Enhancing Chemotherapy Response with Sustained EphA2 Silencing Using Multistage Vector Delivery

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Abstract

Purpose: RNA interference has the potential to specifically knockdown the expression of target genes and thereby transform cancer therapy. However, lack of effective delivery of siRNA has dramatically limited its in vivo applications. We have developed a multistage vector (MSV) system, composed of discoidal porous silicon particles loaded with nanotherapeutics, that directs effective delivery and sustained release of siRNA in tumor tissues. In this study, we evaluated therapeutic efficacy of MSV-loaded EphA2 siRNA (MSV/EphA2) with murine orthotopic models of metastatic ovarian cancers as a first step toward development of a new class of nanotherapeutics for the treatment of ovarian cancer.

Experimental Design: Tumor accumulation of MSV/EphA2 and sustained release of siRNA from MSV were analyzed after intravenous administration of MSV/siRNA. Nude mice with metastatic SKOV3ip2 tumors were treated with MSV/EphA2 and paclitaxel, and therapeutic efficacy was assessed. Mice with chemotherapy-resistant HeyA8 ovarian tumors were treated with a combination of MSV/EphA2 and docetaxel, and enhanced therapeutic efficacy was evaluated.

Results: Treatment of SKOV3ip2 tumor mice with MSV/EphA2 biweekly for 6 weeks resulted in dose-dependent (5, 10, and 15 mg/mice) reduction of tumor weight (36%, 64%, and 83%) and number of tumor nodules compared with the control groups. In addition, tumor growth was completely inhibited when mice were treated with MSV/EphA2 in combination with paclitaxel. Furthermore, combination treatment with MSV/EphA2 and docetaxel inhibited growth of HeyA8-MDR tumors, which were otherwise resistant to docetaxel treatment.

Conclusion: These findings indicate that MSV/EphA2 merits further development as a novel therapeutic agent for ovarian cancer. Clin Cancer Res; 19(7); 1806–15. ©2013 AACR.

Introduction

The EphA2 gene encoding the epithelial cell receptor protein–tyrosine kinase is overexpressed in multiple cancer types (1–3). Its expression level is associated with aggressive features of tumor growth (4) and is a predictive marker for tumor recurrence and patient survival (5, 6). We have previously shown that EphA2 expression was associated with angiogenesis in ovarian tumors (7) and that knockdown of EphA2 expression with gene-specific siRNA oligos delivered in dioleoyl phosphatidylcholine (DOPC) neutral liposomes led to inhibition of tumor growth in orthotopic mouse models of ovarian cancer (3). In an effort to develop a tumor-specific delivery system with sustained release of siRNA oligos, we loaded liposomal siRNA into the 40 to 65 nm size pores inside the 1.6 μm hemispherical porous silicon particles to create a nanoparticle-in-microparticle multistage vector (MSV) delivery system (8). Once delivered in vivo via intravenous administration, the silicon particles travel in circulation and settle at the tumor vasculature...
where the liposomal siRNA gets released when the porous silicon degrades (9, 10). In a proof-of-principle study, we have shown that knockdown of EphA2 expression lasted for as long as 3 weeks from a single administration of this new formulation, which resulted in reduced tumor cell proliferation and tumor angiogenesis and eventually tumor growth (8). Our results also indicated that the MSV delivery system did not cause significant toxicity to major organs such as liver and kidney (8).

The micrometer-sized, nanoporous silicon particles were designed to accumulate in tumor endothelial and perivascular depots (11). Unlike most nanocarriers for drug delivery that rely on the leaky vasculature to accumulate in tumor tissues, these porous silicon particles do not solely exploit the enhanced permeability and retention (EPR) effect of the tumor vasculature. Instead, they travel hematogenously and interact with endothelial cells taking advantage of the optimal hydrodynamic force and interfacial interaction within the tumor vasculature. Our recent studies have revealed that size, shape, and surface chemical property of the porous silicon particles are major determinants of tissue distribution (12–14). More discoidal particles accumulate in tumor tissues than the spherical or cylindrical particles in mouse model of hemispherical tumors that were otherwise resistant to taxanes. Our study provides a strong rationale for the development of EphA2 siRNA therapeutics for the treatment of ovarian cancer in clinic.

