Therapeutic EphA2 Gene Targeting In vivo Using Neutral Liposomal Small Interfering RNA Delivery

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Abstract

Inducing destruction of specific mRNA using small interfering RNA (siRNA) is a powerful tool in analysis of protein function, but its use as a therapeutic modality has been limited by inefficient or impractical delivery systems. We have used siRNA incorporated into the neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) for efficient in vivo siRNA delivery. In nude mice bearing i.p. ovarian tumors, nonsilencing siRNA tagged with the fluorochrome Alexa 555 was encapsulated into DOPC liposomes and shown to be taken up by the tumor as well as many major organs. Furthermore, DOPC-encapsulated siRNA targeting the oncoprotein EphA2 was highly effective in reducing in vivo EphA2 expression 48 hours after a single dose as measured by both Western blot and immunohistochemistry. Therapy experiments in an orthotopic mouse model of ovarian cancer were initiated 1 week after injection of either HeyA8 or SKOV3ip1 cell lines. Three weeks of treatment with EphA2-targeting siRNA-DOPC (150 μg/kg twice weekly) reduced tumor growth when compared with a nonsilencing siRNA (SKOV3ip1: 0.35 versus 0.70 g; P = 0.020; HeyA8: 0.98 versus 1.51 g; P = 0.16). When EphA2-targeting siRNA-DOPC was combined with paclitaxel, tumor growth was dramatically reduced compared with treatment with paclitaxel and a nonsilencing siRNA (SKOV3ip1: 0.04 versus 0.22 g; P < 0.001; HeyA8: 0.21 versus 0.84 g; P = 0.0027). These studies show the feasibility of siRNA as a clinically applicable therapeutic modality. (Cancer Res 2005; 65(15): 6910-8)

Introduction

Since its description in Caenorhabditis elegans (1) and mammalian cells (2), use of small interfering RNA (siRNA) as a method of gene silencing has rapidly become a powerful tool in protein function delineation, gene discovery, and drug development (3). The promise of specific RNA degradation has also generated much excitement for possible use as a therapeutic modality, but its use as a therapeutic modality has been limited by inefficient or impractical delivery systems. We have previously used liposomes composed of the neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) to deliver antisense oligonucleotides in vivo (27). Here, we sought to determine the feasibility and effectiveness of delivering EphA2-targeting siRNA in DOPC. Therapeutic delivery of siRNA directed against EphA2 resulted in decreased protein expression in the tumor and remarkably decreased tumor growth when combined with chemotherapy in an orthotopic mouse model of ovarian cancer.

Materials and Methods

Cell lines and culture. The ovarian cancer cell lines HeyA8 and SKOV3ip1 (28) were maintained in RPMI 1640 supplemented with 15% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). All in vitro experiments were conducted at 60% to 80% confluence. For in vivo injection, cells were trypsinized and centrifuged at 1,000 rpm for 7 minutes at 4°C, washed twice, and reconstituted in serum-free HBSS (Life Technologies, Carlsbad, CA) at a concentration of 5 × 10⁸ cells/mL (SKOV3ip1) or 1.25 × 10⁹ cells/mL (HeyA8) for 200 μL i.p. injections.

Small interfering RNA constructs and in vitro delivery. siRNA was purchased from Qiagen (Valencia, CA) in three formulations. A nonsilencing siRNA sequence, shown by BLAST search to not share sequence homology not clinically practical. The only study to show orthotopic in vivo uptake and target down-regulation of an endogenous protein after normal systemic dosing required chemical modulation of siRNA that will have unknown toxicities and may affect siRNA activity or longevity (10).

We have used an ovarian cancer xenograft mouse model to examine the efficacy of in vivo gene silencing by siRNA. Ovarian cancer is associated with the highest mortality among all gynecologic malignancies, with an estimated 22,220 cases and 16,210 deaths in the United States in 2005 (11). The majority of ovarian cancer patients respond to initial therapy of tumor cytoreductive surgery and platinum-based chemotherapy, but of these, ~70% will recur and succumb to disease (12). Therefore, novel therapeutic strategies are urgently needed to improve the outcome of women with ovarian cancer. Fortunately, ovarian cancer has a favorable mouse model. i.p. injected ovarian cancer cells form tumors resembling human cancer in growth pattern, and their response to therapy tends to be predictive of response in human patients (13, 14).

