A Phase I Trial of Retroviral BRCA1sv Gene Therapy in Ovarian Cancer

David L. Tait, Patrice S. Obermiller, Sheryl Redlin-Frazier, Roy A. Jensen, Piri Welsh, Jamie Dann, Mary-Claire King, David H. Johnson, and Jeffrey T. Holt


ABSTRACT

Gene transfer of BRCA1sv (a normal splice variant of BRCA1) into ovarian cancer cells produces growth inhibition in vitro and tumor suppression in nude mouse xenografts. As an initial step toward gene replacement therapy for ovarian cancer, we conducted a Phase I trial to assess the pharmacokinetics and toxicity of i.p. BRCA1sv retroviral vector therapy.

Following placement of an indwelling Port-a-Cath in patients, a dose escalation study was performed of four daily i.p. infusions spanning doses from 3 to 300 ml (i.e., 10^10 viral particles) at half-log intervals (23 cycles in 12 patients). Gene transfer and expression were documented by PCR, Southern blot, reverse transcription-PCR, and nuclease protection assays. Pharmacokinetics were assessed by PCR and Southern blots detecting vector DNA, and toxicity was evaluated by clinical exam and fluid analysis.

Three of 12 patients developed an acute sterile peritonitis, which spontaneously resolved within 48 h. Plasma and peritoneal antibodies to the retroviral envelope protein were detected only in patients treated with the highest dose levels but not in others, despite repeat dosing for an interval of up to 4 months. Eight patients showed stable disease for 4–16 weeks, and three patients showed tumor reduction with diminished miliary tumor implants at reoperation (two patients) and radiographic shrinkage of measurable disease (one patient).

The vector-related complication of peritonitis was observed in three patients but resolved quickly as in preclinical mouse studies. Ovarian cancer may provide an important model for retroviral gene therapy studies due to vector stability, minimal antibody response, and access to tumor by i.p. therapy.

INTRODUCTION

Cancer gene therapy approaches use a number of distinct strategies including tumor suppressor gene replacement, onco-gene inactivation, immunotherapy, and introduction of drug sensitivity or resistance gene (1). Because the BRCA1 gene is mutated in the majority of cases of hereditary breast and ovarian cancer and exhibits loss of heterozygosity and/or decreased expression in many sporadic cases (2–10), we have explored the possibility of BRCA1 gene replacement as a potential gene therapy for ovarian cancer. Multiple studies have demonstrated that overexpression of the BRCA1 gene results in growth inhibition and/or cell death, suggesting that BRCA1 can function directly as a growth inhibitor or tumor suppressor (6, 11–15). Alternatively, growth inhibition may not be the major or sole mechanism for tumor suppression (16) because BRCA1 also functions during differentiation (17–19) and may modulate DNA repair (16, 20).

The peritoneal cavity is both the first site of metastasis of most ovarian cancers and potentially a permissive environment for retroviral vectors. Retroviruses are rapidly inactivated by complement in human sera (21–25) but are considerably more stable in anatomical compartments, such as the peritoneal cavity (22), with lower complement levels due to complement inhibitors in peritoneal fluid (21). These ideas led to the hypothesis that i.p. BRCA1 retroviral gene therapy could inhibit invasive ovarian cancer through gene replacement of a tumor suppressor gene or by its more generalized growth-inhibitory properties.

Preclinical studies have demonstrated that i.p. injection of retroviral vectors expressing BRCA1sv can inhibit the growth of established i.p. tumors, using a nude mouse xenograft model (11). Control retroviral vectors do not exhibit a significant antitumor effect, indicating that this is an effect of BRCA1 gene transfer and not merely a nonspecific effect of retroviral injection (11). These studies showed transduction of tumor cells and expression of BRCA1 mRNA and protein following i.p. injection of established i.p. tumors (11). Several different BRCA1 viral vectors have been produced and tested for efficacy in preclinical xenograft models of breast and ovarian cancer. Attempts to generate an adenoviral vector expressing BRCA1 have been unsuccessful to date, and identification of high-titer retroviral producer clones has required screening of numerous clones.
(presumably due to the growth-inhibitory effects of BRCA1). Although retroviral producer clones expressing wild-type BRCA1 may be obtained at moderate titer and their viral stocks produce a small but significant tumor reduction compared to control vector, i.p. injection of a retroviral vector expressing a normal BRCA1 splice variant LXSN-BRCA1sv\(^1\) produces marked tumor reduction and even cure in some ovarian cancer models.\(^4\) For example, treatment of established PA-1 ovarian cancer nude mice xenografts with retroviral vectors show the following survival results. LXSN control vector-treated mice died 60–70 days after tumor cell injection; LXSN-BRCA1-treated mice died at 76–79 days; and LXSN-BRCA1sv-treated mice died at 78–131 days. Necropsies showed that mice treated with control media or low-dose LXSN-BRCA1sv died with large intra-abdominal tumors and ascites, whereas mice with high-dose LXSN-BRCA1sv treatments died of lung metastasis with significantly smaller abdominal tumors.\(^4\) The antitumor effect may be due to secretion of the BRCA1 gene product and hence a paracrine inhibitory effect (26), although the precise biochemical function of BRCA1 is controversial and may involve nuclear functions (27, 28) such as DNA repair (20) or transcriptional activation (29, 30). Toxicity of i.p. LXSN-BRCA1sv vector therapy was evaluated in preclinical mouse models as we have described previously (31) and revealed a dose-related peritonitis in mice at high-vector doses but only in those mice in the highest dose group, which were previously treated with oyster glycogen to induce peritonitis.

