed on all of the patients before initiating therapy to allow assessment of peritoneal disease and detection of loculated ascites. A standard Tenckhoff peritoneal dialysis catheter was inserted and placed in the pelvis. Before therapy, peritoneal fluid samples were obtained from all of the patients (patients without ascites had peritoneal lavage through the catheter). The E1A-lipid complex was administered i.p. through the catheter over 5–15 min at a rate of 25 ml/min, and the drug administration was completed within 4 h of preparation. During infusion, patients were asked to change positions every 15 min for 1 h to assist uniform distribution of E1A-lipid complex in the peritoneal cavity.

At least 3 patients were recruited at each dose level. A starting dose of 1.8 mg of DNA/m² was chosen because it was equivalent (by standard scaling formula) to the efficacious, nontoxic dose of 15 μg DNA/mouse used in the orthotopic ovarian cancer mouse model (33). Subsequent cohorts of patients received 100% dose increments (3.6 mg DNA/m² escalated to 7.2 mg DNA/m²). Each cycle of therapy consisted of weekly i.p. infusion for 3 weeks followed by 1 week of rest. A maximum of six cycles was allowed for each patient with
long-term follow-up thereafter. Response evaluation was performed at 12 weekly intervals.

**Evaluation Protocol at Baseline and during Therapy**

Safety and biological activities were assessed during and after each course of therapy (Table 1). Patients had physical examination, biochemical evaluation (serum Na, K, glucose, urea, creatinine, alkaline phosphatase, total bilirubin, alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase), hematological evaluation (full blood counts and coagulation profile), immunological testing (ratio of B:T cell lymphocytes CD4 and CD8 in the blood and incidence of CD4 and CD8 T lymphocytes in ascites or peritoneal washings), urinalysis, and peritoneal fluid sampling (for CA125 levels, HER-2/neu, E1A gene transfection analysis, immunology, and bacteriological cultures). Radiological assessment (computed tomography scans) was performed at baseline, before cycle 4, and at the end of therapy.

Toxicity during therapy was categorized as unrelated to, probably, possibly, or definitely related to E1A lipid complex. The dose-limiting toxicity was defined as the highest dose at which at least 2 of the 6 patients experienced National Cancer Institute Common Toxicity Criteria grade 3 or 4 drug-related toxicity during the course of therapy. Maximum tolerated dose was defined at one dose level below dose-limiting toxicity.

Complete response was defined as disappearance of all of the clinical evidence of active tumor for a minimum of 4 weeks, and normalization of tumor markers and tumor-related biochemical abnormalities. Partial response was defined as ≥50% decrease in the sum of the diameters of measured lesions. Stable disease was defined as <25% decrease or <25% increase in the sum of the longest perpendicular lesion diameter. An increase of >25% in the size of any measured lesion or appearance of new lesions was defined as progressive disease.

**Laboratory Procedures**

Immunohistochemistry for HER-2/neu expression, PCR for E1A gene transfer, reverse transcription-PCR (RT-PCR) for E1A gene transcription, and immunohistochemistry/flow cytometry for E1A gene translation were performed.