Preparation of siRNA DOPC nanoliposomes and assembly into MSV

siRNA oligos were incorporated into 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, INC) by lyophilization. Briefly, 15 μg siRNA and DOPC were mixed in the presence of excess t-butanol to a ratio of 1:10 (w/w) as described previously (3, 7, 8). After Tween-20 was added, the mixture was frozen in an acetone-dry ice bath and lyophilized. Nanoliposomes were reconstituted by adding 40 μL water into the vial followed by a brief mix. The nanoliposome suspension was then mixed with 6 × 10^8 discoidal porous silicon particles and sonicated for 5 minutes.

Tumor cells, culture condition, and in vitro release of MSV/siRNA

The human ovarian cancer cell lines SKOV3ip2 and HeyA8-MDR have been described previously (17). Cells were maintained in RPMI-1640 medium supplemented with 10% FBS in 5% CO2/95% air at 37°C.

To measure siRNA release from MSV in cancer cells, SKOV3ip2 and HeyA8 cells were seeded in 4-chamber tissue culture slides and MSV/Alexa555-labeled siRNA particles were added into cell culture 24 hours later at a ratio of 1:50 (cell/MSV). Cells were washed the next day to get rid of free particles and fluorescence intensity from Alexa-555 siRNA was visualized with a confocal microscope (cell/MSV). Cells were also stained with 4′,6-diamidino-2-phenylindole (DAPI) for visualization of the nuclei. To measure sustained knockdown of EphA2 expression in tumor cells in vitro, SKOV3 cells were incubated with MSV/scramble siRNA or MSV/EphA2 siRNA. Samples were collected on days 3, 5, 7, and 9, and processed for Western blot analysis. EphA2 expression level was detected with an
anti-EphA2 antibody from Invitrogen. Time-dependent cell viability was also measured with the Cell Counting Kit-8 from Dojindo.

Orthotopic tumor implantation
Female athymic nude mice (NCr-nu, 8–12 weeks old) were purchased from Taconic (Hudson). To generate tumors, SKOV3ip2 [1 × 10⁶ cells/0.2 mL Hank’s balanced salt solution (HBSS)] or HeyA8-MDR (5 × 10⁵ cells/0.2 mL HBSS) cells were injected into the peritoneal cavity of nude mice. For efficacy evaluation, mice were treated with therapeutics 2 weeks after tumor implantation.

In vivo distribution of siRNA
Porous silicon particles were covalently conjugated to the Dylight-488 fluorescent dye and Cy3-labeled siRNA oligos were used to prepare nanoliposomes. Nude mice bearing SKOV3ip2 tumors were treated intravenously with 15 μg siRNA in Dylight-MSV/Cy3-siRNA. Animals were sacrificed 12, 24, 72, 120, or 168 hours later. Tumor nodules and major organs including kidney, spleen, liver, lung, and heart were collected, and tissues with equal size and shape were deposited into a 96-well plate for measurement of fluorescent intensities. Images were captured with a Xenogen IVIS Spectrum imaging system (Caliper Life Sciences) with a 535 nm excitation filter and 580 nm emission filter for Cy3, and 605 nm excitation filter and 660 nm emission filter for Dylight.

Therapeutic activity of MSV/EphA2
Paclitaxel (PTX) and docetaxel (DTX) were purchased from M D Anderson Cancer Center (Houston, TX) pharmacy and diluted in PBS to 2 and 3 mg/kg before use. To assess the therapeutic activity of MSV/EphA2 alone or in combination with paclitaxel, nude mice bearing SKOV3ip2 tumors were randomly divided into 8 groups (10 mice/group) and treated with: (i) empty MSV, (ii) MSV/scramble siRNA (MSV/Scr, 15 μg siRNA), (iii) MSV/EphA2 (5 μg siRNA), (iv) MSV/EphA2 (10 μg siRNA), (v) MSV/EphA2 (15 μg siRNA), (vi) PTX (75 μg), (vii) MSV/Scr plus PTX, and (viii) MSV/EphA2 (15 μg) plus PTX. Paclitaxel was intraperitoneally dosed weekly, and empty MSV and MSV/siRNA were intravenously dosed biweekly. Animals were sacrificed after 6 weeks of treatment, and tumor samples were removed and processed for immunohistochemical (IHC) analyses. To assess gene expression by Western blot analysis, the mice with tumor were treated with the indicated therapeutics once and biweekly and docetaxel was intraperitoneally dosed weekly.

