Targeting Melanoma Growth and Metastasis with Systemic Delivery of Liposome-Incorporated Protease-Activated Receptor-1 Small Interfering RNA

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Abstract

The thrombin receptor [protease-activated receptor-1 (PAR-1)] is overexpressed in highly metastatic melanoma cell lines and in patients with metastatic lesions. Activation of PAR-1 leads to cell signaling and up-regulation of genes involved in adhesion, invasion, and angiogenesis. Herein, we stably silence PAR-1 through the use of lentiviral short hairpin RNA and found significant decreases in both tumor growth (P = 0.01) and metastasis (P < 0.001) of highly metastatic melanoma cell lines in vivo. The use of viruses for therapy is not ideal as it can induce toxic immune responses and possible gene alterations following viral integration. Therefore, we also used systemic delivery of PAR-1 small interfering RNA (siRNA) incorporated into neutral liposomes [1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC)] to decrease melanoma growth and metastasis in vivo. Significant decreases in tumor growth, weight, and metastatic lung colonies (P < 0.001 for all) were found in mice treated with PAR-1 siRNA-DOPC. The in vivo effects of PAR-1 on invasion and angiogenesis were analyzed via immunohistochemistry. Concomitant decreases in vascular endothelial growth factor, interleukin-8, and matrix metalloproteinase-2 expression levels, as well as decreased blood vessel density (CD31), were found in tumor samples from PAR-1 siRNA-treated mice, suggesting that PAR-1 is a regulator of melanoma cell growth and metastasis by affecting angiogenic and invasive factors. We propose that siRNA incorporated into DOPC nanoparticles could be delivered systemically and used as a new modality for melanoma treatment. [Cancer Res 2008;68(21):9078–86]

Introduction

The thrombin receptor is a seven-pass transmembrane G protein–coupled receptor. Unlike typical ligand-receptor interactions, thrombin does not activate protease-activated receptor-1 (PAR-1) on NH2-terminal binding. Rather, it cleaves the NH2-terminal peptide to initiate cellular signaling (1).

The pathway of cellular activation and induction of mitogenesis by thrombin involves increases in Ca2+ and activation of protein kinase C via the second messengers, inositol 1,4,5-triphosphate and diacylglycerol (2). Activation of PAR-1 can lead to cell signaling and up-regulation of genes involved in adhesion (α5β1, α6β1, and αvβ3 integrins; refs. 3–5), invasion [matrix metalloproteinase (MMP)-2; ref. 6], and angiogenesis [interleukin-8 (II-8) and vascular endothelial growth factor (VEGF); refs. 7–10]. This suggests that activation of PAR-1 may facilitate tumor invasion and metastasis through induction of cell adhesion molecules, matrix-degrading proteases, and stimulating the secretion of angiogenic factors, thus contributing to the metastatic phenotype of melanoma.

PAR-1 can also be activated by ligands other than thrombin, such as factor Xa, granzyme A, trypsin, and plasmin (11–13). Furthermore, studies suggest that PAR-1 in breast cancer cells can be activated by MMP-1 (14). PAR-1 was also described as a rate-limiting factor in thrombin-enhanced experimental pulmonary metastasis, attesting to the role of the thrombin receptor in melanoma metastasis (15). In addition to melanoma, overexpression of PAR-1 has been identified in various cancers, including breast (16, 17), lung (18), colon (19–21), and prostate (22, 23).

Our laboratory has previously shown that PAR-1 is differentially expressed in melanoma cell lines with overexpression being found in highly metastatic cells compared with nonmetastatic cell lines (22, 24). Overexpression of PAR-1 is predominantly seen in patients with malignant melanoma and in metastatic lesions compared with common melanocytic nevi and normal skin (25). Furthermore, our laboratory has found a significantly higher percentage of PAR-1–positive cells in metastatic melanoma than in both dysplastic nevi and primary melanoma (26). We also reported that the expression of PAR-1 in melanoma cells is regulated by the activator protein-2α (AP-2α) transcription factor and showed an inverse correlation between the expression of AP-2α and PAR-1 in metastatic melanoma specimens (24, 26). Herein, we hypothesize that PAR-1 is a major contributor to the metastatic process of human melanoma. Therefore, we sought to silence PAR-1 in vivo by stably transfecting cells using lentiviral-based short hairpin RNA (shRNA) as well as using neutral liposomes to deliver PAR-1 small interfering RNA (siRNA) systemically to decrease melanoma growth and metastasis.