**HER-2/neu Expression Analysis by Immunohistochemistry.** Paraffin-embedded tissue blocks were dewaxed in xylene and descending grades of alcohol, incubated in 3% hydrogen peroxide for 30 min, followed by microwave oven antigen retrieval in 0.01 M citrate buffer (pH 6.0) for 15 min at 750 W to enhance the affinity of the antigen-antibody reaction before immunostaining. Smears of cell suspensions prepared from peritoneal samples were fixed in absolute alcohol for 10 min followed by Papanicolaou staining to assess the tumor cell density. Endogenous peroxide was blocked using 3% hydrogen peroxide in PBS/methanol for 10 min. Immunostaining using a rabbit polyclonal antibody to HER-2/neu from DAKO (High Wycombe, United Kingdom) at a dilution of 1:1000 was used (incubated overnight at 4°C) followed by biotinylated swine antirabbit IgG (DAKO) incubated for 30 min at room temperature. The section was later incubated with streptavidin-peroxidase conjugate (Amersham International, Little Chalfont, United Kingdom). The peroxidase reaction was developed with 0.01% hydrogen peroxide in 0.05% diaminobenzidine tetrahydrochloride (Sigma Chemical Co., Poole, United Kingdom). The sections were counterstained in hematoxylin, and dehydrated in ascending grades of alcohol and xylene before mounting. Sections from breast carcinoma tissue known to be overexpressing HER-2/neu were used as a positive control. For negative controls, normal rabbit serum (1:2000) was used. The slides were then visualized under a light microscope. Percentage of tumor cells with membranous expression of HER-2/neu (≥20% tumor cells was scored as 0, 21–40% tumor cells was scored as 1, 41–60% tumor cells was scored as 2, and >60% tumor cells was scored as 3) and the intensity of the observed staining (no staining, score 0; weak, score 1; moderate, score 2; and strong, score 3) were analyzed, and the assigned numerical values were multiplied to give a combined score. A combined score of ≥1 was considered as HER-2/neu overexpression.

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* Before infusion.
* 4 weekly for 24 weeks and 6 months thereafter.
* + = performed/assessed.
* Before cycle 4 and after cycle 6.
E1A Gene Expression Analysis by RT-PCR. A semi-quantitative RT-PCR plus nested PCR was performed on cells collected from the peritoneal cavity. Total cellular RNA was extracted using the Qiagen RNeasy mini kit and QIA shred kit. First-strand cDNA synthesis was performed using SUPERSCRIPT II (SUPERSCRIPT II RNAse H Reverse Transcriptase; Life Technologies, Inc.). A positive control using RNA from human embryo kidney cells transformed with E1A adenovirus (HEK 293 cells) and a negative control without reverse transcriptase was also set up. The primers AdE1A-EN1 5' AT GAGACATATTATCGCCACG 3', AdE1A-EN2 5' GTCA ATCCTTCTGTCACC 3', and AdE1A-EN3 5' GGACCA GCTGATCGAAGAG 3' were used in a series of PCRs. The first set of PCR amplification was performed by mixing cDNA with AdE1A-EN1 and AdE1A-EN2 primers. A nested-PCR amplification was performed by mixing the PCR product of the first reaction with AdE1A-EN2 and AdE1A-EN3 primers. The resultant PCR product was resolved on 2% agarose gels.

E1A Gene Expression Analysis by in Situ Hybridization to Cellular mRNA. RNA probes were generated by inserting E1A gene fragment into pGEM (Promega), and the resultant pGEM-E1A recombinant was cut with either HindIII and transcribed with T7 RNA polymerase to generate the 35S radiolabelled antisense RNA probe or cut with EcoRI and transcribed with SP6 RNA polymerase to generate 35S radiolabelled sense RNA probe, which was used as a control. Harvested cells were fixed with 4% paraformaldehyde (for 20 min), dehydrated in a series of graded alcohols, and air-dried. Before hybridization the slides were permeabilized in proteinase K for 10 min at 37°C and later washed twice with PBS containing 2% glycine, post-fixed in 4% paraformaldehyde for 20 min, acetylated with 500 ml 0.1 M Triethanolamine and 1.25 ml acetic anhydride for 10 min, dehydrated through graded alcohol, and allowed to dry. The slides were immersed in a rack containing 500 ml of 50% formamide buffer (prewarmed to 55°C) over 3–4 h and then washed in 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA pH 7.5 (TNE) buffer nine times. The slides were incubated with 100 μg/ml RNase A in TNE buffer for 1 h at 37°C. The slides were then washed with 0.5× SSC, 0.1× SSC, and 0.1× SSC serially for 30 min at 65°C with agitation, and passed through graded ethanol containing 0.3 M ammonium acetate, air-dried, subjected to autoradiography, and examined under bright field microscope to assess the percentage of cells showing evidence of in situ hybridization (silver granules). The preparations were scored as follows: < 5% cells positive = –, 5–30% cells positive = +, 30–70% cells positive = ++, > 70% cells positive = +++.