A Phase I study of LXSN-BRCA1sv administered as a single agent i.p. for four consecutive daily doses was conducted to evaluate toxicity in patients and to determine the pharmacokinetics of i.p. retroviral vector therapy. The aims of this study were to identify the maximal tolerated dose and to study the peritoneal pharmacokinetics, transduction rate, and expression of retroviral vector in relation to toxicity.

**PATIENTS AND METHODS**

**Patient Selection and Eligibility Criteria.** Patients with recurrent or persistent metastatic epithelial ovarian cancer treated previously with standard surgery and chemotherapy were considered for study. The study was approved by the Vanderbilt Institutional Review Board and the Vanderbilt Institutional Biosafety Committee and was reviewed by the Office of Recombinant DNA Activities and declared exempt from Recombinant Advisory Committee of the NIH review in February 1996 but was then reviewed and approved in April 1997 after Recombinant Advisory Committee of the NIH reorganization. An investigational new drug application was filed with the FDA,\(^5\) and the study was allowed to proceed once FDA questions and concerns were satisfied. Written informed consent was obtained from all patients before investigations to determine eligibility were performed. Separate written informed consent was obtained prior to BRCA1 gene sequencing of patient samples. All patients were significantly chemoresistant because they had been treated with multiple chemotherapeutic agents (median, three) prior to entry to the study. Inclusion criteria included measurable tumor in two dimensions confined to the peritoneal cavity, age &gt;18 and &lt;75 years, Gynecological Oncology Group performance status of &lt;2, life expectancy of greater than 3 months, 4-week interval from previous surgery and/or cancer therapy, adequate hematological (WBC &gt;4000 mm\(^{-3}\)), hepatic (bilirubin &lt;2 mg/dl; aspartate aminotransferase &lt;2X normal), and renal (creatinine &lt;1.5 mg/dl) functions. Exclusion criteria included: active bacterial infections; concomitant experimental or other alternative therapies; heart failure (New York Heart Association class 4), recent myocardial infarction, respiratory insufficiency, or hematological, hepatic, or renal dysfunction; or concomitant anticoagulant or antiplatelet drug therapy. For all patients, cDNA of BRCA1 from lymphoblast lines was screened for mutations by direct sequencing and by single-strand conformational polymorphism, as described previously (5). These studies would not have detected rare somatic mutations in BRCA1 within a tumor, because DNA sequencing was not performed on tumor DNA samples.

**Vector Structure, Production, and Testing.** LXSN-BRCA1sv vector was constructed by cloning a BRCA1 cDNA into the well-characterized retroviral vector LXSN (Fig. 1) (11). The LXSN-BRCA1 retroviral construct incorporates a BRCA1 splice variant (BRCA1sv, GenBank accession no. AF 005068), which functioned successfully as a growth suppressor of tumor cells in vitro and in vivo (11). Compared to the wild type, this BRCA1, derived from human testicular RNA, contains three splicing differences [nucleotide numbering is from the BRCA1 genomic sequence (GenBank accession no. L78833; Smith et al., Ref. 32)]: (a) exon 1c is nucleotides 3447–3553 of genomic BRCA1 with two substitutions, G3465T and A34700, in the noncoding sequence; (b) exon 5 is nucleotides 22201–22256 (compared to wild-type exon 5 at nucleotides 22201–22278), which is spliced to wild-type exon 6. The contiguous cDNA sequence of the splice variant, corresponding to wild-type BRCA1 cDNA sequence (HSU14680) nucleotides 300–309 and 332–337 is TGTCCTTTATGAGC-CTA. Hence, BRCA1sv has a TGA stop in frame 1 (the ultimate cysteine of the ring finger), followed by a cryptic start in frame 3, from which the proper reading frame would be restored and

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\(^1\) A normal splice variant of the BRCA1 gene. The complete sequence of the BRCA1sv cDNA is in GenBank, accession number AF 005068.