Although lentiviral technology is a proven tool in the laboratory setting, the use of viruses for treatment has several adverse effects, such as toxic immune responses and genetic alterations. Therefore, in the present study, we also used systemic delivery of liposome-incorporated siRNA, which is a much safer alternative to viral technology for the treatment of melanoma.

Recently, siRNA incorporated into neutral 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) liposomes has been used effectively in vivo (27–29). Evidence from these experiments...
suggests that there was higher tumor uptake of DOPC liposomes compared with cationic liposomes and that neutral liposomes were less toxic. In fact, it is expected that liposomal packaging of siRNA will work best in solid tumors, such as melanoma, as there is increased vessel permeability and reduced lymphatic drainage, thereby allowing for increased accumulation of siRNA-incorporated liposomes (30). Here, we show for the first time that systemic treatment of melanoma-bearing mice with PAR-1 siRNA-DOPC resulted in inhibition of melanoma growth and metastasis.

Materials and Methods

Cell lines and culture conditions. A375SM human melanoma cell line was established from pooled lung metastases produced by A375-P cells injected i.v. into nude mice (31). These cells were maintained in Eagle's MEM supplemented with 10% fetal bovine serum (FBS), as previously described (32). The C8161 is an aggressive amelanotic human melanoma cell line maintained in DMEM-F12 supplemented with 5% FBS, as previously described (33). The 293FT cells (Invitrogen), used to make lentiviral shRNA, are a clonal isolate derived from transformed embryonal kidney cells containing the large T antigen for high-level expression of the packaging proteins, thereby resulting in higher viral titers. These cells were maintained in DMEM supplemented with 10% FBS, according to the manufacturers' instructions.

Transient transfection of siRNA. PAR-1 siRNA purchased from Dharmacon was used to silence PAR-1 expression in melanoma cell lines (target sequence: AAGAUAGCUCCAUCAUAU). Nontargeting (NT) siRNA (Qiagen), with no sequence homology to any known human mRNA, was used as control (target sequence: UUCCUGAACGUGUCCGU). A375SM cells were grown to 60% confluency in six-well plates and transiently transfected with PAR-1 siRNA or NT siRNA using RNAiFect Reagent (Qiagen) according to the manufacturers’ instructions. The cells were analyzed for PAR-1 expression 72 h after transfection.

Western blot analysis. PAR-1 was detected in cell extracts by Western blot, as previously described (22). Briefly, proteins of total cell extracts (40 μg) were separated by SDS-PAGE and transferred to Immobilon-P transfer membrane (Millipore Corp.). The membranes were washed in Tris-HCl–buffered saline [10 mmol/L Tris-HCl (pH 8) containing 150 mmol/L NaCl] and blocked with 5% nonfat milk in TBS for 1 h at room temperature. The blots were then probed overnight in Tris-HCl–buffered saline with the monoclonal antibody for PAR-1 (WDE15, Immunotech Coulter) at a dilution of 1:2,000. α-Actin antibody (Sigma) was used as a loading control.

Semiquantitative reverse transcriptase-PCR. One microgram of total RNA was reverse primed with an oligo(dT) primer and extended with Moloney murine leukemia virus reverse transcriptase (Clontech). The PCR was performed, using the Clontech Advantage cDNA PCR kit, in a 50 μL reaction mixture containing 1× PCR buffer, 5 μL cDNA, 0.2 mmol/L deoxynucleotide triphosphate, and 2.5 units of Taq polymerase. For PAR-1 quantification, specific primers (5'-GCAGACCCGGGACATGGGG-3' and 5'-AGATGCGGACAACTGG-3') were used. Glyceraldehyde-3-phosphate dehydrogenase cDNAs were amplified by PCR in the same reaction mixture and carried out by an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1 min. A final elongation step was carried out at 72°C for 10 min.