E1A Protein Expression Analysis. E1A protein expression analysis by immunohistochemistry and flow cytometry was performed using anti-E1A monoclonal antibody (E1A M73 monoclonal antibody; Oncogene Science, Inc., Cambridge, MA). Staining of cells within a lymphocyte gate and within a tumor gate were analyzed to study the specificity of E1A incorporation into tumor cells.

Immunological Investigations. To investigate immunological responses to E1A therapy, peripheral blood mononuclear cells and ascitic fluid cells were stained with fluorochrome-conjugated monoclonal antibodies. To test activation of T lymphocytes, double staining was performed for expression of HLA-DR/CD4 and HLA-DR/CD8 molecules on T lymphocytes. Incorporation of the E1A plasmid into cells of ascitic fluid was examined by intracellular staining. In brief, cells were fixed with 4% paraformaldehyde in PBS for 5 min at 4°C. After washing them twice in PBS, they were incubated for 30 min at room temperature in the dark, either with primary control MSA3 IgG2a antibody or with E1A antibody (5–20 μg/ml), together with 0.5% saponin and 1% FCS diluted in PBS. The same procedure was repeated when the cells were stained with 20 FITC antimouse antibody. Cells were acquired and analyzed by flow cytometry (Becton Dickinson).

RESULTS

Patient Characteristics

A total of 75 patients diagnosed previously with stage III/IV ovarian cancer were screened for the HER-2/new status from existing pathology specimens using either formalin-fixed, paraffin-embedded tissue sections (n = 52) or cytospin sections from ascitic fluid (n = 23). These were selected patients who had recurrent ovarian cancer. Overall, 21 of 52 (paraffin blocks) and 16 of 23 (ascites) were positive for HER-2/new. Fifteen patients with advanced ovarian cancer not amenable to conventional therapy participated in this multicenter Phase I trial between January 1998 and September 1999. Patient characteristics are summarized in Table 2. Median age was 57 years (range, 43–81). Most patients had good performance status with Zubrod score of 0 (2 patients), 1 (9 patients), and 2 (4 patients) before entry. Eight patients had stage III and 7 had stage IV disease at baseline. Ten of the 15 patients had clinical ascites before starting therapy. None of the patients had any loculation of ascites as assessed under laparoscopy. Most of the patients had been heavily pretreated with surgery, chemotherapy, and/or hormone therapy, and/or radiotherapy, and/or other trial medications with up to 80% of patients having received platinum-based therapy and 53% having received Taxol therapy before E1A (prior lines of treatments ranged from 1 to 5). Histopathological samples from initial surgery were assessed for HER-2/new expression and was confirmed to be overexpressed in all of the patients [(combined scores of 9 (3 patients), 6 (6 patients), 4 (4 patients), and 1 (2 patients)]. After obtaining informed written consent, a Tenckhoff catheter was placed i.p.

Treatment

Cohorts of at least 3 patients received ascending doses of E1A-lipid complex starting at 1.8 mg DNA/m² (n = 3 patients), and then escalated to 3.6 mg DNA/m² (n = 4 patients) and 7.2 mg DNA/m² (n = 8 patients). All of the patients were closely assessed during therapy (Table 1). A total of 91 infusions were administered with an average of 6 infusions per patient (range, 1–18). Seven patients received at least 6 E1A infusions. All of the patients except patient 1A stopped treatment before a maximum of six cycles (18 E1A infusions) due to progressive disease or adverse events.