IHC analysis of tumor samples
EphA2 expression was analyzed by IHC analysis using paraffin-embedded tumors as described previously (3, 8). IHC analysis for CD31 (microvessel density) was done on freshly cut frozen tissue with the anti-CD31 antibody (platelet/endothelial cell adhesion molecule-1, rat IgG; BD Pharmingen). To quantify microvessel density (MVD), 5 random fields at ×20 final magnification were examined for each tumor and the number of microvessels per field was counted.

Assessment of cell apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was carried out by IHC analysis using freshly cut frozen tissue as described previously (3, 7, 8). To quantify TUNEL-positive cells, the number of positive cells was counted in 5 random fields at ×20 magnification.

Statistical analysis
For in vivo experiments, differences in continuous variables (tumor weight, MVD, and TUNEL staining) were analyzed using the Student t test for comparing 2 groups and by ANOVA for multiple group comparisons with P < 0.05 considered statistically significant and P < 0.01 very significant.

Results
Characterization of porous silicon microparticles
The 1,000 × 400 nm discoidal porous silicon particles were fabricated by a combination of electrochemical etching and photolithography (15). The unmodified particles had a negative surface charge (−30 mV) as there was a thin-layer of oxidized silicon on the surface as a result of the fabrication process and were vulnerable to degradation in aqueous solution. In serum, most particles were dissolved after several hours of incubation (15). We modified the silicon particles by chemically conjugating APTES onto the surface (16). APTES modification not only stabilized the particles but also converted the otherwise negatively charged surface to slightly positive (ξ potential +3–+7 mV), which facilitated loading of the slightly negative to neutral liposomes (30–35 nm in diameter) into the nanopores (average diameter 60 nm).

Stability of the APTES-modified porous silicon particles were tested in PBS containing 10% FBS. SEM images showed that the newly fabricated discoidal silicon particles consisted of a thin, high-density top layer and a thick, high-porosity bottom layer (Fig. 1A). The nanoliposomes were loaded into the high-porosity layer. The uniformly looking particles had clear edges and well-defined nanoporous structure. After 24-hour incubation in FBS, the particles had lost part of the porous structure already. After 3 days in FBS, most particles had lost a substantial proportion of the high-porosity layer. Only the high-density layer
was left with some particles. However, some particles still retained part of the porous structure after 10 days of incubation. Particle degradation was also confirmed by size measurement with a Multisizer based on particle diameter (Fig. 1B, top) and volume (Fig. 1B, bottom). While the median sizes of particles were close between the preincubation and day 1 samples, a 32% drop on median particle size (from 0.13 to 0.088 μm³) was noticed after 3 days of incubation. By day 10, most particles were much smaller than the preinoculation samples with a 46% drop on median particle size.

**Characterization of MSV/siRNA**

To prepare MSV/siRNA, DOPC liposomes incorporated with siRNA oligos were loaded into the 1,000 /C2 400 nm discoidal porous silicon particles. Six hundred million porous silicon particles were needed to load 15 mg siRNA oligos. We have previously shown that 15 mg siRNA per injection per mouse is needed to ensure sufficient knockdown of EphA2 expression in tumor samples (8). To assess siRNA release inside cells, we prepared DOPC liposomes with the fluorescent Alexa555 siRNA oligos and loaded them into porous silicon. MSV/Alexa555 siRNA was added into culture of human SKOV3ip2 and HeyA8 cancer cells and fluorescence intensity inside the cells was monitored. Multiple silicon particles could be found within each cell one day after incubation (Fig. 2A). We have previously shown that particles are internalized by phagocytosis and/or macropinocytosis (18). All particles were localized in the cytosol where the liposomal siRNA got released. Bright fluorescent intensity could still be detected on day 2 and day 7, indicating that the particles were stable inside the cell and that sustained release of siRNA could be achieved. It is interesting to note that the cells still carried MSV/Alexa555 siRNA by day 7, when the original cells should have gone through several cycles of cell division (Fig. 2A). The result implies that the MSV particles were partitioned evenly during cell division.

