Nanoparticle-Delivered Suicide Gene Therapy Effectively Reduces Ovarian Tumor Burden in Mice

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Abstract

There is currently no effective therapy for patients with advanced ovarian cancer. To address the need for a more effective treatment for this deadly disease, we conducted preclinical tests in ovarian tumor–bearing mice to evaluate the therapeutic efficacy of using a cationic biodegradable poly(β-amino ester) polymer as a vector for nanoparticulate delivery of DNA encoding a diphtheria toxin suicide protein (DT-A). The promoter sequences of two genes that are highly active in ovarian tumor cells, MSLN and HE4, were used to target DT-A expression to tumor cells. Administration of DT-A nanoparticles directly to s.c. xenograft tumors and to the peritoneal cavity of mice bearing primary and metastatic ovarian tumors resulted in a significant reduction in tumor mass and a prolonged life span compared to control mice. Minimal nonspecific tissue and blood chemistry toxicity was observed following extended treatment with nanoparticles. DT-A nanoparticle therapy suppressed tumor growth more effectively than treatment with clinically relevant doses of cisplatin and paclitaxel. Our findings suggest that i.p. administration of polymeric nanoparticles to deliver DT-A encoding DNA, combined with transcriptional regulation to target gene expression to ovarian tumor cells, holds promise as an effective therapy for advanced-stage ovarian cancer. [Cancer Res 2009;69(15):6184–91]

Introduction

The standard treatment for patients with advanced-stage epithelial ovarian cancer is optimal surgical debulking followed by chemotherapy with paclitaxel plus a platinum-based therapy (cisplatin or carboplatin). While ~80% of patients receiving this therapeutic regimen have an initial favorable response, recurrent disease will occur in a majority of cases. Regrettably, there are currently no effective therapies for those patients with advanced-stage ovarian cancer who either do not respond to initial therapy or for those who develop recurrent disease. There is an immediate need for a more effective treatment for this deadly disease.

Gene therapy holds promise as an alternative treatment for metastatic ovarian cancer. Metastatic tumors in this disease are nearly always confined to the peritoneal cavity, so i.p. delivery of therapeutic DNA allows for direct treatment of the tumors. In addition, this delivery route would presumably protect healthy organs outside the cavity from harmful nonspecific toxic side effects. In theory, the ability to target the delivery of DNA to tumor cells, as well as the ability to control its expression once inside the cell, provides an added level of therapeutic efficiency and specificity that is difficult to achieve using chemotherapy. In practice, however, the full potential of these advantages of DNA therapies has yet to be achieved and remains a goal of preclinical and clinical studies.

An important consideration in any gene therapy protocol is the choice of vector used to deliver the DNA to cells. With few exceptions, viral vectors for DNA delivery have been used in clinical trials for the treatment of ovarian cancer.7 Recently, however, the use of nonviral vectors for the delivery of therapeutic genes is receiving wide attention by the research community, particularly in light of the serious consequences that have occurred in association with the use of viral vectors in patients. There is one published report of phase I clinical trial in which cationic liposomes were used for gene transfer into peritoneally disseminated tumors; the therapy had limited antitumor activity and the vector-DNA complex was associated with adverse effects at all dose levels (1).

We have previously reported the use of nanoparticles, formed by complexing diphtheria toxin (DT-A)–encoding DNA with a biodegradable cationic poly(β-amino ester) polymer, to deliver a prostate-specific antigen enhancer/promoter-regulated DT-A gene to prostate cancer cells (2, 3). This toxin is an especially potent inhibitor of protein synthesis; its expression in tumor cells results in their death and a reduction in tumor mass.