EphA2 is a tyrosine kinase receptor in the ephrin family that plays a key role in neuronal development (15, 16). In adults, it is expressed to a low degree, primarily in epithelial cells (17). Several investigators have reported EphA2 overexpression in human cancers (18–21), and we have shown that the high rate of overexpression in ovarian cancer is associated with poor clinical outcome (22). EphA2 can function as an oncoprotein (23), and down-regulation reduces tumorigenicity in preclinical studies of breast and pancreatic cancer (24–26), making it an ideal therapeutic target.

We have previously used liposomes composed of the neutral lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) to deliver antisense oligonucleotides in vivo (27). Here, we sought to determine the feasibility and effectiveness of delivering EphA2-targeting siRNA in DOPC. Therapeutic delivery of siRNA directed against EphA2 resulted in decreased protein expression in the tumor and remarkably decreased tumor growth when combined with chemotherapy in an orthotopic mouse model of ovarian cancer.

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with any known human mRNA (target sequence 5′-AATCTTCCGAAGGTG-3′ and tagged with Alexa 555), was used to determine uptake and distribution in various tissues when given in vivo. siRNA with the target sequence 5′-AATGACATGCCGACTCATACGT-3′, designed and shown (26) to target mRNA of the receptor tyrosine kinase EphA2, was used to down-regulate EphA2 in vitro and in vivo. A nonsilencing siRNA construct (sequence as above without an Alexa 555 tag) was used as control for EphA2-targeting experiments. For in vitro delivery, siRNA (5 μg) was incubated with 30 μL RNAiFect transfection reagent (Qiagen) for 10 minutes at room temperature and added to cells in culture at 80% confluence in 35 mm culture plates. The medium was changed 6 hours later, and cells collected after 48 hours as lysate for Western blot analysis.

Liposomal preparation. siRNA for in vivo delivery was either given naked (without transfection agent), incorporated into N-[1-(2-dioleoyloxy-ethyl)propyll]-N,N,N-trimethylammonium methyl sulfate (DOTAP; Roche, Indianapolis, IN), or incorporated into DOPC. DOPC and siRNA were mixed in the presence of excess tertiary butanol at a ratio of 1:10 (w/w) siRNA/DOPC. Tween 20 was added to the mixture in a ratio of 1:19 Tween 20siRNA/DOPC. The mixture was vortexed, frozen in an aceton/dry ice bath, and lyophilized. Before in vivo administration, this preparation was hydrated with normal 0.9% saline at a concentration of 15 μg/mL to achieve the desired dose in 150 to 200 μL per injection. To estimate the quantity of siRNA not taken up by liposomes, free siRNA was separated from liposomes using 30,000 nominal molecular weight limit filter units (Millipore Corp., Billerica, MA). The liposomal suspension was added to the filters and centrifuged at 5,000 × g for 40 minutes at room temperature. Fractions were collected, the material trapped in the filter was reconstituted with 0.9% saline, and siRNA of the collected fraction and the elute were measured by spectrophotometry.

Orthotopic in vivo model of advanced ovarian cancer and tissue processing. Female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and housed in specific pathogen-free conditions. They were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the USDA "Policy on Human Care and Use of Laboratory Animals," and all studies were approved and supervised by The University of Texas M.D. Anderson Cancer Center Institutional Animal Care and Use Committee. Tumors were established by i.p. injection of cells prepared as above. This model reflects the i.p. growth pattern of advanced ovarian cancer, as intraperitoneal spread is the main mechanism of ovarian cancer metastasis (12–14). Studies to determine uptake of single-dose fluorescent siRNA in tissue or silencing potential of single-dose siRNA against EphA2 were initiated once i.p. tumors reached a size of 0.5 to 1.0 cm³ as assessed by palpation (> 17 days after injection). Liposomal siRNA (5 μg) was given as a 200 μL i.v. bolus into the tail vein under normal pressure, and tumor and other tissues were harvested at various time points after injection (1 hour, 6 hours, 48 hours, 4 days, 7 days, or 10 days). Tissue specimens were snap frozen for lysate preparation, fixed in formalin for paraffin embedding, or frozen in OCT medium for frozen slide preparation. For long-term experiments to assess tumor growth, therapy began 1 week after tumor cell injection. Paclitaxel (100 μg) or vehicle was injected i.p. once weekly; siRNAs (nonspecific or EphA2 targeting, 150 μg/kg) in liposomes, or empty liposomes, were injected twice weekly i.v. in 150 to 200 mL volume (depending on mouse weight) with normal pressure. Mice (n = 10 per group) were monitored for adverse effects, and tumors were harvested after 4 weeks of therapy or when any of the mice began to appear moribund. Mouse weight, tumor weight, and distribution of tumor were recorded. Vital organs were also harvested and necropsies were done by a board-certified pathologist for evidence of tissue toxicity.