Lentiviral shRNA to PAR-1. Sense and antisense oligonucleotides from the PAR-1 siRNA target sequence and the NT sequence were designed with a hairpin and sticky ends (Clai and MluI) for use with the lentiviral system developed and kindly provided by Didier Trono (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland; ref. 34). The oligonucleotides were annealed into the lentiviral gene transfer vector, pLVTHM, using the Clai and MluI restriction enzyme sites. Competent Escherichia coli bacteria were transformed with the annealed lentiviral vector and grown overnight. Several bacterial colonies were isolated and grown in Luria broth overnight. DNA was isolated from the bacteria using a Maxi plasmid DNA purification kit (Qiagen). The DNA was sequenced to test for proper insertion and length of the inserts. Lentivirus was then produced by transfecting human embryonic kidney cells (293FT; Invitrogen) with the sequence-verified pLVTHM vector containing the PAR-1 shRNA sequence, the packaging plasmid (MD2G), and envelope plasmid (PA2X) required for viral production. Three days later, the viral supernatant was collected and filtered to remove cellular debris. The highly metastatic and PAR-1–positive A375SM and C8161 cell lines plated at 70% confluency in six-well plates were transduced with the virus. After 16 h, the virus-containing medium was removed and replaced with normal growth medium.

Fluorescence-activated cell sorting analysis. These data were obtained in a BD FACSaria flow cytometer (BD Biosciences). A375SM and C8161 cells transduced with lentiviral constructs were washed with PBS and detached with 1% EDTA. Cells were centrifuged at 1,000 rpm, the supernatant was removed, and cells were incubated on a rotating shaker with PAR-1 antibody (ATAP2; Santa Cruz Biotechnology) at 1:50 dilution in PBS with 2% FBS (PBS-FBS) for 45 min at 4°C. Cells were washed with PBS-FBS, centrifuged, and incubated on a rotating shaker with a phycoerythrin (PE)–conjugated secondary antibody (PE anti-mouse; Jackson Immunoresearch) at a 1:100 dilution in PBS-FBS for 30 min at 4°C. Cells were washed thrice and analyzed.

In vivo experiments. Female athymic nude mice (Ncr-nu) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center. All studies were approved and supervised by The University of Texas M. D. Anderson Cancer Center Institutional Animal Care and Use Committee. For experiments using stably transfected cells with PAR-1 or NT shRNA, 5 × 105 transduced cells were injected s.c. into the right flank of mice (n = 10 per group) to analyze tumor growth. Tumors were measured twice weekly in two dimensions with a caliper and mice were sacrificed after 6 wk. Tumor volumes for all tumor growth experiments were calculated using the formula \( V = a \times b^2 \), as described previously (35), where \( a \) is the largest diameter and \( b \) is the smallest. To analyze for metastatic potential of lentiviral transduced cells, 1 × 105 PAR-1 or NT shRNA-transduced cells were injected i.v. into the tail vein of mice (n = 10 per group). The mice were sacrificed after 6 wk, at which time the lungs were fixed in Bouin’s solution and the metastatic colonies were counted as we previously described (36). To determine whether siRNA delivered by neutral liposomes could reach the s.c. tumors, a NT siRNA sequence was tagged with Alexa Fluor 555 and incorporated into DOPC liposomes as described previously (28). A375SM cells were implanted s.c. into mice. After the tumors reached a size of 5 mm3, the mice were administered a single injection of 5 μg of Alexa Fluor 555 siRNA incorporated in DOPC liposomes into the tail vein. Mice (n = 5 per time point) were sacrificed 2, 4, and 6 d after injection of siRNA. To determine the amount and frequency of injections of PAR-1 siRNA-DOPC to silence PAR-1 in vivo, mice were s.c. injected with A375SM cells, and after the tumors grew to a size of 5 mm3, a single administration of PAR-1 siRNA-DOPC (5 or 10 μg) or NT siRNA-DOPC (5 or 10 μg) was administered into the tail vein of nude mice. Five mice per day were then sacrificed at 2, 4, and 6 d after treatment for each treatment group. To determine the effects of liposome-delivered PAR-1 siRNA on tumor growth, 1 × 106 A375SM cells were injected s.c. into nude mice (n = 10 per group). After the tumors reached a size of 3 to 5 mm3, mice were randomized into the two treatment groups and administered 10 μg of PAR-1 siRNA-DOPC or NT siRNA-DOPC into the tail vein. Systemic administration of DOPC-incorporated siRNA was performed twice weekly for 4 wk. Mice were measured in two dimensions with a caliper twice weekly. Mice were sacrificed after 4 wk, at which time s.c. tumors were harvested. To determine metastatic potential, 1 × 105 A375SM cells were injected into the tail vein of nude mice (n = 10 per group). As with the tumor growth experiment, mice were randomized into two treatment groups, and after 7 d, treatments began with 10 μg of either NT or PAR-1 siRNA-DOPC. After 4 wk of biweekly treatments, the lungs were fixed in Bouin’s solution and metastatic colonies were counted.