In the present study, we explore the therapeutic efficacy of using poly(β-amino ester) polymers to deliver DT-A DNA to ovarian tumors in mice, an approach that we recently reviewed (4). We generated nanoparticles by complexing DNA with either one of two different poly(β-amino ester) polymers—C32 (2, 5), or C32-117, henceforth called 117, a modified version of C32 containing aminocapped ends (6). We have previously shown that 117-nanoparticles dramatically enhance DNA delivery to ovarian tumors as compared to C32 (7). In this study, we develop ovarian-specific antitumor constructs using the promoters of two genes, HE4 (WFDC2) and MSLN, to target the expression of DNA to ovarian cancer cells in vitro and to tumor cells in mouse models. Activity of these

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7 http://clinicaltrials.gov
promoters is significantly enhanced in ovarian cancer cells relative to normal ovarian cells and cells in other tissues (8–11). In ovarian cancer samples where there is little or no CA125 expression, MLSN and HE4 protein proved to be the most reliable biomarkers for ovarian cancer, as compared to a number of other candidate proteins, when reactivity with normal tissues was considered (10, 12).

Here, we show that injection of poly(β-amino ester)-DNA nanoparticles injected into the peritoneal space significantly reduces the size of tumors resulting in an increase in life span in mice. Furthermore, we show that the growth of ovarian tumors in mice treated with DT-A nanoparticles is suppressed more effectively, and with minimal nonspecific cytotoxicity, than the growth of tumors in mice treated with clinically relevant doses of cisplatin and paclitaxel. These findings suggest that the use of biodegradable poly(β-amino ester) polymers to deliver transcriptionally targeted DT-A DNA to tumor cells may offer significant advantages and improved efficacy compared to chemotherapeutic therapies that are the current standard treatment for ovarian cancer patients.

Materials and Methods

Plasmid Constructions

pMSLN/Fluc and pMSLN/EGFP. pMSLN/Fluc, containing human MLSN promoter–regulated firefly luciferase, was constructed as follows: The 1,850-bp MLSN promoter was PCR amplified from human genomic DNA using the oligonucleotide sequence 5′-GCT ACT AGT GTT TTC ATC-3′ as the forward primer and 5′-amino ester)-DNA (reverse primer). These primers were analogous to those previously described (11), except that they contain nucleotides corresponding to restriction enzyme sites at their 5′-ends. PCR cycling conditions were as follows: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 2 min at 68°C, using Platinum Pfx Taq polymerase (Invitrogen). The (Sepl + BglII)–digested PCR product was ligated to (NheI + BglII)–digested pGL4.10(luc2) plasmid (Promega).

To make pMSLN/EGFP, pMSLN/Fluc was digested with BglII and XbaI to remove the luciferase sequence, and ligated to a 749-bp EGFP fragment obtained by (BamHI + XbaI) digestion of pEGFP-1.

pHE4/Fluc and pHE4/EGFP. To construct pHE4/Fluc, containing human HE4 promoter–regulated firefly luciferase, a 530-bp HE4 promoter sequence was PCR amplified from pHE4-652 (gift from Kenneth Nephew, Indiana University, Bloomington, IN). Oligonucleotide sequences for PCR amplification were 5′-GCTCTCGAGTTAATAATAAGTGCTATTTC-3′ (forward primer) and 5′-amino ester)-DNA -CTGACTAGTGTTCATGCCTGCCC-3′ (reverse primer). PCR cycling conditions were as follows: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 68°C, using Platinum Pfx Taq polymerase (Invitrogen). The (XhoI + BglII)–digested PCR product was ligated and digested with (XhoI + BglII)–digested pGL4.10(luc2) plasmid (Promega).

To make pHE4/EGFP, pHE4/Fluc was digested with BglII and XbaI to remove the luciferase sequence, and ligated to a (BamHI + XbaI) 749 bp EGFP digestion fragment from pEGFP-1.

pMSLN/DT-A. To construct pMSLN/DT-A, containing human MLSN promoter–regulated DT-A, a 1,334-bp (BglII + NotI)–digestion fragment of p22ED1T (gift of A. Francis Stewart, European Molecular Biology Laboratory, Heidelberg, Germany) was ligated to (BglII + NotI)–digested pMECA to generate pMECA/DT-A. Following NheI and NotI digestion of pMECA/DT-A, the released DT-A sequence was ligated to (NheI + NotI)–digested pND (Clonetech) to form pND/DT-A. To replace the luciferase sequence in pMSLN/Fluc with DT-A, luciferase was excised by digestion with BglII and XbaI and the remaining vector containing the MLSN promoter was ligated to a 1,351 bp DT-A (BglII + XbaI) digestion fragment from pND/DTA.