\(^4\) P. S. Obermiller and J. T. Holt, unpublished data; data on file at the FDA.

\(^5\) The abbreviations used are: FDA, Food and Drug Administration; GAPD, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; CH50, activity of complement producing 50% hemolysis.
translation would generate wild-type BRCA1 sequence to codon 1863. Translation of amino acids 72–1863 (Mr 200,000 predicted size) was confirmed by tagging the LXSN-BRCA1 construct with an epitope in exon 11. 3’ of the cryptic start. MCF-7 cells transfected with this construct produced a Mr 190,000 band on a Western blot stained with hemagglutinin antibody, which was not present in untransfected cells (Fig. 2c of Ref. 26). In addition, reticulocyte translation of BRCA1sv initiated translation at amino acid 72, producing an N-terminally truncated protein 95% of the size of wild-type BRCA1 (data on file at the FDA). Growth inhibition of breast and ovarian tumor cells in culture and of tumors in mice transduced with LXSN-BRCA1sv is, therefore, likely to be due to a BRCA1 protein with initiation of translation 3’ of the ring finger.

Retroviral vector was manufactured under cGMP (current Good Manufacturing Practices) conditions using a CellCube (Corning-Costar, Cambridge, MA) apparatus perfused with Aim V media (Life Technologies, Gaithersburg, MD) under continuous monitoring of pH and O2. Once the oxygen and glucose consumption were consistent and appropriate, supernatant was collected as long as the oxygen and glucose levels assured optimal vector production. No postproduction manufacturing was performed on the supernatants collected in Aim V media, which were frozen and stored in aliquots at −70°C. The titer of the vector preparations was determined by counting the number of particles present that conferred G418 resistance to transduced MCF-7 cells, using appropriate dilutions. Vector from this production lot tested negative for bacterial, Mycoplasma, and viral contamination and was endotoxin negative. Replication-competent retroviruses could not be detected using PG4 indicator cells following amplification on Mus musculus. In addition to the tests performed on the clinical grade vector described above, a number of tests had been performed previously on the producer cells in the master cell bank, including tests for pathogenic viruses and replication-competent retroviruses. A toxicity study was done in mice: 92 Balb/C female mice were injected with either 300 ml, depending on the dose escalation) was given with 1.5 ml of sterile saline i.p., and the three subsequent doses were given with sterile saline to a total volume of 100–300 ml.

**Vector Administration.** Aliquots of vector were thawed, and 8 μg/ml of polybrene were added in a sterile manner. Infusions of vector into patients were always initiated within 1 h of thawing the vector aliquot. The initial dose (between 3 and 8 p.g/ml of polybrene were added in a sterile manner.

**Study Design.** Patients underwent initial placement of a peritoneal Port-a-Cath for access to the peritoneal cavity followed by admission to the Clinical Research Center at Vanderbilt University Medical Center for treatment. Patients were treated in the Clinical Research Center for 4 consecutive days with i.p. LXSN-BRCA1sv gene therapy. The starting dose level in patients was that dose which corresponded to the no effect dose in mice (106), and a half-log dose escalation was performed up to the dose that corresponded to the LD10 dose in mice (1010). Five dose levels were studied: 105, 3.3 × 105, 106, 3.3 × 106, and 1010 viral particles. Objective end points to assess toxicity included: daily blood and peritoneal sample to evaluate peritoneal fluid cell counts; hematological cell counts; serum chemistries; bacterial cultures as needed; vector stability; viral uptake by cells; expression of BRCA1 gene; and the presence of antibodies to vector envelope proteins. Measurable tumor dimensions were determined at study entry by computed tomography scan or physical exam. At 4-week intervals, patients were evaluated for response to therapy; and if tumor measurements were stable or decreased, retreatment was allowed. The first three patients were treated at the first dose level. After the next higher dose level was tolerated by a new patient, any repeat patients were graduated to that dose. The dose was again elevated after three patients had tolerated it without toxicity. Patients who demonstrated tumor progression were evaluated at monthly intervals until death, at which time autopsy was requested to evaluate for the systemic presence of retroviral particles and sites of tumor progression.