Immunofluorescence and confocal microscopy. Tissue for immunofluorescence was collected from sacrificed mice, immediately placed in OCT medium, and rapidly frozen. Frozen sections were cut at 8 μm sections for conventional microscopy and 30 μm sections for confocal microscopy. Tissue was fixed with acetone and either examined immediately or stunned for Fl/80 (to detect scavenging macrophages) or CD31 (to detect endothelial cells). For immunofluorescence detection, slides were blocked with 5% normal horse serum and 1% normal goat serum (Invitrogen, Carlsbad, CA) in PBS, exposed to 10 μg/mL anti-fluorescein isothiocyanate (FITC) secondary antibody (Sigma, St. Louis, MO) or 0.625 μg/mL anti-CD31 antibody in blocking solution overnight at 4°C, washed with PBS, and exposed to 4 μg/mL anti-rat antibody-Alexa 488 (Molecular Probes, Eugene, OR) in blocking solution for 1 hour at room temperature. Slides were washed with PBS, exposed to either 1.0 μg/mL Hoechst (Molecular Probes, in PBS) or 10 mmol/L Sytox green (Molecular Probes, in PBS) for 10 minutes to stain nuclei, washed, and covered with propylgallate and coverslips for microscopic evaluation. Conventional microscopy was done with a Zeiss Axiophot 2 microscope (Carl Zeiss, Inc., Germany), Hamamatsu ORCA-ER Digital camera (Hamamatsu Corp., Japan), and ImagePro software (Media Cybernetics, Silver Spring, MD). Fluorescence in three dimensions within 30 μm sections was examined with a Zeiss LSM 510 confocal microscope and LSM 510 Image Browser software (Carl Zeiss).

Western blot. Cultured cell lysates were prepared by washing cells with PBS followed by incubation in modified radioimmunoprecipitation assay buffer (RIPA) lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton, 0.5% deoxycholate plus 25 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate; Sigma Chemical Co., St. Louis, MO) for 10 minutes at 4°C. Cells were scraped from plates and centrifuged at 13,000 rpm for 20 minutes at 4°C and the supernatant was stored at −80°C. To prepare lysate from snap-frozen tissue samples, 200 μg/cm² cuts of tissue were incubated on ice in RIPA for 3 hours, mortes and pestel disrupted and homogenized, and centrifuged, and the supernatant was stored at −80°C. Samples from three regions of the tumors were collected and tested individually. Protein concentrations were determined using a BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL) and subjected to 10% SDS-PAGE separation. Samples transferred to a nitrocellulose membrane by semidy electrotransfer to Bio-Dad Laboratories, Hercules, CA) were incubated with 0.625 μg/mL anti-EphA2 antibody (Upstate, Lake Placid, NY) overnight at 4°C, detected with 1 μg/mL horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Amersham, Piscataway, NJ), and developed using enhanced chemiluminescence detection kit (Pierce Biotechnology). Membranes were tested for β-actin (0.1 μg/mL anti-β-actin primary antibody; Sigma Chemical) to confirm equal loading.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were deparaffinized by sequential washing with xylene, 100% ethanol, 95% ethanol, 80% ethanol, and PBS. Antigen retrieval was done by heating in steam cooker in 0.2 mol/L Tris-HCl (pH 9.0) for 20 minutes. After cooling and PBS wash, endogenous peroxide was blocked with 3% H₂O₂ in methanol for 5 minutes. Nonspecific proteins and exposed endogenous mouse IgG antibodies were blocked with 0.13 μg/mL mouse IgG Fc blocker (The Jackson Laboratory, Bar Harbor, ME) in 0.5% blocking agent (TSA Biotin System kit, Perkin-Elmer, Boston, MA) overnight at 4°C. Slides were incubated in primary antibody (5 μg/mL mouse anti-EphA2 clone aE5, a kind gift of Dr. Michael Kinch, MedImmune, Inc., Gaithersburg, MD) for 4 hours at 4°C and washed followed by 1.5 μg/mL biotinylated horse anti-mouse (Vector Labs, Burlingame, CA) for 1 hour at room temperature. The secondary antibody signal was enhanced with 0.75 μg/mL streptavidin-HRP (DakoCytomation, Carpinteria, CA) for 30 minutes, detected with 3,3′-diaminobenzidine (DAB; Phoenix Biotechnologies, Huntsville, AL) substrate for 7 minutes, and counterstained with Gill no. 3 hematoxylin (Sigma Chemical) for 20 seconds.