We incubated SK-OV-3 cells with MSV/Scramble siRNA or MSV/EphA2 siRNA and monitored knockdown of EphA2 expression by Western blot analysis. EphA2 knockdown was apparent on day 3 and maximum knockdown was reached on day 7 (Fig. 2B). Knockdown of EphA2 expression reduced cell viability by 25% on day 5 and 40% by day 9 (Fig. 2C).

**Biodistribution of MSV/siRNA**

To determine siRNA distribution in vivo, we packaged Cy3-siRNA into DOPC liposomes, loaded them into Dylight-conjugated MSV particles, and administrated MSV/siRNA intravenously into the SKOV3ip2 orthotopic tumor mice. Mice were sacrificed at different time points, major organs and tumor tissues were collected, and fluorescence from Cy3 and Dylight was measured with a Xenogen IVIS Spectrum imaging system. Liver and tumor accumulation of MSV particles was apparent 12 hours after administration based on fluorescence intensity (Fig. 3A). Dylight accumulation in the kidney was also observed at the 12-hour time point, suggesting that a substantial amount of the fluorescent dye had already been cleaved from the particle. A 5-fold increase of Cy3 fluorescence intensity was observed in tumor tissues from mice in the treatment group when compared with the untreated mice at the 12-hour
Fluorescence intensity from Cy3-siRNA in tumor tissues was maintained at 2-fold above the background level over the next 4 days (24–120-hour time points) and started to drop on day 7 (Fig. 3B). This result indicates that a relatively high level of siRNA in tumor tissues can be maintained by sustained release of siRNA from MSV. Interestingly, Cy3 intensity was comparable in the major organs including the liver between control and treated mice, although more liver accumulation of MSV particles could be detected (Fig. 3A). It is possible that liver served as a depot of MSV/siRNA. As soon as liposomal siRNA was released from MSV, they entered the circulation and accumulated in the tumor tissues as a result of the EPR effect of the tumor (19).

Dose-dependent inhibition of tumor growth by MSV/EphA2 siRNA

Mice with metastatic SKOV3ip2 tumors were treated biweekly with MSV/EphA2 siRNA at 5, 10, or 15 μg...
siRNA per mouse per treatment for 6 weeks. In the control groups, animals were treated either with empty MSV or MSV/Scr. At the end of the treatment, all animals were sacrificed, and the number of tumor nodules and total weight of tumor tissues per mouse were measured. Overall, treatment with MSV/EphA2 resulted in significantly reduced tumor growth (Fig. 4). Dose-dependent inhibition was apparent among the EphA2 siRNA groups. Treatments with 5, 10, or 15 μg siRNA resulted in 36% (P < 0.05), 64% (P < 0.05), and 83% (P < 0.01) reduction of total tumor weight, respectively, when compared with the MSV/Scr group (Fig. 4). Dose-dependent inhibition was mostly reflected in the tumor weight, as the average number of tumor nodules per mouse was close in the 3 treatment groups.

Pathologic analysis of tumor samples revealed that treatment with MSV/5 μg EphA2 siRNA only partially inhibited gene expression (Fig. 5A). Increasing siRNA to 10 and 15 μg/treatment dramatically reduced EphA2 expression. To confirm knockdown of EphA2 expression, we carried out another experiment by treating mice with tumor once instead of 3 times so that there would be enough tumor nodules left for Western blot analysis (Fig. 5B). Although there was still EphA2 expression in the 5 μg EphA2 siRNA treatment group, no EphA2 expression was detected in tumor samples treated with 10 or 15 μg EphA2 siRNA.

Knockdown of EphA2 expression resulted in reduction of total number of microvessels in tumor samples (Fig. 5C). Multiple apoptotic cells could be identified in MSV/EphA2-treated tumors but not in samples from the control groups (Fig. 5D). Dose-dependent cell apoptosis was apparent in the MSV/EphA2 treatment groups (Fig. 5E). These results indicate that MSV/EphA2 siRNA is an effective agent to treat metastatic ovarian tumor.