Real-time PCR. RNA (20 ng/μL) from s.c. tumors was harvested using RNeaQueous kit (Ambion) according to the manufacturers’ instructions. The RNA was then made into cDNA using Taqman reverse transcriptase reagents (Applied Biosystems). The primers and fluorescence probes were obtained from Applied Biosystems (PAR-1: Hs00189298) specific for human melanoma.
PAR-1. Reaction components for reverse transcription-PCR (RT-PCR) included Taqman Universal PCR Master Mix (Applied Biosystems), 20× Assay Mix (Applied Biosystems), RNase-free water, and the diluted cDNA (1:5). The amplifications were carried out in an Applied Biosystems 7700 Prism RT-PCR device using the following temperature profile: denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min for 40 cycles. Amplifications were run in triplicates and averages were obtained after normalization with 18S rRNA (Applied Biosystems).

**Immunohistochemistry and immunofluorescence.** Formalin-fixed, paraffin-embedded sections were deparaffinized by sequential washing with xylene, graded ethanol, and PBS. Antigen retrieval was done by heating in steam cooker in 1× Target Retrieval Solution (Dako) for 30 min. After cooling and washing with PBS, endogenous peroxide was blocked with 3% hydrogen peroxide inhibitor in PBS for 12 min. Nonspecific proteins were blocked in 5% horse serum and 1% goat serum for 20 min. Slides were incubated with VEGF (1:200; Santa Cruz Biotechnology), IL-8 (1:25; Biosource International), and MMP-2 (1:400; Chemicon) antibodies overnight at 4°C followed by a peroxidase-labeled anti-rabbit antibody (1:500; Jackson ImmunoResearch) for 1 h at room temperature. Signal was detected with 3,3’-diaminobenzidine (DAB; Phoenix Biotechnologies) substrate for 6 min and counterstained with Gill's no. 3 hematoxylin (Sigma) for 20 s. Immunohistochemistry for PAR-1 (1:20; Immunotech Coulter) also included an overnight blocking step with an anti-mouse IgG antibody (1:10; Jackson ImmunoResearch) before incubating with primary antibody overnight. For CD31 staining, frozen sections were fixed with cold acetone each for 5 min. Nonspecific proteins were blocked in 5% horse serum and 1% goat serum for 20 min. Overnight incubation at 4°C with anti-mouse CD31 antibody (1:800; PharMingen) was performed followed by a 1-h incubation with peroxidase-labeled anti-rabbit antibody (1:200; Jackson ImmunoResearch) incubation at room temperature. Signal was detected with DAB substrate for 5 min and counterstained with Gill's no. 3 hematoxylin for 20 s. For detection of scavenging macrophages, frozen slides were fixed and nonspecific proteins were blocked as per CD31 protocol. Overnight incubation with anti-mouse F4/80 (1:600; Serotec) at 4°C (with no exposure to light as the slides had fluorescence from the Alexa Fluor 555–tagged siRNA) was followed by anti-rat Alexa Fluor 488 antibody (1:1,000; Molecular Probes) for 1 h at room temperature. After washing, Hoechst (1:10,000; Molecular Probes) was added for visualization of cell nuclei.

**Statistics.** Statistical analysis on animal studies was performed using the Mann-Whitney tests. All statistical tests were two sided.

**Results**

**Development of PAR-1 shRNA.** We have previously shown that metastatic melanoma cells express high levels of PAR-1 compared with nonmetastatic melanoma cells (24). PAR-1 siRNA constructs targeting the human PAR-1 gene were purchased from Dharmacon and tested to determine effective gene silencing. A NT construct with no homology to any known human gene was also used as a negative control (28). We observed down-regulation of both mRNA and protein levels of PAR-1 by ~80%, 72 hours after transfection of one of the PAR-1 siRNA constructs (Fig. 1A and B).