pHE4/DT-A. pHE4/DT-A plasmid, containing human HE4 promoter–regulated DT-A, was constructed as follows: To replace the luciferase coding sequence in pHE4/Fluc with DT-A, luciferase was excised by digestion with BglII and XbaI and the remaining vector containing the HE4 promoter was ligated to a 1,351 bp DT-A (BglII + XbaI) digestion fragment from pND/DTA.

Cell Culture and Cell Transfection

A2780 cells (T. Hamilton, Fox Chase Cancer Center, Philadelphia) were grown in 1640 medium containing 10% fetal bovine serum (FBS), 0.3 mg/mL t-glutamine, and 0.25 units/mL porcine insulin. ES-2 (ovarian), HepG2 (liver), A549 (lung), and PC3 (prostate) cell lines were purchased from American Type Culture Collection (ATCC) and grown in media as recommended by ATCC. All cell lines were transiently transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's directions. For each cell line, MLSN or HE4 promoter–regulated luciferase activity was normalized to luciferase activity of cells transfected with pCAG/luc. CAG is a strong, ubiquitously active promoter/enhancer sequence (13).

The derivation of the ID8-Fluc cells and their maintenance was as described (14). According to an approved Institutional Review Board protocol, ascites fluid was drawn from patients (n = 5; all had poorly differentiated serous adenocarcinoma) during the course of their normal treatment and collected in sterile centrifuge bottles. The deidentified samples were centrifuged at 1,500 rpm, and the pelleted cells were washed 1× in PBS and then resuspended and grown in DMEM + 4% FBS + insulin, transferrin, and selenium (Cellgro). Cells were transfected with C32-117 nanoparticles as previously described (15).

Flow Cytometry

Transfected ascites cells were analyzed for green fluorescent protein expression using a BD FACSCaneto II flow cytometer equipped with a 488-μm laser and BD FACS Diva Software, version 502.

Cell Injection to Generate Tumors

To generate xenografts, 2 × 10⁶ A2780 or 5 × 10⁶ ES-2 cells in 100 μL PBS containing 20% cold Matrigel (BD Biosciences) were injected s.c. into the flank of 8 wk-old female athymic nude-Foxn1−/mice (Harlan). To generate tumors in the peritoneum of nude mice, 5- to 8-wk-old female mice (Harlan) were injected i.p. with 5 × 10⁶ ID8-Fluc cells suspended in 200 μL DMEM containing no additives.

Transgenic Mice

MISIIR/TAg transgenic mice were bred and genotyped as described (16).

Intratumoral and I.p. Injection of Nanoparticles and Chemotherapeutics

Nanoparticles, resulting from complexation of plasmid DNA with a poly(β-amino ester), were prepared as previously described (2). For administration of nanoparticles to xenografts and ovarian tumors in MISIIR/TAg mice, 50 μg complexed DNA/60 μL total volume were delivered directly to the tumor by injection using a 30G needle. MISIIR/TAg mice were first anesthetized with Avertin and a small abdominal incision was made to expose the ovarian tumor. Following injection of the nanoparticles, the incision was closed with a stainless steel clip.

In some experiments, nanoparticles (100–150 μg DNA) or chemotherapeutics (3.3 mg cisplatin/kg/injection, 10 mg/kg paclitaxel/injection) were injected i.p. into tumor-bearing MISIIR/TAg females.

All procedures performed on mice in this study were done in accordance with protocols approved by the Lankenau Institutional Animal Care and Use Committee.

Imaging. Optical imaging to detect luciferase activity in mice was performed using an IVIS 100 series Bioimaging Imaging System (Caliper Life Sciences) as described previously with a 5-min integration time for image acquisition (17).