Toxicity

The most frequently reported adverse events “possibly” or “probably” related to E1A-lipid complex were asthenia (incidence 80%), abdominal pain (incidence 87%), nausea and/or...
vomiting (incidence 93%), and fever (incidence 40%). A majority of the side effects occurred within 24–48 h after infusion. Toxicity data expressed as number of patients at each dose level with the worst grade of toxicity due to E1A are summarized in Table 3. Twelve patients were evaluable for toxicity. A total of 28 serious adverse events were recorded, out of which 5 events were attributed to the study drug [abdominal pain with or without other symptoms (4) and intestinal obstruction (1)] and the rest attributed to disease progression. There were no treatment-related deaths. Abdominal pain was the dose-limiting toxicity. No patient experienced grade 3 abdominal pain in cohort 1. On the other hand, 2 patients in cohort 2 and 4 patients in cohort 3 experienced severe abdominal pain. In patient 10C, treatment had to be withdrawn due to severe abdominal pain. Patient 9C required a dose reduction to 5.4 mg DNA/m² after first infusion. In patient 10C, in addition, patients without ascites were more likely to experience abdominal pain, and buscopan given either orally or i.m. was found to relieve the pain rapidly. Therefore, subsequent patients received prophylactic buscopan (20 mg given i.m.) 30 min before infusion continued and 6 hourly for the first 24 h, and in addition, patients without ascites received 1.5 liters of 5% dextrose infusion before E1A. Nausea and vomiting responded to antiemetic therapy. Fever resolved either spontaneously or in response to antipyretics. As a consequence of or related to the use of the i.p. catheter, several adverse events were reported including catheter dysfunction (40%), catheter site infection (27%), catheter site inflammation (27%), and infection of ascitic fluid (27%). There was no evidence of a relationship between catheter events and dose level or time after infusion. However in 3 patients (5B, 8C, and 9C) treatment had to be withdrawn due to catheter-related problems.

No clinically significant abnormalities in hematology, coagulation, serum biochemistry, and urine were detected directly attributable to trial medication.

### Table 3 Patient demographics and response data

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</table>

* A, cohort 1 (1.8 mg DNA/m²); B, cohort 2 (3.6 mg DNA/m²); C, cohort 3 (7.2 mg DNA/m²).

* RT-PCR, reverse transcription-PCR; PD, progressive disease; AE, adverse event; NA, not available; E1A gene expression [+ (low), ++ (moderate), +++ (high)]; ↓ (decrease); ↑ (increase); ⇨ (no significant change).

* These patients were not included in the safety committee assessment (7B was recruited on compassionate grounds, 8C inadvertently received 7.6 mg DNA/m², and 9C received dose reduction to 5.4 mg DNA/m² after first infusion).

### Table 3 Toxicity data (expressed as number of patients with worst grade of toxicity “probably or possibly” related to E1A lipid complex)

<table>
<thead>
<tr>
<th></th>
<th>Cohort 1 (n = 3)</th>
<th>Cohort 2 (n = 4)</th>
<th>Cohort 3 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>Grade 2</td>
<td>Grade 3</td>
<td>Grade 4</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Astenia</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Fever/chills/flu</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Headache</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Anorexia</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Intestinal obstruction</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Other toxicities “probably or possibly” related to E1A lipid complex were grade 2 skin rash (1), grade 1 ascites (1), grade 1 hypotension (1), grade 2 anemia (1), grade 2 pleural effusion (1), Cohort 1 (1.8 mg DNA/m²), Cohort 2 (3.6 mg DNA/m²), Cohort 3 (7.2 mg DNA/m²).
Biological and Disease Response to E1A-Lipid Complex

**E1A Analysis (Table 2).** Each patient’s tumor showed E1A gene expression levels ranging from + to ++++. There was no correlation between the intensity of expression and dose level. The RT-PCR was negative at baseline and was positive after starting E1A therapy (Fig. 1A). Three patients (4B, 5B, and 7B) showed baseline low level of E1A gene expression in the peritoneal sample suggestive of adenoviral infection before initiating gene therapy. Interestingly, in each of these patients E1A expression increased measurably from the base level, indicating expression from the exogenous gene delivered in the E1A-lipid complex. 