**Detection of Vector Stability and Expression.** DNA was prepared from cell samples by hypotonic lysis, digestion with proteinase K (Boehringer Mannheim, Indianapolis, Indiana), and SDS, followed by phenol/chloroform extraction and ethanol precipitation. DNA was prepared from tissue or tumor samples by freezing samples at −70°C and then finely mining cold samples with a blade, prior to treatment with proteinase K as described above. RNA were purified from both cells and tumors by lysis in guanidinium isothiocyanate by our prior cited methods (6). PCR primers specific for the neo sequences within the LXSN-BRCA1sv vector were used for determination of vector presence and stability within patient samples. The primers were 5’-CCGCCCGTTGGGTGAGA-3’ and 5’-CAGGTAACGGGATACAAAGGCTATGC-3’ (Ransom Hill, Ramona, CA) and were by prior published methods (11). RT-PCR was performed by our published methods (6). The following primers were used for RT-PCR studies on patient samples: LXSN-BRCA1sv primers designed to span the LXSN LTR and BRCA1 sequences, 5’-CCCTCCCTGTTGCTAAAGCCCTTTATCA-3’ and 5’-TTCAACGCGAAGAAGCAGATAATCAT-3’; and control primers for GAPD with sequences S’-CCCTCCCTGTTGCTAAAGCCCTTTATCA-3’ and 5’-AGCCCCAGCTTCTTCCCTAT-3’ (Ransom Hill, Ramona, CA).

Southern blotting of AvaI-digested DNA was performed with a human BRCA1 probe that was directed against exon 24, producing a 1.8-kb fragment from the vector and a 900-bp fragment from normal genomic DNA. The percentage of transduction was estimated by quantitating hybridization with the phosphoimager and then comparing hybridization of the presumed haploid vector lower band to that of the diploid BRCA1 upper band (percentage of transduction = 2 × vector signal/genomic signal × 100). Nuclease protection assays were performed with mRNA isolated from patient samples and then probed with a T7 polymerase-generated probe from a BRCA1 DNA template digested with HinII, which spans nucleotides 25 to 360. Radiolabeled probe was hybridized with patient mRNA.6 Data on file at the FDA.
samples for 8 h at 52°C in 80% formamide and then digested for 30 min with RNase A and RNase T1 at 25°C, and then products were resolved on a 10% denaturing polyacrylamide gel as described (6). Thus, this probe would protect a 210-bp fragment from LXSN-BRCA1sv splice variant mRNA (100-310). These size variations result because other patient. Although several patients were found to have first- or second-degree relatives with ovarian or breast cancer, no clearcut hereditary cancer syndromes were identified by pedigree analysis. Although no disease-associated mutations were detected in germ-line DNA samples from any patient, a patient had the polymorphism A-G at nucleotide 3667, leading to a substitution of arginine for lysine. This is a fairly common germline polymorphism and has not been demonstrated to be disease-associated in family studies. Germ-line BRCA1 coding sequence and splice junctions were wild type in all other patients.

### Immunological Studies

Patient plasmas and peritoneal fluids were frozen and then used for measurements of CH50 or Western blotting to detect for envelope antibodies. CH50 was performed following manufacturer’s instructions on plasma and peritoneal samples, using antibody-sensitized sheep erythrocytes (Sigma Chemical Co., St. Louis, MO). Basically, patient peritoneal fluid or sera were incubated with antibody-sensitized sheep erythrocytes in sodium barbital buffer for 30 min at 37°C. The extent of antibody-dependent lysis was then determined by pelleting unlysed red cells and measuring hemolysis in the supernatant by spectrophotometry against a standard curve. Standard complement serum (Sigma) was used as a control standard. Microbiological Associates (Rockville, MD) performed the Western blotting for envelope antibodies on patient samples.

### RESULTS

Twelve patients with recurrent or persistent epithelial ovarian cancer were treated with one to three cycles of i.p. vector. The starting dose was 10^8 vector particles, the highest dose administered was 10^10 vector particles, and a total of 23 treatment cycles were administered. The mean patient age was 51 years (range, 29–70). The original stage at diagnosis was III or IV in 11 patients and IC with a subsequent recurrence in the other patient. Although several patients were found to have first- or second-degree relatives with ovarian or breast cancer, no clearcut hereditary cancer syndromes were identified by pedigree analysis. Although no disease-associated mutations were detected in germ-line DNA samples from any patient, a patient had the polymorphism A-G at nucleotide 3667, leading to a substitution of arginine for lysine. This is a fairly common germline polymorphism and has not been demonstrated to be disease-associated in family studies. Germ-line BRCA1 coding sequence and splice junctions were wild type in all other patients. BRCA2 has not yet been sequenced. Clinical features of individual patients are presented in Table 1.