MicrovesSEL density. To determine microvesSEL density (MVD), collected tissue was frozen in OCT with liquid nitrogen. Eight-micron sections were fixed in acetone for 10 minutes, washed in PBS, and blocked with 10% fish gelatin for 10 minutes. Slides were exposed to rat anti-mouse CD31 for 2 hours at room temperature, diluted in blocking solution, and exposed, after three PBS washes (5 minutes each), to anti-rat IgG conjugated to HRP. HRP was detected with DAB for 10 minutes and counterstained with Gill no. 3 hematoxylin for 20 seconds. To calculate MVD, five representative photographs were taken of each slide (1 slide per mouse, 5 slides per group), and the number of vessels per field (final magnification, > 100) was counted.
by an examiner blinded to treatment group. A vessel was defined as an open lumen with at least one adjacent CD31-positive cell. Multiple positive cells beside a single lumen are counted as one vessel.

Statistical considerations. For in vivo therapy experiments, 10 mice in each group were used as directed by a power analysis to detect a 50% reduction in tumor size (β error = 0.2). Continuous variables (tumor size and MVD) were analyzed for statistical significance (achieved if P < 0.05) with Student’s t test for two-group comparisons and ANOVA for multiple-group comparisons. If values were not normally distributed, the Mann-Whitney rank sum test was used using Stata 8 software (College Station, TX).

Results

Incorporation of small interfering RNA into liposomes. An efficient delivery vehicle is necessary for in vivo delivery. Cationic liposomes, while efficiently taking up nucleic acids, have had limited success for in vivo gene down-regulation perhaps because of their affinity for endothelial cells, their stable intracellular nature, or their failure to release siRNA contents. We selected DOPC because we have successfully used this molecule to deliver antisense oligonucleotides in vivo (27). When mixed together, >90% of liposomes spontaneously incorporate fluorescently tagged siRNA when microscopically examined for fluorescence (Fig. 1A and B). To estimate the quantity of siRNA not taken up by liposomes, free siRNA was separated from liposomes with siRNA by column filtration and residual siRNA was measured by spectrophotometry. It is estimated that ~65% of the siRNA are incorporated into liposomes. In our experience, the liposomal-nucleic acid complexes are stable for at least 4 weeks when stored at ~20°C.

Delivery of small interfering RNA into orthotopically implanted ovarian tumor. To examine whether siRNA could be effectively delivered into tumor cells, we used a nonsilencing siRNA tagged with the fluorochrome Alexa 555 in DOPC complexes. Mice with HeyA8 orthotopic tumors (15 days after i.p. inoculation of tumor cells; Fig. 1C) were i.v. injected with 5 μg DOPC-conjugated nonsilencing siRNA/Alexa 555. Tumors were harvested at 1 hour and at 4, 7, and 10 days and examined for fluorescence. As early as 1 hour after injection, punctuated emissions of the siRNA were noted in the perinuclear region of individual cells (Fig. 1E) that were absent in the emission pattern of tumor injected with nonfluorescent siRNA (Fig. 1D). siRNA was seen in 80% of fields examined (original magnification ×400) and an estimated 30% of all tumor cells. This distribution can be appreciated by a low-power view (Fig. 1H), although the signal strength is weaker at this magnification.

To confirm that the siRNA was present in tumor cells and not simply scavenged by macrophages, separate slides were stained for f4/80 to identify scavenging macrophages. These macrophages are seen to surround nests of tumor cells that contain perinuclear siRNA and have about the same rate of siRNA uptake as tumor cells (Fig. 1F), suggesting that siRNA is delivered directly into the tumor cells. To confirm that the fluorescent signal was not a contaminating secondary antibody or an artifact of processing, 30 μm sections were examined with confocal microscopy. This technique permitted signal detection within the middle of tissue rather than from surface emissions alone. After evaluating emissions at multiple depths, a three-dimensional cross-section was created. Lateral views clearly show the presence of fluorescently tagged siRNA within tissue parenchyma (Fig. 1G). In this view, fluorescent emission from macrophage staining (green) was noted only at the surface, because the detecting antibody is too large to penetrate tissue. Emission from nuclear staining (blue) is the result of a dye (Hoescht) small enough to penetrate tissue. Tumors collected at 4, 7, and 10 days after a single injection were also noted to retain siRNA fluorescence. However, because this fluorescently tagged siRNA should be a nonsilencing construct, longevity after
administration of a mRNA-targeting construct is likely to be of shorter duration.