For microCT scans, mice were injected i.p. with 250 μL OptiTag Pharmacy Ioversol Injection 74% contrast medium (741 mg ioversol/mL). Ten minutes later, mice were anesthetized and underwent computed tomography (CT)
scanning using a MicroCAT 1A scanner (ImTek, Inc.; 40 kV, 600 μA, 196 rotation steps; total angle of rotation, 196). Images were reconstructed using RVAS software program (ImTek, Inc.). Tumor volumes were determined using Amira 3.1 software (Mercury Computer Systems).

**Blood Analysis and Histology**

Cardiac puncture was performed, and whole blood and serum underwent complete blood count (CBC) analysis and assay of metabolic proteins (LabCorp). Multiple organs were collected, fixed in formalin for 2 h, washed thrice in PBS, and processed for paraffin embedding. Five-micrometer sections were prepared, H&E stained, and examined microscopically.

**Apoptosis**

Apoptotic cells were identified by terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) assay using an In Situ Detection Kit (Roche Boehringer Mannheim) as previously described (3).

**Statistics**

Time to death (or euthanization) was completed using Kaplan-Meier estimates of survival time, stratified by treatment group. Equality of survival was determined using the two-sided log-rank test. Median survival times with 95% confidence limits for each treatment group were also determined. Statistical significance of differences in tumor growth rates were determined by a multivariate repeated measures ANOVA using PROC GLM from SAS 9.2 and differences in tumor mass were determined using the unpaired two-tailed student t test.

**Results**

**Transcriptional targeting of diphtheria toxin activity.** The promoters of two genes, the whey-acidic protein human epididymis protein 4 (HE4) gene and the mesothelin (MSLN) gene, have been reported to be highly active in ovarian cancer cells (18, 19). Compared to other potential markers of ovarian cancer, HE4 and MSLN are the most intensely expressed proteins in cancer cells as compared to normal tissues (10).

We made two DNA constructs: pHE4-530/Fluc and pMSLN/Fluc in which expression of firefly luciferase (Fluc) was placed under control of an HE4 or MSLN promoter sequence. The pHE4-530/Fluc construct contained a 530 bp sequence upstream of the HE4 ATG translation initiation site. To test the function of these promoters, as well as that of a longer HE4 promoter sequence consisting of the 530-bp upstream sequence plus 122 bp of intron 1 immediately following the ATG site (18), we transiently transfected A2780 human ovarian tumor cells with each of these constructs and assayed luciferase activity 48 hours later. The shorter 530-bp HE4 sequence (henceforth referred to simply as HE4) yielded >3× higher luciferase expression than the longer sequence and was used in all subsequent experiments (Fig. 1). Following transfection of cells in culture, both the HE4 and MSLN promoters had higher activity in A2780 cells (~8× and ~5×, respectively) than in other cell types of human origin including lung, liver, and prostate (Fig. 1B). Thus, both of these promoter sequences exhibited ovarian cell–specific activity.

We made two additional DNA constructs, pHE4/D T-A and pMSLN/D T-A, in which the HE4 and MSLN promoters regulate expression of the A chain of diphtheria toxin (DT-A). To determine how effectively DT-A inhibited protein synthesis in ovarian tumor cells in culture, we measured luciferase enzyme activity 48 hours after cotransfection of A2780 and ES2 cells with pCAG/Fluc (CAG is a very strong, ubiquitously expressed regulatory sequence) and either pMSLN/D T-A or pHE4/D T-A. For both cell lines, luciferase activity was reduced to background levels as compared to cells

![Figure 1. HE4 and MSLN promoter activity.](image-url)
Figure 2. Nanoparticle delivery of DNA to ovarian xenografts. A, optical images of mice 6 and 48 h after intratumoral injection of 117-MSLN/Fluc nanoparticles with C32-HE4/Fluc, C32-MSLN/Fluc, and PEI-MSLN/Fluc DNA nanoparticles. In each panel, pseudocolor images representing emitted light are superimposed over grayscale images of mice. Photons per second are indicated in the color scale bar. Fold increase in tumor volume (B) and the growth rate of tumors (C) following intratumoral injection of A2780 xenografts with 117-MSLN/Fluc (n = 9) and 117-MSLN/DT-A nanoparticles (n = 10), or with Na acetate buffer (n = 10). Tumors were injected every other day starting at day 0. The mean body weight of mice on treatment days is also plotted in C.