Taxanes are widely used for the treatment of ovarian cancer in clinic. We treated SKOV3ip2 tumor-bearing mice with paclitaxel and compared therapeutic efficacy between monotherapy and combination therapy with MSV/EphA2 siRNA. As expected, paclitaxel treatment alone significantly inhibited tumor growth, which was reflected by the 90% reduction in tumor weight and dramatic reduction in the number of tumor nodules (Fig. 4). Interestingly, paclitaxel treatment also caused slight reduction of EphA2 expression in the residual tumor nodules (Fig. 5A). This probably reflects the fact that the majority of tumor cells had been killed after 6-week treatment with this chemotherapy drug, as a single treatment with paclitaxel did not alter EphA2 expression (Fig. 5B). The greatest therapeutic benefit was from combination treatment with paclitaxel and MSV/EphA2. Almost all tumor nodules were eliminated, so that there was no tumor sample available from this treatment group for pathologic analysis at the end of the 6-week treatment. This result provides support for an effective therapy strategy in clinic to target the EphA2 gene in combination with standard of care chemotherapy drugs.

**Enhanced therapeutic efficacy in taxane-resistant tumor**

While taxanes are effective drugs to treat ovarian cancers in clinic, most cancers eventually develop therapy resistance. We generated HeyA8-MDR mice to explore the possibility of fighting taxane-resistant tumor by knocking down EphA2 expression. The HeyA8-MDR tumor cells overexpress drug efflux transporters (20) and have crossresistance to cisplatin, adriamycin, and many other chemotherapy drugs (17). As expected, no therapeutic efficacy was observed in mice treated with 50 μg docetaxel biweekly for 6 weeks (Fig. 6A). As with the SKOV3ip2 tumor mice, there was an 80% reduction of tumor weight in the HeyA8-MDR
mice treated with MSV/15 μg EphA2 siRNA compared with those in the control groups (docetaxel and MSV/Scr). Docetaxel and MSV/EphA2 combination treatment further reduced tumor weight but not the number of tumor nodules (Fig. 6A).

As expected, MSV/EphA2 treatment resulted in knockdown of EphA2 expression, and consequently reduced number of microvessels and increased cell apoptosis in HeyA8-MDR tumor (Fig. 6B and C). In contrast to the SKOV3ip2 tumors, treatment with the chemotherapy drug did not significantly alter EphA2 expression level (Fig. 6B). Docetaxel treatment reduced the number of microvessels (Fig. 6B and C); however, the result did not have any significant impact on overall tumor weight or the number of tumor nodules (Fig. 6A). Combination treatment reduced the number of microvessels by 80% and dramatically increased the number of apoptotic cells in tumor tissues (Fig. 6B and C).

**Discussion**

We have shown here the efficient multistage delivery of siRNA to tumor tissues. The delivery system allowed for sustained release of nanoliposomal siRNA from the primary uptake sites of MSV and subsequent siRNA accumulation into tumors. Treatment with MSV/EphA2 siRNA sensitized tumors to chemotherapy and overcame drug resistance in mice bearing tumors overexpressing multidrug-resistant genes.

Most newly diagnosed patients with ovarian cancer are treated with surgery and chemotherapy (21–23). Taxanes and cisplatin analogues are the first-line chemotherapeutic agents of choice for advanced ovarian cancer (22). Initial responsiveness to drug treatment is high; however, the majority of patients will relapse with resistant diseases. The poor patient survival is directly attributable to advanced and metastatic disease and the lack of effective treatment strategies. Drug resistance is the leading cause of therapy failure in the treatment of ovarian cancer (23). There are many potential mechanisms for resistance to chemotherapy treatment. Among them is P-glycoprotein–mediated efflux to reduce cellular accumulation of the drug (24). It has been suggested that the current therapy regimens only eliminate vulnerable cells, with the remaining cells eventually developing a drug-resistant phenotype, promoting tumor growth and metastasis. So there is an urgency in developing novel treatment strategies that will lead to better outcomes in patients with ovarian cancer. In this study, we showed effective siRNA delivery to tumor tissues with the multistage system and synergy between MSV/EphA2 siRNA and chemotherapy drugs in the treatment of ovarian cancer with both taxane-sensitive and -resistant tumor mouse models.