**HER-2/neu Analysis (Table 2).** At baseline, all of the patients had HER-2/neu overexpression. With E1A gene therapy 2 of the 15 patients showed a consistent (i.e., more than two consecutive measurements) decrease in HER-2/neu expression detected by immunohistochemistry of cells taken from the peritoneal cavity. Patient 1A received the lowest dose of E1A (1.8 mg DNA/m²), and the HER-2/neu levels remained negligible for the last four cycles (Fig. 2A). Patient 14C, who received the highest E1A dose of 7.2 mg DNA/m², also showed a consistent HER-2/neu down-regulation.

**Immunological Analysis.** *In vitro* monitoring of tumor-specific immune response frequently involves phenotypic analysis of the patient lymphocytes, before and after the treatment. Expression of activation markers on T cells has been examined in peripheral blood mononuclear cells (35, 36), but mostly on tumor-infiltrating lymphocytes (TILs; Refs. 37–39). Whereas expression of markers such as CD69 demonstrates early activation, HLA-DR expression occurs at a later stage.

We analyzed DR expression on CD4⁺ and CD8⁺ T cells in the ascites and in peripheral blood of ovarian cancer patients with advanced metastasis. Our results demonstrate that E1A
treatment triggered DR-expression in all but 1 patient, although to different degrees. We analyzed the effects of the E1A plasmid vaccination on the number of double-positive CD4/DR and CD8/DR cells in the ascites and in the peripheral blood of the patients. Fig. 3, A and B, demonstrates that already after the first treatment, there was a major increase in the percentages of DR-expressing lymphocytes. After the last treatment, a significant expansion of the DR-expressing CD4⁺ and CD8⁺ was observed. Additional analysis of changes in mean fluorescence intensity of DR molecule expression by ascitic fluid in three different patients was performed and indicated marked increase of DR expression (Fig. 3C). In summary, our results indicate that treatment with E1A-plasmid up-regulated expression of HLA-DR on T lymphocytes of ovarian cancer patients. We also observed that within the same patient, DR expression was stronger in peritoneal fluid lymphocytes (Fig. 3B) than in those in peripheral blood (Fig. 3A).

CA-125 Analysis (Table 2). Seven patients received at least 2 months of therapy, of whom 4 patients showed stabilization of CA-125 during therapy. Patient 1A showed a 50% decrease in CA-125 levels after the first cycle followed by stabilization for the next four cycles and an additional fall to <25% of the starting value for the last course.

Ascitic fluid CA-125 levels consistently fell in 3 patients (patients 1A, 4B, and 7B), and in the rest, the values increased or were inconclusive.

**Disease Response (Table 2).** Six patients were available for radiological tumor response evaluation. No antitumor responses were seen. Patient 1A achieved stable disease after 3 cycles but progressed after 6 cycles of therapy. All of the other patients had progressive disease.

**DISCUSSION**

This Phase I clinical trial involved heavily pretreated patients with advanced ovarian cancer not amenable to standard therapy. Assessment of toxicity was difficult, as many of the symptoms could have been related to progressive disease. Abdominal pain evolved rapidly in patients soon after E1A-lipid complex administration. Although this was tolerated differently by individual patients, dose-limiting toxicity was observed at 7.2 mg DNA/m². Abdominal pain was more likely in the absence of ascites. During the course of the trial we found that treatment with buscopan effectively controlled the abdominal pain. Therefore, prophylactic buscopan was given to all of the subsequent patients. The success of this strategy could not be evaluated fully in this small group of patients. Although the mechanism of abdominal pain remains obscure, it is more likely to be related to the lipid component rather than the plasmid DNA. The presence of an indwelling catheter was the reason for several adverse events such as catheter dysfunction, catheter site infection, catheter site inflammation, and infection of ascitic fluid. Mechanical dysfunction due to blockage and one-way “valving” are a frequent consequence of peritoneal loculation, and/or obstructive growth of malignant adhesions observed commonly in late-stage ovarian cancer. Guidelines for prevention of catheter-related issues were distributed to patients, their relatives, study nursing team, and medical staff. Briefly, the guidelines for patients focused on catheter hygiene, precautions during activities of daily living, and instruction for daily dressing of the catheter (dressing packs were provided to patients). In addition, an information sheet highlighting the potential problems and early recognition of these were provided to the patients. The medical staff was issued guidelines for catheter insertion including the use of prophylactic antibiotics (e.g., i.v. cephalaxin, metronidazole, and gentamicin as stat doses) and detailed guidelines for drainage of ascites and i.p. administration of fluids before E1A lipid complex administration to patients without ascites. However, the study was terminated before any change in the frequency of catheter-related adverse events were noted. It is also likely that cancer-related immune suppression could have significantly contributed to these catheter-related events. Moreover, similar symptoms are observed in i.p. administration of conventional cytotoxic drugs.