To determine whether nanoparticles effectively deliver DNA to human ovarian tumor cells, as well as whether the MSLN promoter sequence is active in primary ovarian tumor cells, we complexed polymer 117 with pCAG/GFP and pMSLN/Fluc DNA and incubated the nanoparticles for 3 hours with cultured tumor cells obtained from ascites fluid drawn from patients with advanced ovarian cancer. Control cells were incubated with 117-pMSLN/X nanoparticles (only the MSLN promoter, no coding sequence). Approximately 20% of primary ovarian cells expressed GFP following transfection with 117-pCAG/GFP as determined by FACScan analysis and confirmed by fluorescent microscopy (Fig. 1D). Luciferase expression in cells transfected with 117-MSLN/Fluc was ∼15× higher than in cells transfected with 117-MSLN/X control DNA, indicating MSLN promoter function following nanoparticle delivery of DNA (Fig. 1E).

Xenograft studies. To test whether polymeric nanoparticle-delivered DNA would be expressed by ovarian tumor cells in vivo, C32-pMSLN/Fluc and C32-pHE4/Fluc DNA nanoparticles were injected directly into A2780 xenografts. Upon optical imaging of the mice, robust bioluminescence was observed in all tumors at 6 and 48 hours after nanoparticle injection (Fig. 2A). In contrast, luciferase expression in tumors injected with PEI-MSLN/Fluc nanoparticles was only observed at 48 hours after injection. These results demonstrate that polymeric nanoparticles delivered DNA to the tumor cells and that, as expected, the MSLN and HE4 promoters function in these cells.

To test the therapeutic efficacy of nanoparticle-delivered DT-A–encoding DNA, we injected 117-MSLN/DT-A nanoparticles directly into A2780 xenografts every other day over a 14-day period. Control tumors were injected with 117-MSLN/Fluc or with buffer. Calipers were used to measure tumor size before each injection. The mean fold increase in tumor volume (from day 0 to day 14) of DT-A–treated tumors (2.0 - 1.0-fold) was significantly reduced compared to Fluc (4.1 - 1.1-fold) and buffer (6.0 - 2.0-fold) control tumors (P < 0.0005). Four of the 10 DT-A–treated tumors failed to grow at all, while all control tumors got larger (Fig. 2B). The growth rate of tumors injected with DT-A nanoparticles was also significantly reduced compared to Fluc-nano–treated (P = 0.0002) and buffer-treated (P > 0.0001) control tumors (Fig. 2C), while there was no significant difference between the body weights of mice treated with DT-A nanoparticles and control mice, and none of the mice showed outward signs of physical distress over the course of the experiment. Whole blood and blood serum were collected at the termination of experiment. CBC and levels of several metabolic enzymes were within the normal range (Supplementary Table S1). Nearly identical results were obtained when ES2 xenografts were injected with 117-HE4/Fluc nanoparticles (data not shown).

MISIIR/TAg transgenic mouse studies. MISIIR/TAg transgenic mice develop bilateral epithelial ovarian tumors as a consequence of SV40 T antigen expression in ovarian epithelial cells (16). Following injection of 117-MSLN/Fluc and 117-HE4/Fluc nanoparticles directly into ovarian tumors in MISIIR/TAg mice, we optically imaged mice to test for luciferase expression. All injected tumors emitted bioluminescence, indicating that the DNA was delivered to ovarian tumor cells and was expressed (Fig. 3A). Upon ex vivo imaging of mice after 24, 72 hours, and 1 week after injection, we did not observe bioluminescence in other organs in the peritoneum. Bioluminescence was observed in ovarian tumors following i.p. injection of 117-MSLN/Fluc nanoparticles into tumor-bearing MISIIR/TAg mice (Fig. 3B). In these mice, however, bioluminescence was also observed associated with the uterus, abdominal fat, and the intestines. Very low bioluminescence...
(~ 5000 relative light units/mm$^3$) was consistently observed in several organs in the peritoneal space following optical images of i.p.-injected nontumor-bearing C57BL/6 mice with 117-MSLN/Fluc nanoparticles, indicating that there is some nonspecific gene expression.