### Table 1 Patients in Phase I trial: Clinical characteristics

<table>
<thead>
<tr>
<th>Pt*</th>
<th>Age</th>
<th>Stage</th>
<th>Initial histology</th>
<th>Family history</th>
<th>Prior chemotherapy regimens</th>
<th>Dose levels (viral particles) Cycles</th>
<th>Total dose (viral particles)</th>
<th>Toxicity</th>
<th>Clinical response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49</td>
<td>IV</td>
<td>Papillary serous</td>
<td>Negative</td>
<td>&gt;3 10^8</td>
<td>4 × 10^8</td>
<td>None</td>
<td>Progression</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>IIIB</td>
<td>Papillary serous</td>
<td>Br: maternal aunt</td>
<td>&gt;3 10^8</td>
<td>8 × 10^8</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>IV</td>
<td>Papillary serous</td>
<td>Br: maternal cousin</td>
<td>2 10^9</td>
<td>3.8 × 10^9</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>IV</td>
<td>Papillary serous</td>
<td>Br: mother, maternal aunt</td>
<td>&gt;3 3 × 10^8</td>
<td>1.2 × 10^8</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>49</td>
<td>IC</td>
<td>Clear cell, grade 3</td>
<td>Br: mother</td>
<td>3 10^8</td>
<td>3 × 10^8</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>62</td>
<td>IIC</td>
<td>Papillary serous</td>
<td>Br: mother, 2 sisters, paternal cousin</td>
<td>1 10^8</td>
<td>9.2 × 10^9</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>52</td>
<td>IIC</td>
<td>Papillary serous</td>
<td>Br: maternal aunt</td>
<td>&gt;3 10^9</td>
<td>8 × 10^9</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>47</td>
<td>IIC</td>
<td>Papillary serous</td>
<td>Negative</td>
<td>2 10^9</td>
<td>1.2 × 10^9</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>47</td>
<td>IIIB</td>
<td>Papillary serous</td>
<td>Ov: maternal aunt; Br: 2 maternal aunts, 2 maternal cousins</td>
<td>&gt;3 3 × 10^9</td>
<td>5.2 × 10^10</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>58</td>
<td>IIC</td>
<td>Clear cell features, grade 3</td>
<td>Negative</td>
<td>3 10^10</td>
<td>4 × 10^10</td>
<td>None</td>
<td>Progression</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>55</td>
<td>IIC</td>
<td>Papillary serous, grade 3</td>
<td>Ov: paternal cousin; Br: maternal cousin</td>
<td>3 10^10</td>
<td>8 × 10^10</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>70</td>
<td>IIC</td>
<td>Adenocarcinoma, grade 3</td>
<td>Ov: maternal grandmother; Br: self (45 yr), mother</td>
<td>3 10^10</td>
<td>4 × 10^10</td>
<td>None</td>
<td>Stable disease</td>
<td></td>
</tr>
</tbody>
</table>

* Pt, patient.

* Br, breast cancer; Ov, ovarian cancer.

* Apparent reduction in number of peritoneal tumor nodules (<5 mm) at reoperation for complications related to large tumor progression.

* Congestive heart failure exacerbation (respondent to diuretics).
Fig. 2  PCR detection of vector DNA in patient samples. Five ml of peritoneal fluid samples were analyzed by 30 cycles of PCR with the primers and conditions listed in "Patients and Methods." The patient samples are flanked by a 1-kb ladder DNA marker on both sides. Samples 1–15 were from patient 3; samples 16–23 were from patient 6. The strong signal in all sample lanes (lower band) and present in the negative control lane represents the PCR primers. Positive bands that co-migrate with the positive control signal are present in samples 14, 18, 21, and 22. Positive control shows detection of 1 ng of vector plasmid DNA.

for clinical and laboratory signs of acute peritonitis. Three of the 12 patients (patients 3, 5, and 9) developed peritonitis as evidenced by patient discomfort, fever, peritoneal fluid counts, and bacterial cultures (negative), which resolved within 24–48 h after treatment was stopped. Patient 3 was re-treated with a lower dose of vector and showed no recurrence of peritonitis, even after two additional dose escalations. In retrospect, patient 5 was an obese patient with a loculated peritoneal space and may have received a larger than anticipated local dose. Catheter placement is clearly an important consideration in i.p. therapy because delivery of an agent into a confined space likely decreases efficacy and increases risk of local toxicity. Other toxicities in the trial included fever in four patients and nausea in two patients from the abdominal distension produced by the i.p. infusion of vector.