**Tissue distribution of small interfering RNA after delivery by conventional methods.** To compare tumor delivery of siRNA with other methods, we injected siRNA i.v. either without a transfection agent (naked) or complexed in DOTAP. After administration of naked fluorescent siRNA, fluorescence was rarely observed (2% of ×40 fields, <1% of cells; Fig. 2A) although notably present in the desired perinuclear location in positive cells. Administration of siRNA complexed with DOTAP showed sporadic presence of fluorescence within tumor tissue (7% of all fields examined). However, the observed fluorescence was primarily adjacent to CD31-positive endothelial cells (shown green in Fig. 2B), bringing into question whether the liposomal contents were released or trapped in the vasculature. Tissue obtained after siRNA delivery in DOPC and stained with CD31 showed that delivery was not restricted to the vasculature and is efficiently delivered deep into the tumor parenchyma. Our DOPC liposome preparation was associated with an estimated 10-fold improvement in delivery of siRNA compared with DOTAP and 30-fold improvement over naked siRNA.

**Tissue distribution of small interfering RNA throughout vital organs.** To examine the distribution of delivery to other organs, sections of the liver, kidney, spleen, heart, lung, and brain were examined for fluorescence after a single dose of Alexa 555 siRNA in DOPC. Specimens from mice treated with nonfluorescent siRNA were examined to determine background fluorescence. There was significant siRNA uptake and cytoplasmic distribution in the liver, kidney, and lung (Fig. 3A-C, respectively). There was a small amount of uptake in the heart but significantly greater than that of untreated heart tissue. The fluorescence emitted by endogenous protein products in the spleen, pancreas, and brain...
made evaluation more difficult and precludes a definitive conclusion that liposomes are incorporated in these tissues.

Similar patterns of uptake after DOTAP-complexed and naked siRNA administration were seen in other organs as was seen in the tumor. DOTAP complexes formed multiple large fluorescent signals near the vasculature without perinuclear punctuations in the liver. There was a high level of uptake in the kidney both near the vasculature and by individual cells. Naked siRNA administration did result in uptake by a large percentage of liver and kidney cells, but the fluorescent signal was greatly decreased compared with uptake by DOPC-complexed siRNA.

Down-regulation of EphA2 with liposomal small interfering RNA. We have shown previously that EphA2 is overexpressed by a large percentage of patients with ovarian cancer and that overexpression is predictive of poor outcome (22). Furthermore, this protein has low relative expression in the adult and so is an attractive tumor selective target. Therefore, we used EphA2 as a model to test the efficacy of siRNA therapy. In vitro, both HeyA8 and SKOV3ip1 ovarian cancer cell lines transfected with EphA2 siRNA showed a 95% decrease in EphA2 expression compared with transfection with control siRNA as determined by Western blot analysis (data not shown). Subsequently, we tested the ability of DOPC liposomal siRNA to silence EphA2 in an orthotopic in vivo model. EphA2-targeting siRNA-DOPC was given to tumor-bearing mice and tumor collected at various time points. Measurement of EphA2 by Western blot of tumor lysate (Fig. 4A) and by immunohistochemistry (Fig. 4D) showed that tumor collected 48 hours following administration of single-dose anti-EphA2 siRNA had significantly decreased EphA2 expression compared with treatment with a nonspecific siRNA (Fig. 4B) or naked siRNA (Fig. 4C). Expression of EphA2 remained suppressed at 4 days, was recovering after 7 days, and had returned to normal levels by 10 days. Therefore, we used twice-weekly dosing of anti-EphA2 siRNA for subsequent therapy experiments.