To determine whether nanoparticle delivery of DT-A DNA to tumor cells in mice resulted in tumor cell death, we performed TUNEL assays on sections of tumors that had been injected with 117-MSLN/DT-A nanoparticles. Greater than 95% of the tumor cells had undergone apoptosis (Fig. 3C). In contrast, in each of the control tumors injected with 117-MSLN/Fluc nanoparticles, a small locus of densely populated apoptotic cells, probably corresponding to the injection site, whereas <5% of cells had undergone apoptosis in the balance of the tumors.

To determine whether treatment of MISIIR/TAg mice with DT-A nanoparticles prolongs life, 40 mice bearing tumors with a volume of ~ 100 mm$^3$ were identified by palpation. Mice were injected i.p. twice weekly with either 117-MSLN/DT-A nanoparticles ($n = 20$) or with 117-MSLN/Fluc nanoparticles ($n = 20$). A third control group received no treatment ($n = 14$). The median survival of DT-A–treated mice is significantly longer than either the Fluc-treated mice or untreated mice (78 versus 64 or 52 days; Fig. 4A). The log-rank test of significance across the three groups was a $P$ value of <0.001, indicating a significant difference across the three treatment groups in survival. The median survival of DT-A–treated animals was significantly longer than either Fluc or untreated animals; there was no significant difference in median survival of the two control groups. Significantly, some mice in both the DT-A and Fluc groups withstood nanoparticle treatment for nearly three months. This indicates that peritoneal administration of the given dose of DT-A nanoparticles is tolerated quite well by mice. A histopathologic analysis of multiple tissues (liver, kidney, spleen, lung, stomach, small intestine, large intestine, abdominal wall with peritoneum, diaphragm, urinary bladder, pancreas, and uterus) from two mice treated with DT-A nanoparticles revealed minimal to mild chronic inflammation along the serosal surfaces of abdominal organs in both animals (low-grade chronic peritonitis). The mice had enlarged spleens due to extramedullary hematopoiesis. Extramedullary hematopoiesis was also observed in the livers of these animals (Fig. 4B). No differences in CBCs and levels of several metabolic enzymes in blood sera between DT-A nanoparticle–treated and control mice were observed (Supplementary Table S2).

**DT-A nanoparticles versus chemotherapy.** The standard treatment for women with advanced-stage ovarian cancer is optimal surgical debulking followed by chemotherapy, usually a combination of cisplatin and paclitaxel. To compare the therapeutic efficacy of DT-A nanoparticles with that of the combination cisplatin/paclitaxel chemotherapy, tumor-bearing MISIIR/TAg mice were injected i.p. twice a week with either DT-A nanoparticles (100 µg DNA/injection) or a cisplatin/paclitaxel drug combination (3.3 mg/kg cisplatin; 10.0 mg/kg paclitaxel) over a 3-week period. The total cisplatin (20 mg/kg) and paclitaxel (60 mg/kg) doses were equivalent to therapeutic doses used in patients (i.e., cisplatin,