After obtaining a functional PAR-1 siRNA construct, the targeting sequence was used to develop lentiviral shRNA to stably silence PAR-1 using the system developed and kindly provided by Didier Trono (34). Fluorescence-activated cell sorting analysis was used after transduction of the two most highly metastatic and aggressive melanoma cell lines we have available (A375SM and C8161). Cells transduced with PAR-1 shRNA are green fluorescent protein (GFP) positive and PE negative (after using the PAR-1 antibody and a second PE-tagged secondary antibody), whereas those transduced with a NT sequence control are GFP positive and PE positive. In both A375SM (Fig. 1C) and C8161 (Fig. 1D) metastatic melanoma cells, there is almost complete silencing of PAR-1 after transduction with lentiviral PAR-1 shRNA. Next, we sought to

![Figure 1. Silencing of PAR-1 by siRNA and shRNA. A. Western blot depicting silencing of PAR-1 in A375SM transfected with PAR-1 siRNA compared with parental and nontargeting control. α-Actin is used as loading control. Approximately 80% PAR-1 down-regulation was observed in PAR-1 siRNA-transfected cells compared with NT siRNA as determined by densitometry (0.2 and 1, respectively). B. Semiquantitative RT-PCR depicting a decrease of PAR-1 (by ~90%) in A375SM, A375SM (C) and C8161 (D) cells were transduced with lentivirus containing either NT siRNA or PAR-1 shRNA. The lentiviral vectors have GFP expression, and therefore, transduced cells will have green fluorescence. Cells were incubated with a PAR-1 antibody and a secondary PE-tagged antibody (red fluorescence). Top, cells transduced with NT shRNA showing GFP positive/PE positive (presence of PAR-1); bottom, cells transduced with PAR-1 shRNA showing GFP positive/PE negative (decreased PAR-1 expression).](https://cancerres.aacrjournals.org)
determine tumorigenicity and metastatic potential of these cells with silenced PAR-1 expression in an in vivo setting.

**Tumor growth and metastatic potential of PAR-1 shRNA-transduced melanoma cells.** To test the effect of PAR-1 on tumor growth, stably transduced highly metastatic cells were injected s.c. into nude mice (n = 10). Figure 2A shows significant inhibition of tumor growth in A375SM cells stably transduced with PAR-1 shRNA compared with NT shRNA-transduced cells (41.1 and 534.3 mm³, respectively; *P < 0.01). This difference was also observed in C8161 cells transduced with PAR-1 shRNA compared with NT shRNA-transduced cells (median, 159.7 and 813.5 mm³, respectively; *P < 0.01). To ascertain that the changes seen in tumor growth were not due to differences in cell proliferation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assays were performed on both cell lines transduced with PAR-1 or NT shRNA in vitro. No difference in proliferation rates was observed (data not shown).

We sought to determine if silencing of PAR-1 would also limit experimental lung metastasis of these transduced cell lines. Nude mice (n = 10) were i.v. injected with A375SM or C8161 cells transduced with either PAR-1 or NT shRNA. As with tumor growth, there was also significant inhibition of lung metastasis in both A375SM (median: NT shRNA, 37; PAR-1 shRNA, 1; *P < 0.001) and C8161 (median: NT shRNA, 70; PAR-1 shRNA, 6; *P < 0.001) 6 weeks after the challenge. Taken together, we herein present data for the first time that silencing of PAR-1 using shRNA results in inhibition of tumor growth and metastatic potential of human melanoma cells, thereby strengthening the hypothesis that PAR-1 is a major contributor to the acquisition of the metastatic phenotype of human melanoma.

**Systemic delivery of PAR-1 siRNA using neutral liposomes.**

Next, we determined whether delivery of PAR-1 siRNA was feasible with the use of neutral liposomes in our experimental melanoma mouse model. As a proof of principle, we chose the most metastatic melanoma cell line, A375SM, to continue our in vivo therapeutic experiments. Using liposomes to deliver therapeutic agents is safer than using viruses. Recent evidence shows the usefulness of neutral liposomes in delivering siRNA to i.p. tumors in vivo in various cancers, such as ovarian, colorectal carcinomas, and lymphomas (27–29, 37, 38). By using this liposomal method to package PAR-1–specific siRNAs, we sought to achieve similar results in human melanoma. However, it has not been previously established if i.v. delivery of DOPC liposomes is able to effectively reach s.c. tumors. Therefore, we sought first to determine if i.v. injection of siRNA tagged with Alexa Fluor 555 incorporated in DOPC liposomes could reach s.c. tumors. Results indicate that DOPC-siRNAs were reaching the s.c. tumors at day 2 (Fig. 3A). Even at day 6 after a single i.v. injection, there were siRNA particles (red) in a perinuclear orientation within the s.c. tumor cells (blue Hoechst nuclear staining) almost imperceptible to scavenging macrophages (green; Fig. 3B) as observed previously in studies with i.p. delivery of DOPC-siRNA (28). We concluded that siRNAs will be able to...
reach s.c. tumors effectively by systemic administration of DOPC-incorporated siRNA.