The use of siRNA is an attractive strategy for cancer treatment by targeting essential genes for cancer cell survival. This strategy can be applied to the vast majority of cancer targets that are either considered nontargetable by conventional drug development approaches or their small-molecule inhibitors are yet to be developed (25). However, application of siRNA therapeutics in clinic has proven to be challenging so far due to the lack of effective delivery vectors to overcome the multiple biologic barriers (9, 10). Our MSV delivery system serves as a bridge from bench to clinic. In comparison with the hemispherical silicon particles used in our previous study (8), the discoidal silicon particles used in this study have...
optimal hemodynamic properties in circulation and larger surface interaction area with the tumor vasculature, and consequently, improved tumor accumulation (12, 13). In a recent study, we have shown that the tumor accumulation of discoidal particles was dramatically improved over spherical particles of similar sizes (15). Interestingly, the 1,000 $\mu$g/C2400 nm discoidal particles had the best tumor accumulation when compared with bigger (1,800 $\mu$g/C2600 nm) or smaller (600 $\mu$g/C2200 nm) particles and exhibited the best tumor-to-liver ratio, as the smaller particles were more extensively uptaken in the liver and spleen (13). Toxicity evaluation revealed that discoidal MSV loaded with siRNA oligos did not trigger acute innate immune responses judged by changes in levels of 32 serum cytokines, chemokines, and colony-stimulating factors (26). Repetitive treatments with escalating dosages of MSV/siRNA did not cause subacute toxicities either including body weight changes, morphologic changes of major organs, blood chemistry on liver and kidney functions, and hematology (26). These studies have shown that discoidal silicon particles loaded with siRNA oligos in nanoliposomes are safe to use as cancer therapeutics.

We have also developed a protocol for large-scale fabrication of discoidal silicon particles. Instead of fabricating particles one layer at the time, we can now manufacture them in stacks of 10 to 20 layers at a time (Unpublished data), with enough flexibility to fabricate silicon particles with any size and porosity. This protocol can be directly translated to large-scale cyclic guanosine 3',5'-monophosphate (cGMP) production of MSV/siRNA. Thus, MSV/...
EphA2 is a novel approach to solid tumor treatment and it warrants further drug development.

Disclosure of Potential Conflicts of Interest
X. Liu has ownership interest (including patents) in Leonardo Biosystems Inc. J. Sakamoto is employed as a consultant, has ownership interest (including patents), and is a consultant/advisory board member of Leonardo Biosystems Inc. M. Ferrari is the founding scientist and a member of the Board of Directors of Leonardo Biosystems, a member of Board of Directors of Arrow Head Research Corporation, and hereby discloses potential financial interests in the companies. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Figure 6. Therapeutic efficacy of MSV/EphA2 in combination with docetaxel on HeyA8-MDR tumors. Nude mice were inoculated intraperitoneally with HeyA8-MDR cells and randomly allocated to 1 of 5 treatment groups (n = 10): (i) docetaxel (DTX), (ii) MSV loaded with nonsilencing scramble siRNA-DOPC (MSV/control, 15 μg), (iii) MSV loaded with 15 μg EphA2-siRNA-DOPC (MSV/EphA2, 15 μg), (iv) DTX and MSV/control combination (DTX + MSV/control), (v) DTX and MSV/EphA2 combination (DTX + MSV/EphA2). Mice were treated biweekly for 6 weeks. At the end of the treatment, all mice were sacrificed, and total tumor weight and the number of tumor nodules were measured. A, distribution of tumor weight and tumor nodules among the treatment groups. B, IHC staining for EphA2 expression, tumor microvessels by CD31, and cell apoptosis by TUNEL assay. C, quantitation of tumor microvessels and apoptotic cells. Five fields per slide and at least 5 slides per group (all from different animals) were counted. **, P < 0.01; ***, P < 0.001.
Multistage Vector Delivery of EphA2 siRNA

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