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Nanoparticle delivery of DNA to ovarian tumor–bearing MISIIR/TAg mice. A, ex vivo optical images of ovarian tumors and other organs 24 h after intratumoral injection of C32-MSLN/Fluc and C32-HE4/Fluc nanoparticles. In the HE4/Fluc panel, part of the tumor broke off during dissection. Color scale bar, photons per second. B, ex vivo optical images of ovarian tumors and other organs 6 h after i.p. injection of C32-MSLN/Fluc nanoparticles. 1, ovary and uterus; 2, spleen; 3, kidney; 4, liver; 5, fat; 6, stomach; 7, intestine; 8, carcass. C, representative TUNEL analysis and DAPI staining of tumor sections 24 h after intratumoral injection of 117-MSLN/DT-A and 117-MSLN/Fluc nanoparticles. Apoptotic cells appear green. The area in the dotted box is enlarged in the inset. Larger bar, 100 µmol/L; shorter bar, 20 µmol/L (inset).
60 mg/m²; paclitaxel, 180 mg/m²). MicroCT scans were used to determine the size of tumors before treatment and to monitor tumor size during the course of treatment (Fig. 5A). The mean fold increase in tumor volume of DT-A–treated tumors over the 3-week period was significantly reduced compared to untreated tumors ($P = 0.005$) and to drug-treated tumors ($P = 0.05$; Fig. 5B). One week after the last treatment, mice were CT scanned and tumors were removed and weighed (Fig. 5C). The mean tumor weight of tumors removed from the DT-A nanoparticle–treated mice at the time of sacrifice (0.85 ± 0.41 grams) was significantly less than the mean weight of tumors from the drug-treated mice (1.94 ± 0.85 grams; $P = 0.02$). No significant differences in histopathology in various organs in areas away from tumor involvement, in CBC results, and in levels of several blood serum enzymes between drug-treated and DT-A–treated or untreated mice, were observed (Supplementary Tables S2 and S3).

To explore further the effectiveness of DT-A nanoparticle therapy in reducing tumor load and compare it to standard chemotherapy, we treated nude mice that had been i.p. injected with ID8-Fluc ovarian tumor cells. These mice develop extensive tumors throughout the peritoneum. Following optical imaging of these mice, emitted bioluminescence can be quantified and is a measure of the tumor load (Fig. 6A). Eight weeks after injection of the ID8-Fluc cells, mice were optically imaged to define the baseline tumor load (relative light units), stratified into three treatment groups having equivalent tumor load profiles ($n = 5$), and injected i.p. with either 117-DT-A nanoparticles (100 μg DNA), a combination of cisplatin and paclitaxel, or with 50 mmol/L sodium acetate buffer (pH 5.0). Mice received two treatments per week for 3 weeks and were optically imaged once a week. After 3 weeks of treatment, the tumor load in mice treated with either 117-DT-A nanoparticles was not significantly different from the tumor load of mice treated with the cisplatin/paclitaxel drug combination, but the tumor load in mice in both these treatment groups was significantly less than control mice treated with buffer (Fig. 6B). Every mouse in the buffer control group either died during the course of the treatment or developed ascites. None of the mice treated with 117-DT-A nanoparticles or with the drugs developed ascites. Following the final imaging of mice, they were euthanized. Upon gross visual inspection of the peritoneum, numerous tumors were observed in control mice, whereas there were far fewer tumors in the DT-A and drug-treated mice. CBC assays showed no differences between DT-A and drug-treated mice (Supplementary Table S4). Levels of several blood serum enzymes in DT-A–treated, drug-treated, and control mice were tested. Creatinine levels were normal in control and DT-A–treated mice, but slightly elevated in drug-treated mice, indicative of kidney toxicity (Supplementary Table S4). Eighty percent (4 of 5) of drug-treated mice lost significant weight (mean loss, 18.5%) over the course of treatment, whereas no weight loss was observed in DT-A–treated and control mice.
Discussion

The urgent need for a better therapy for advanced ovarian cancer, and our previous work demonstrating the effective delivery of DNA encoding diphtheria toxin (DT-A) to prostate tumor cells in mice following intratumoral injection of nanoparticles formulated with the poly(γ-amino ester) C32 (2, 3), led us to explore the therapeutic efficacy of this nanotherapy for the treatment of ovarian cancer. We reasoned that i.p. delivery of DT-A nanoparticles would avoid biodistribution complications associated with i.v. injection of nanoparticles and would therefore allow their access to metastatic tumors in the peritoneal cavity where most metastatic ovarian tumors are located. i.p. administration of paclitaxel nanoparticles has been shown to inhibit the progression of ovarian carcinoma in rats (20). It is noteworthy that a recent report indicating that i.p. chemotherapy for advanced ovarian cancer improves overall and disease-free survival (21) was the basis for a rare Clinical Announcement posted by the National Cancer Institute in January 2006 recommending that physicians use this mode of drug administration in treating ovarian cancer patients.