In vivo therapy experiments with liposomal anti-EphA2 small interfering RNA. Female nude mice (n = 50 per cell line, 10 per group) were injected with HeyA8 or SKOV3ip1 cells into the peritoneal cavity. One week after tumor cell injection, animals were randomly allocated to five treatment groups: (a) empty liposomes, (b) nonspecific siRNA-DOPC, (c) EphA2-targeted siRNA-DOPC, (d) paclitaxel and nonspecific siRNA-DOPC, and (e) paclitaxel and EphA2-targeting siRNA-DOPC. After 4 weeks of therapy, the animals were sacrificed and necropsies were done. Tumors were excised and weighed. Treatment with anti-EphA2 siRNA, paclitaxel plus control siRNA, and paclitaxel plus anti-EphA2 siRNA were all effective in reducing tumor size (overall ANOVA, P < 0.001 for both cell lines), with combination therapy leading to 86% to 91% reduction compared with treatment with control siRNA alone (Fig. 5A and B). Targeting EphA2 with siRNA alone diminished tumor growth in both lines when compared with control siRNA alone (HeyA8: 0.98 versus 1.51 g, respectively; P = 0.155; SKOV3ip1: 0.35 versus 0.70 g, respectively; P = 0.020). EphA2-targeting siRNA in combination with paclitaxel significantly reduced tumor growth by 67% to 82% compared with nonspecific siRNA and paclitaxel (HeyA8: 0.21 versus 0.84 g, respectively; P < 0.003; SKOV3ip1: 0.04 versus 0.22 g, respectively; P < 0.001). This pattern of tumor growth inhibition (moderate inhibition with EphA2 targeting alone, marked inhibition in combination with paclitaxel) is similar to that seen with antibody-based EphA2 down-regulation in this mouse model (data not shown).

Data from other measured variables of these therapy experiments are shown in Table 1. The incidence of tumor formation was not significantly different among the five groups in either cell line. However, the number of nodules formed was reduced by both EphA2 siRNA and paclitaxel individually, and further reduced by the combination, in both cell lines. Therapy was not continued long enough to allow development of ascites, which is typical in the SKOV3ip1 line 5 to 6 weeks after injection.

Interestingly, administration of nonspecific siRNA-DOPC resulted in some reduction in tumor growth, although statistically significant only in the HeyA8 model, when compared with empty liposomes. These data may support prior reports that siRNA without a specific mRNA target may have nonspecific effects that affect tumor growth (3) and further support our hypothesis that siRNA-DOPC is delivered to the tumor parenchyma.

Effects of EphA2-targeted therapy on vascular density. To explore the mechanisms involved in the reduced tumor formation with this therapy, we first confirmed that EphA2 levels remained low with long-term therapy. Tumors harvested at the conclusion of therapy trials were subjected to immunohistochemistry for EphA2 (Fig. 6A). Both groups treated with EphA2-targeting siRNA showed ~50% decrease in distribution of expression compared with control siRNA or control siRNA plus paclitaxel. The intensity of staining was not significantly different among the groups, suggesting that gene silencing is effective if delivery is achieved.

EphA2 has been shown to play a role in migration, invasion, and angiogenesis (21). To assess possible antiangiogenic effects of EphA2 down-regulation, tissues obtained at the conclusion of long-term therapy trials were subjected to immunohistochemistry for
CD31, and MVD was calculated for each group. Representative sections are shown in Fig. 6B, with the mean number of vessels for each group in Fig. 6C. MVD was unaffected by control siRNA (15.7 versus 17.9; \( P = 0.29 \)) but was reduced by 52% with EphA2-targeting siRNA alone (8.6 versus 17.9; \( P < 0.001 \)). Paclitaxel did not affect MVD (18.2 versus 17.9; \( P = 0.80 \)). The addition of EphA2 siRNA to paclitaxel similarly reduced MVD by 74% compared with paclitaxel and control siRNA (4.7 versus 18.2; \( P < 0.001 \)).

No toxicities were observed by behavioral changes, such as eating habits and mobility in animals treated with liposomal siRNA preparations, both those that are nonsilencing and those targeting EphA2. Mouse weights were not significantly different among the five groups of animals, suggesting that eating and drinking habits were not affected. Organ sections were reviewed by a board-certified pathologist, and after 5 weeks of therapy, no histologic toxicities were detected in the liver, kidney, heart, lung, or brain. A slight increase in the size of the white pulp of the spleen was noted in all four siRNA groups, which may be indicative of a general inflammatory response.