Pharmacokinetics of i.p. Vector Therapy. Reombinant DNA methods such as Southern blotting and PCR permit sensitive and specific detection of retroviral vectors in patient fluids and biopsied tissues. Multiple PCR analyses on plasma samples showed no detectable vector distribution to the systemic circulation, even in patients treated at the highest dose. Twenty-four h after each infusion (just prior to the next dose), we sampled peritoneal fluid to assess stability and uptake of the retroviral vector. An example of the PCR analysis is presented in Fig. 2. Although more frequent sampling might have provided a more accurate pharmacokinetic analysis, this protocol involved no additional catheter access and demonstrated the amount of vector that persisted for 24 h, a stringent test for vector stability. Fluid samples were centrifuged to obtain distinct samples for stable vector in peritoneal supernatant (vector in peritoneal fluid, Table 2), as well as vector that had entered cells within peritoneal fluid (vector in peritoneal pellet, Table 2). Because PCR fluid determinations were performed on 5 ml of peritoneal supernatant and PCR cell pellet determinations were performed on cells from as much as 10 ml of peritoneal fluid, the PCR cell pellet assay has greater sensitivity (can detect smaller quantities of vector). Because PCR analysis can detect either transduction-capable vector or degraded vector DNA, we assayed 200 µl of patient peritoneal fluid for the capacity to transduce MCF-7 target cells (viable vector recovery, Table 2). Results of this study demonstrate that LXSN-BRCA1sv vector from some patient samples is still transduction-capable 24 h after infusion. Table 2 shows results from three different PCR-based methods for assessing vector stability and gene transfer. The results of these assays were quite consistent, despite the fact that each measured something slightly different. We consistently observed that vector assays were much more likely to be positive during the later days of treatment than during the first two days of treatment (Table 2).

The presence of antibodies or other immune responses was presumed to limit vector stability and prevent effective retreatment of patients. Antibodies could also effect vector stability; therefore, patient sera and peritoneal fluid were tested for the development of antibodies to the amphotropic envelope. Patients treated at the first two dose levels did not develop antibody, even 6 months after treatment. One patient (patient 3) treated at dose levels 1–3 developed antibodies after 3 months in both plasma and peritoneal fluid. Antibodies did not eliminate vector from the peritoneal fluid in patient 3, as indicated by positive PCR samples after the development of antibodies. Two of four patients at dose level 5 developed antibodies, and patient 11 developed a strong antibody in peritoneal fluid and plasma within 1 month of treatment. A representative Western blot showing the development of envelope antibodies in patients is presented in Fig. 3.

Because complement is known to inactivate retroviruses and vector stability did not correlate perfectly with vector dose, we assayed complement levels in patient fluid samples and compared these with vector stability evaluated by PCR. These results showed a loose association between complement level and vector stability. For example, patient 6 had the most samples positive for retrovirus and consistently had undetectable CH50 levels in peritoneal fluid. Although there is no obvious correlation between initial CH50 or mean CH50 and vector stability in patients, samples with low CH50s were more likely to be positive than were those samples with higher CH50s.
Table 2 Pharmacokinetics of LXSN-BRCA1sv: Gene transfer and stability

<table>
<thead>
<tr>
<th>Presence of vector in patient specimens (detected by PCR)</th>
<th>Complement activity (CH50)</th>
<th>Antibody development (from 1st treatment)</th>
<th>% transduction in tumor tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose level</td>
<td>Plasma</td>
<td>Peritoneal fluid</td>
<td>Peritoneal pellet</td>
</tr>
<tr>
<td>Pt*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0/14</td>
<td>0/14</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0/14</td>
<td>+1/14</td>
</tr>
<tr>
<td>3</td>
<td>1–3</td>
<td>0/18</td>
<td>+1/18</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0/8</td>
<td>0/14</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>0/8</td>
<td>0/14</td>
</tr>
<tr>
<td>6</td>
<td>2–3</td>
<td>0/8</td>
<td>+3/12</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>9</td>
<td>4–5</td>
<td>0/14</td>
<td>+1/11</td>
</tr>
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<td>10</td>
<td>5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>0/6</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* Pt, patient.

* Numerator, number of positive samples; denominator, total number of samples assayed from this patient at different time points.

* Biopsy samples not obtained (patients had no subsequent surgery).