We have been able to show E1A gene transduction into tumor cells at all of the dose levels confirming the transfection efficiency of the cationic liposome. We have also convincingly demonstrated HER-2/neu down-regulation in 2 patients. However, the rest of the patients did not show HER-2/neu down-regulation despite having had E1A gene transduction. It is interesting that, despite excellent preclinical data, down-regulation of HER 2/neu was not observed in all of the patients. This
might be due to several factors, including that suppression by E1A is dependent on an adequate amount of E1A being delivered and persisting in tumor cells. Because only 6 patients received 2 months of therapy, it is possible that this may be a factor.

Immunological monitoring in this study included measuring DR expression levels on CD4+ and CD8+ T cells in peripheral blood and in ascitic fluid. DR expression on TILs and on peripheral blood T lymphocytes have been largely investigated in various tumor models. Expression of DR molecule on T cells is normally low, and we found 5–8% expression in 5 different normal individuals. Studies have demonstrated that in vitro expansion of TILs in the presence of interleukin 2 and tumor necrosis factor α (37, 40) or after stimulation with anti-CD3 (36, 38) up-regulated expression of HLA-DR on these cells. Other studies compared DR expression on peripheral blood and on TIL CD3+ cells, and showed that such expression was higher on CD3+ TILs (35, 39). Van den Hove et al. (36,
38) showed that stimulation with anti-CD3 increased DR- mean fluorescence intensity on TIL-CD3+CD8+ and CD3+CD4+ cells. However, unlike other studies, our study elaborated on the effects of a specific antitumor treatment on DR expression in different T-cell subtypes. Our immunological monitoring indicated that in ovarian cancer patients, E1A treatment triggered DR expression on T lymphocytes. This expression was higher on TIL than on peripheral blood T cells. Specifically, we observed that in some patients, the differences between DR expression on CD8+ cells and on CD4+ cells increased during the treatment. Additional investigation, such as of CTL killing capacity and T-cell receptor analysis, are required to determine whether oligoclonal expression of antigen-activated T cells or nonspecific cytokine-mediated effects were responsible for these findings.

All of the patients had a heavy disease burden before starting therapy. It is perhaps not surprising that no partial or complete responses were seen. Interestingly, Patient 1A, who completed the trial, showed stable disease after three courses of therapy and had an impressive reduction in serum CA-125 levels. This may represent a favorable disease response to E1A. In addition at least 4 of 6 patients had stabilization of serum CA-125.