**Discussion**

In this study, we describe the therapeutic delivery of gene-specific siRNA into the tumor using DOPC liposomes, with subsequent reduced protein expression and reduced tumor growth. To the best of our knowledge, this is the first study to collectively show the direct delivery, gene targeting, and growth attenuation after systemic delivery of siRNA in an orthotopic model of ovarian cancer. The significance of this work is that packaging of siRNA into liposomes is rapidly transferable to a clinical setting. Previously, the most reproducible systemic delivery of siRNA *in vivo* has been rapid injection of high volume of material (i.e., 2 mL into a mouse with estimated 4 mL total blood volume over 5 seconds), hydrodynamically forcing siRNA into the liver (29). Such a technique would not

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**Table 1. Characteristics of tumors after siRNA/paclitaxel therapy**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Groups</th>
<th>Incidence (%)*</th>
<th>No. nodules</th>
<th>( P ) for no. of nodules</th>
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<tbody>
<tr>
<td>SKOV3ip1</td>
<td>Liposomes</td>
<td>100</td>
<td>16.3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Control siRNA</td>
<td>100</td>
<td>17.8</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>EphA2 siRNA</td>
<td>100</td>
<td>6</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Paclitaxel + control siRNA</td>
<td>100</td>
<td>4.3</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Paclitaxel + EphA2 siRNA</td>
<td>90</td>
<td>2.2</td>
<td>0.001</td>
</tr>
<tr>
<td>HeyA8</td>
<td>Liposomes</td>
<td>100</td>
<td>4.8</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Control siRNA</td>
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</tr>
<tr>
<td></td>
<td>Paclitaxel + EphA2 siRNA</td>
<td>80</td>
<td>1.5</td>
<td>0.034</td>
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</table>

*There was no significant difference (\( P < 0.05 \)) between any two compared groups (by Student’s *t* test) or all groups (by ANOVA) for incidence of tumor formation.
be feasible in a clinical setting, whereas liposomes have been used extensively clinically for chemotherapy and other delivery systems. Because delivery in this study was efficient to other vital organs, most notably in the liver and kidney, this method may be used in noncancerous conditions shown to be amenable to siRNA therapy in preclinical models, such as viral hepatitis (9, 29) and HIV (30). However, this mode of delivery is not tissue specific, so it will be important that the gene chosen to down-regulate with siRNA is not crucial to function by normal cells. Alternatively, further modifications of the liposome may allow tumor-selective delivery (31, 32).

The first demonstration that siRNA had activity in vivo was in the hydrodynamic injection of naked siRNA that effectively decreased luciferase expression in the livers of mice (29). Along with confirmatory reports of high-pressure i.v. injection (33, 34), others have shown that siRNA has activity in vivo using delivery in viral vectors (8, 9), retinal electroporation (35), and direct intracellular (36), intratumoral (37), intravitreal (38), intranasal (39), and intrathecal (40) administration. Although these methods are useful in a preclinical setting, their delivery methods and the climate of viral gene therapy make clinical applicability limited. Sorensen et al. effectively reduced tumor necrosis factor-α expression in the liver and spleen by delivering siRNA packaged in cationic liposomes (DOTAP), protecting mice from a lethal dose of lipopolysaccharide (41). We have found that DOTAP accumulates near the vasculature and is preferentially taken up by the liver and spleen, limiting its effectiveness in systemic or antitumor therapy. Soutschek et al. have reported that siRNA conjugated with cholesterol improved delivery to multiple organs and that down-regulation of ApoB was achieved in liver and jejunum (10). However, the effects of cholesterol conjugation on siRNA activity and duration of effect, efficiency of uptake in tumors, and toxicities are not known. Duxbury et al. have shown that systemic delivery of naked siRNA-targeting FAK (42), EphA2 (26), or CEACAM6 (43) down-regulated protein expression and decreased growth of a single s.c. injected malignant pancreatic cell line. It is possible that naked siRNA may be effectively delivered to s.c. sites, but not to orthotopic sites, as supported by our results. To the best of our knowledge, others have not reported successful therapy with naked unaltered siRNA in other cancer models.