Fig. 3 Western blots to detect patient production of antibodies to retroviral envelope proteins. a, patient 3. Lanes 1, 3, and 5, peritoneal fluid from posttreatment days 59, 61, and 62, respectively; Lanes 2 and 4, plasma from posttreatment days 61 and 62, respectively. Neg, negative assay control. Pos samples are positive sera diluted 1000, 5000, and 10,000, respectively. b, patient 9 (6–10) and patient 10 (11–13). Lanes 6 and 7, peritoneal fluid (6) and plasma (7) from posttreatment day 39. Lanes 8 and 9, peritoneal fluid and plasma from posttreatment day 67. Lanes 10, 11, and 12, peritoneal fluid and plasma from posttreatment day 68. Lanes 11 and 12, pretreatment samples of peritoneal fluid (11) and plasma (12); Lane 13, plasma from posttreatment day 4, c, patient 11 (14–19) and patient 12 (20–22). Lanes 14 and 15, pretreatment samples of peritoneal fluid (14) and plasma (15). Lanes 16 and 17, peritoneal fluid and plasma from posttreatment day 28. Lanes 18 and 19, peritoneal fluid and plasma from posttreatment day 44. Lanes 20 and 21, pretreatment peritoneal fluid and plasma samples. Lanes 22, 23, and 24, plasma from posttreatment day 4. Cross-reactive bands at the lower portion of the gel (Mr >60,000) are commonly seen in untreated controls and are present in pretreatment samples (e.g., Lanes 20 and 21). The strong molecular weight bands Mr >60,000 (e.g., Lanes 16–18) are those that represent envelope antibodies.

DNA was purified from peritoneal fluid cells and analyzed by PCR, which demonstrated transfer of vector into cells within the malignant effusion. Because sampling cells within peritoneal fluid would not necessarily predict gene transfer into malignant
Fig. 4  Efficiency of gene transfer into patient tissues. DNA and RNA were purified from patient samples obtained at surgery or autopsy. For a, b, and c: Lane 1, negative control DNA from an untreated patient; Lanes 2–4, samples from patient 3 taken 6 weeks after treatment cycle 3, including normal peritoneum (Lane 2), surface of 6 × 6-cm ovarian tumor mass (Lane 3), and center of 6 × 6-cm ovarian tumor mass (Lane 4); Lane 5, normal peritoneum from patient 3 at biopsy 2 months after samples in Lanes 2–4; Lane 6, tumor from patient 4 taken 1 week after cycle 1; Lane 7, tumor from patient 7 taken 2 weeks after cycle 2; Lanes 8 and 9, autopsy of patient 6 eight weeks after cycle 3; Lane 8, normal peritoneum; Lane 9, tumor; Lanes 10 and 11, biopsy of patient 10 taken 2 weeks after cycle 1; Lane 10, tumor; Lane 11, normal peritoneum. a, Southern blot of AvaI-digested DNA purified from tissue samples; predicted genomic BRCA1 band is 900 bp, and the predicted vector band is 1.8 kb (see "Patients and Methods"). The bands correspond to or normal tissues, biopsies were obtained from patients who had laparotomies following i.p. treatment. Because retroviral vectors integrate as only a single copy and multiply transduced cells are rarely seen at less than 50% transduction efficiencies, one can estimate the transduction rate of a sample by Southern blot with comparison to genomic levels of DNA (31). These Southern blot results are presented in Fig. 4a and show more efficient integration of vector into the tumor surface than into inner regions of the tumor (Fig. 4a, compare Lanes 3 and 4) and show greater transduction into tumor tissue than into normal tissues (Fig. 4a, compare Lanes 1 and 2, and Lanes 8 and 9). We estimate that in samples with the strongest signals, 5–10% of cells were transduced with vector.

To assess expression of the retroviral vector, we designed PCR primers to detect only transcripts initiated in the retroviral vector; then we used RT-PCR as a semiquantitative measure of BRCA1 hsv vector expression. Vector was comparatively strongly expressed shortly after treatment in samples from patients with significant vector transduction (Fig. 4b, Lanes 3 and 10). Because the LXSN-BRCA1 hsv vector uses a splice variant, it was possible to distinguish vector mRNA from endogenous genomic mRNA, allowing a quantitative determination of vector mRNA within patient samples. The nuclease protection assay shows that endogenous BRCA1 mRNA levels were highest in samples from normal peritoneum, whereas vector mRNA levels were highest in patients who had recent treatment with vector (Fig. 4c, Lanes 3 and 10).

Clinical Response. Although this Phase I study was not designed to evaluate efficacy, we did note stable disease in eight patients. Stable disease was defined as no interval increase in measurable disease 4 weeks after treatment. Patients with stable disease were then eligible for re-treatment. Three of these eight patients demonstrated evidence of tumor reduction. At reoperation for cancer-related complications, patients 3 and 10 were found to have fewer miliary peritoneal implants. One patient demonstrated a partial response; this was defined as a 50% decrease in the sum of the products of the greatest perpendicular tumor dimensions determined radiographically. Histological examination of samples from the two patients with a decrease in miliary disease showed tumor necrosis and granulation tissue in peritoneal tumor implants. However, there was no necrosis of tumor from an intrasplenic site obtained at autopsy for patient 10 (Fig. 5). These results are compatible with i.p. LXSN-BRCA1 hsv having a local inhibitory effect on tumor growth and little or no direct or indirect systemic effect. Tumor necrosis can also be compatible with tumor progression in some settings.
DISCUSSION