A Phase I trial of E1A lipid complex (administered either intrapleurally or i.p.) in patients with recurrent breast or ovarian cancer was reported recently (41). Maximum tolerated dose was defined at 3.6 mg DNA/m2. Most patients receiving the highest dose of 7.2 mg DNA/m2 reported grade 2 or 3 nausea, vomiting, and discomfort at the site of injection. Six patients (5 breast cancer and 1 ovarian cancer) in whom samples were available had E1A gene transduction and HER-2/neu down-regulation. This was associated with increased apoptosis and reduced proliferation in tumor cells. Improvement in performance status and brief period of disease stabilization was seen in 3 patients. In another Phase I study, E1A lipid complex was administered by intratumoral injection in recurrent head, neck, and breast cancer patients. No dose-limiting toxicity was observed. E1A gene transduction was seen in most patients. Two of 5 patients had HER-2/neu down-regulation. Interestingly, eight patients had stable disease, and 2 achieved minor responses (42). Our study and those reported by other investigators confirm the safety, biological activity, and gene transfection efficiency of E1A lipid complex. A recent multicenter Phase II study in head and neck cancer (involving intratumoral injections) reported a complete response in 1 patient, minor responses in 2 patients, and stable disease in 7 patients (43). Whereas locoregional E1A gene therapy in ovarian cancer seems promising, it involves indwelling catheter insertions or intratumoral injections and is unlikely to be effective for disseminated disease. Systemic E1A gene therapy is an attractive treatment strategy. In fact, tumor xenograft studies suggest that this approach is feasible, and systemic E1A therapy produces antitumor responses in mice (44). Whether this could be translated into human studies remains to be established. Evolving preclinical data indicate that E1A could enhance the cytotoxicity of chemotherapy (45). E1A enhances the sensitivity of paclitaxel in Taxol-resistant HER-2/neu-overexpressing human ovarian cancer cell lines. Nude mice bearing i.p HER-2/neu-overexpressing human ovarian cancer cells treated with paclitaxel and E1A survive significantly longer than those treated with either E1A or paclitaxel alone (46). The recent development of a “mini E1A” gene, which is equally effective preclinically, is important for future investigations (34).

Rapid advances in biology have revealed the diverse functions of the ErbB family of receptors and the complex signaling network they initiate (2). Accordingly, several strategies have been developed to target this pathway for antitumor therapy. Immunological manipulation, particularly with a humanized antibody to HER-2/neu, has been extensively investigated and is now approved for the treatment of breast cancer (47). A Phase II trial of Herceptin in ovarian cancer was reported recently. Although the treatment was well tolerated, the low objective response rate (7.3%) seen in this trial is far from encouraging (48). In this context, investigation of other approaches is particularly important. Antigen-specific immunotherapy with HER-2/neu protein for human cancer is also being investigated (49). Low molecular weight chemical inhibitors of the ErbB signaling pathway have been synthesized recently and show significant antitumor effects in preclinical studies (50). In fact several reversible and irreversible tyrosine kinase inhibitors are in various stages of clinical development (51, 52). Assessing their role in ovarian cancer is likely to produce interesting results. Moreover, farnesyl transferase inhibitors, mitogen-activated protein kinase inhibitors, and Akt inhibitors block activated ErbB pathways, and they might have therapeutic effects in HER-2/neu overexpressing tumors (53). Finally, several other gene therapy approaches to ovarian cancer have been investigated in recent years (54). Intracellular single chain antibody (scFvs) directed to ErbB2 can block receptor transfer from endoplasmic reticulum to the plasma membrane and, hence, reduce ErbB2 signaling (55). Such ErbB-2 knockout-based gene therapy protocol using anti-erbB-2 scFv can cause tumor regression and prolong survival in mice (56). A human Phase I gene therapy protocol for anti-erbB-2 scFv has now been developed based on preclinical data (57), and results are awaited.

We conclude that single-agent E1A lipid complex gene therapy is feasible and safe. On the basis of the clinical trials including this one on E1A gene therapy, we also conclude that loco/regional administration of E1A using cationic lipids is sufficient to induce transduction. In this group of heavily pre-treated patients not only was reduction of HER-2 levels seen but clinical responses were also noted for what is essentially a noncytotoxic treatment. However, patients in this study (and in those reported by other investigators) had rapidly progressive advanced disease that might have underestimated the true efficacy of E1A gene therapy. Therefore, future studies should involve patients with less advanced disease (preferably with minimal residual disease). E1A-lipid complex either alone or in combination with chemotherapy is likely to be more promising in this situation.

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A Multicenter Phase I Gene Therapy Clinical Trial Involving Intraperitoneal Administration of E1A-Lipid Complex in Patients with Recurrent Epithelial Ovarian Cancer Overexpressing HER-2/ neu Oncogene

Srinivasan Madhusudan, Ayala Tamir, Nicholas Bates, et al.