Recent studies suggest that the specificity of siRNA may not be as absolute as initially hoped. An analysis of gene expression profiling suggested that RNA down-regulation might occur with as few as 11 complementary base pairs within the 21-bp siRNA sequence (44). A recent study of commonly used siRNA sequences found that ~75% of these sequences had nonspecific targeting (45). Therefore, in siRNA design, a BLAST search for cross-reactive 21-bp sequences is insufficient to have confidence that the mRNA of interest is the only target. Furthermore, siRNAs may bind mRNA of only near-perfect complementarity and prevent translation without degradation (46). This is the mechanism used by endogenously produced microRNAs (miRNA), believed to be another method of natural regulation of gene expression (47). Crossover of siRNA into the miRNA pathway or down-regulation by partial homology seem to be minimal and require participation of several siRNA sequences, but this potential should caution conclusions made regarding the specificity of gene down-regulation. It is difficult at this time to speculate which particular proteins could be "off-site" targets of nonspecific siRNA silencing. Studies with microarray analysis or reporter arrays may allow such projections to be made in the future (44, 48). Another level of questionable specificity of siRNA introduction lies in activation of the innate immune system. siRNA therapy has, in some circumstances, been shown to activate IFN (49, 50). Of course, in the treatment of cancer, IFN induction may be of additional benefit, as long as
Toxins are limited. This is supported by our finding that therapy with a nonspecific siRNA construct results in some reduction in tumor growth compared with empty liposomes.

Toxicities of liposomes are believed to be limited. Liposomal chemotherapy is routinely used in treatment of ovarian and other cancers (51). In a phase I trial with caticionic liposomes carrying a plasmid encoding the E1A gene, fever and pain 3 hours after treatment were the dose-limiting toxicities (52). Although this is the best estimation of side effects we can currently predict, delivery of siRNA is less likely to be recognized as foreign, and host response will almost certainly differ.

The charge of the liposome affects the tissue specificity of liposomal uptake. Macrophages seem to preferentially take up negatively charged liposomes (53). Different malignant cell lines have varying uptake patterns regarding positive, neutral, or negative charges, and in vivo uptake patterns may differ further (53).

Liposomal makeup also influences cellular toxicity, with siRNA delivery using a liposome with a higher proportion of neutral lipids leading to less cellular toxicity without compromising ability to down-regulate gene expression in vitro (54). Clearly, a complete understanding of the best liposomal makeup for delivery of therapeutic substances is still evolving. It is possible that with siRNA delivery the use of a neutral lipid, such as DOPC, allows a balance among efficient uptake of the siRNA into a liposome at preparation, uptake of the liposome into a cell, and breakdown of the intracellular liposome with release of siRNA contents into the cytoplasm.

EphA2 is an attractive target for antitumor therapies. It is minimally expressed in adults, being limited to some epithelial tissues (55), and the EphA2 knockout mouse is phenotypically neutral at birth, being limited to some epithelial cytoplasm. Hence, EphA2 is an attractive target for antitumor therapies. It is minimally expressed in adults, being limited to some epithelial tissues (55), and the EphA2 knockout mouse is phenotypically normal (56). However, EphA2 is overexpressed by several cancers (21), including ovarian, in which it is associated with poorer survival (22). Furthermore, the receptor primarily exhibits carcino-nogenic properties through high levels of the unphosphorylated form. Therefore, decreasing total EphA2 levels are more likely to be effective than attempts to block its activation. We have shown that EphA2-targeting siRNA therapy leads to a decrease in MVD. Others have seen an antiangiogenic effect with EphA2 down-regulation (25), and we have seen this with antibody-based approaches to decrease EphA2 expression in vivo. Delineation of biological pathways dependent on EphA2 is difficult, because EphA2 modulation has little effect on monolayer cell culture properties (21). Further studies of in vivo–treated tissues may help to define other mechanisms affected by EphA2 overexpression.

In vivo delivery of siRNA in experimental models has been shown to provide feasibility for use in humans. Liposomal delivery of drugs is established and safe, and their use for siRNA delivery may make this therapeutic modality clinically attractive. We have shown that using DOPC-complexed siRNA allows delivery to tumor and other tissues, with corresponding gene targeting and reduced tumor growth. With further study and a cautious approach, this is a model that can be taken into a clinical setting for cancer therapy as well as for other conditions amenable to specific gene down-regulation.

Acknowledgments

Received 2/17/2005; revised 5/2/2005; accepted 5/18/2005.

Grant support: The University of Texas M.D. Anderson Cancer Center Bettyann Asche-Murray Fellowship Award (C.N. Landen) and Department of Defense grant W81XWH-04-1-0227 (A.K. Sood).

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5 Unpublished data.
Therapeutic EphA2 Gene Targeting In vivo Using Neutral Liposomal Small Interfering RNA Delivery


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