Our Phase I study of LXSN-BRCA1sv demonstrated that the retroviral vector was reasonably stable in patient peritoneal fluid and transferred the gene into cancer cells that expressed the vector. Peritonitis was observed in three patients but resolved rapidly and was analogous to the peritonitis observed in mouse preclinical models. Re-treatment does not increase toxicity and does not appear to affect vector stability. Vector inactivation by complement and other mechanisms appears to be a problem in vivo, but antibody development occurs rarely and may not eliminate the vector. i.p. infusion of LXSN-BRCA1sv appears to be relatively nontoxic and a technically feasible approach to human gene therapy.

Gene therapy has been heralded as disease-specific therapy with few side effects, but the identification of toxicities specifically associated with gene therapy should not be surprising. The LXSN-BRCA1sv peritonitis observed in mice and in some patients is rapidly reversible and appears to resolve without sequelae. The peritonitis was not clearly dose related in patients (unlike the preclinical mouse studies, where it was dose related), although administration into larger numbers of patients may demonstrate a relationship with dose. Peritonitis did not reproducibly occur, and at least one patient with peritonitis was re-treated without recurrence.

Our protocol used repeat administration in a number of patients for periods ranging from 2 to 4 months. Antibody formation was rarely observed at low doses, and neither antibody production nor repeat administration appeared to decrease vector stability. These data suggest that patients may be given repeat doses of retroviral vectors without development of tolerance or enhanced toxicity. Rapid development of antibodies at the highest dose level and apparent decreased vector stability suggest that dose level 4 may be most appropriate for Phase II testing. Repeat administration increases the cumulative dose of retroviral vector that can be administered and ultimately increases the multiplicity of infection.

Our highest human dose level used four daily injections totaling $4 \times 10^{10}$ vector particles each month. Because i.p. tumor burdens may be as high as $10^{11}$ tumor cells ($10^{12}$ cells is generally believed to cause host death) in different patients, it will be important to increase the dose and/or treat earlier in the disease. The data demonstrating that PCR positivity is more common on days 3 and 4 of the schedule suggests that vector degradation may be saturated by repeated daily doses. Clearly, the development of appropriate dosage schedules will have a major impact on the success of gene therapy and will require ongoing studies of vector pharmacokinetics. It is interesting to note that there has been a report describing a breast cancer family with a DNA mutation producing an RNA identical to the normal splice variant cDNA used in this study (5). This suggests that overexpression of this variant may be required for growth inhibition in vitro and in vivo.

Low levels of complement in peritoneal effusions appear to explain the relative stability of vector in this site; therefore, it is important to consider that vector stability may be a function of both vector dose and complement activity within the patient's peritoneal cavity. However, prior in vitro studies by ourselves (31) and others (34, 35) have demonstrated that complement is not the only factor involved in vector degradation. Anti-galactosyl antibodies to retroviral vector surface proteins also inactivate vector, albeit in a complement-dependent manner. One can envision a number of approaches to enhancing the stability of retroviral vectors including complement blockade with lectins, engineering vector envelopes resistant to complement, or packaging vectors in cells lacking α-galactosyl epitope. These types of approaches could expand the population of patients with stable i.p. vector and might permit stable vector in other sites as well. Although the creation of a more stable vector is a reasonable and important goal, it may prevent repeated administration and/or re-treatment by generating a more rapid immune response. Further testing will be necessary to determine the role of vector stability and treatment schedules in retroviral vector gene therapy.

Retroviral vector therapy with LXSN-BRCA1sv is a rational therapeutic approach that attempts to attack a tumor with the appropriate tumor suppressor gene. A similar approach has been reported using p53 (36). Initial approaches to human gene therapy focused on germ-line inherited diseases. Recently, it has become evident that somatic genetic diseases like cancer represent appropriate targets for somatic gene therapy. i.p. therapy of ovarian cancer with LXSN-BRCA1sv may be an optimal human gene therapy model for several clinical reasons: (a) BRCA1 is a key tumor suppressor gene for ovarian cancer; (b) ovarian cancer is at first confined to the peritoneal cavity; (c) peritoneal treatment permits high-dose delivery and vector stability; (d) regional therapy for ovarian cancer is a well-described therapeutic modality; and (e) current treatments offer little improvement in survival.
from ovarian cancer. Although most genetic diseases will require more complex and sophisticated gene therapy approaches, gene therapy of ovarian cancer should allow testing of improved vectors and development of approaches that may ultimately be applied to many diseases.

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