We investigated the therapeutic efficacy of using polymeric nanoparticles to deliver DNA encoding DT-A to ovarian cancer cells in three mouse models—a xenograft model, a transgenic mouse model, and a cell implantation model. Direct injection of nanoparticles into xenografts, as well as incubation of nanoparticles with primary ascites cells collected from patients, demonstrated the ability of polymeric nanoparticles to deliver DNA to ovarian cancer cells of human origin. Expression of nanoparticle-delivered DT-A DNA suppressed the growth of xenografts in immunosuppressed mice. TUNEL assays done on DT-A nanoparticle–injected tumors in immunocompetent Misiir/TAg mice showed that tumor cells undergo apoptosis following DT-A expression.

MISIIR/TAG transgenic mice and the ID8-Fluc cell implant model were used to compare the therapeutic efficacy of DT-A nanoparticles with that of chemotherapy (cisplatin + paclitaxel). The growth of ovarian tumors in MISIIR/TAG mice treated with DT-A nanoparticles was significantly less than in mice treated with the drugs. In fact, 42% (3 of 7) of tumors in the DT-A–treated mice shrunk in size over the 3-week course of treatment, whereas all of the tumors in the drug-treated mice grew in size. An additional study using the ID8-Fluc cell implant model confirms that DT-A nanotherapy is as effective, if not better, than the combined drug therapy in reducing tumor load. While histologic analyses of multiple nontumorous tissues from drug-treated and from DT-A–treated mice showed no significant nonspecific toxicity, drug-treatment of the ID8-Fluc implant mice did result in significant weight loss, while DT-A–treated mice showed no weight loss.

The promoters of two genes, HE4 and MSLN, are preferentially active in ovarian cancer cells. We used the MSLN promoter to
regulate DT-A expression in the nanotherapy versus chemotherapy studies as well as in a life span study. This promoter appears to target DT-A expression quite effectively because although we have shown that DNA is delivered to multiple organs following i.p. injection of C32-117 nanoparticles (6), very little nonspecific toxicity was observed upon histologic analysis of multiple tissues collected from mice that had received i.p. injections of DT-A nanoparticles for several months. Nevertheless, in further development of this therapy, we plan to make modifications to the nanoparticle formulations that will enhance targeted delivery of DNA to tumor cells. A combination of targeting delivery as well as targeting expression through the use of specific promoters will help ensure the maintenance of noncancerous, healthy tissues.

The therapeutic response of tumors in mice treated with DT-A nanoparticles, in the absence of significant nonspecific toxicity, establishes the utility of a DNA-based nanotherapy for the treatment of ovarian cancer. The C32-117 poly[(β-amino ester) polymer effectively delivers DNA to tumor cells following i.p. delivery, while the use of specific promoter sequences effectively targets expression of the therapeutic DNA to the tumor cells. The acquired resistance of tumor cells to chemotherapeutics is responsible, in large part, for the poor prognosis of patients with advanced ovarian cancer. Given the rapid shutdown of protein synthesis following uptake and expression of DT-A encoding DNA, it is likely that cells will not exhibit resistance to the nanotherapy. Our study suggests that it should be possible to administer DT-A nanotherapy over an extended period of time to suppress tumor growth, and perhaps even reduce tumor burden.

Currently, ovarian cancer patients are treated with chemotherapy following surgical debulking. Further studies using the MISIIR/Tag model will be aimed at assessing the therapeutic efficacy of DT-A nanotherapy as an adjuvant therapy to surgical debulking. DT-A nanotherapy is also likely to be an effective therapy for tumors that develop in other organs in the peritoneum, e.g., pancreatic cancer (15). There is no clinical precedent for the use of C32-117 poly[(β-amino ester) polymer as a delivery vehicle. The National Cancer Institute recently announced that it will sponsor further preclinical development of this therapy for the treatment of solid tumors at the Nanotechnology Characterization Laboratory.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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