Next, a kinetics experiment was performed to determine the amount of siRNA incorporated into DOPC liposomes that needs to be delivered for effective silencing of PAR-1 in vivo. Based on this kinetics experiments, we found that down-regulation of PAR-1, as measured by real-time PCR, peaked 4 days after liposomal injection but recovered by day 6 (Fig. 3B). Therefore, we concluded that the PAR-1 siRNA-DOPC should be administrated twice weekly at a dosage of 10 μg per treatment.

Then, we performed the first in vivo experiments using PAR-1 siRNA-DOPC to inhibit both tumor growth and metastasis of A375SM melanoma cells in nude mice. Mice (n = 10) injected s.c. with A375SM cells were given twice weekly i.v. injections of 10 μg of PAR-1 siRNA-DOPC or NT siRNA-DOPC after tumors grew to a size of 3 to 5 mm³. As can be seen in Fig. 4, there is significant inhibition of both tumor growth (NT siRNA-DOPC, 749.9 mm³; PAR-1 siRNA-DOPC, 176.2 mm³; P < 0.001; Fig. 4A) and tumor weight (median NT siRNA-DOPC, 614 mg; PAR-1 siRNA-DOPC, 106.5 mg; P < 0.001; Fig. 4B). These tumors were analyzed for expression of PAR-1 via immunohistochemistry and quantitative real-time PCR and found that indeed, after treatment with liposome-incorporated PAR-1 siRNA, there was a decrease in PAR-1 expression at the end of treatment (day 27; Fig. 4C and D). These results show that in vivo PAR-1 expression was reduced in PAR-1 siRNA-DOPC–treated tumors 4 weeks after twice weekly i.v. treatments.

Not only was there a significant inhibition of tumor growth with siRNA delivered via neutral liposomes but there was also a robust and significant inhibition in the ability of these cells to form metastatic lung colonies. Figure 5 shows the effects of PAR-1 siRNA treatment on lung metastasis, as mice treated with NT siRNA had a median of 62 lung colonies, whereas PAR-1 siRNA-treated mice had a median of only 12 lung colonies (P < 0.001). There were no obvious toxicities observed as assessed by mobility and feeding behavior in any of the mice used for systemic liposomal delivery of PAR-1 siRNA. Taken together, we now present data that administration of PAR-1 siRNA delivered systemically via neutral liposomes is feasible for in vivo treatment of melanoma.

**Effects of PAR-1 siRNA-DOPC therapy.** To further analyze the effects of PAR-1 silencing on melanoma growth and metastasis, we performed immunohistochemical analysis on tumor samples to determine the expression levels of several invasive and angiogenic factors (MMP-2, IL-8, and VEGF) known to be up-regulated with the activation of PAR-1 (6, 7, 9). There is decreased expression of MMP-2, IL-8, and VEGF after treatment with PAR-1 siRNA-DOPC compared with NT-treated mice (Fig. 6), attesting to the important role PAR-1 plays in the progression of melanoma. Because these factors are major players in angiogenesis, we wanted to determine if there was an overall decrease in angiogenesis with PAR-1 siRNA-DOPC treatment. Indeed, there is an overall decrease in blood vessels (CD31) in PAR-1 siRNA-treated mice (Fig. 6), allowing us to conclude that PAR-1 siRNA affects tumor angiogenesis.

Our results show that silencing of PAR-1 in vivo by shRNA or by systemic treatment of liposome-incorporated PAR-1 siRNA reduces melanoma growth and metastasis. We further show that silencing of PAR-1 expression reduces angiogenesis (IL-8 and VEGF) and invasive (MMPs) factors involved in tumor progression.

**Discussion**

We have previously reported that the loss of transcription factor AP-2α leads to the progression of metastatic melanoma (39–41). Furthermore, there exists an inverse correlation between AP-2α and PAR-1 with an increase in PAR-1 expression in highly metastatic melanoma cells and in melanoma patient samples.
Thrombin is a potent inducer of angiogenesis via PAR-1 activation. PAR-1 is a regulator of several genes and molecules involved in tumor growth and metastatic progression, such as VEGF, IL-8, and MMPs (6–10). Thus, PAR-1 is critical in the transition from radial growth phase to the vertical growth phase of human melanoma. In this study, we sought to determine whether PAR-1 silencing could reduce melanoma growth and metastasis via shRNA and via systemic liposomal delivery of PAR-1 siRNA.

PAR-1 was stably silenced using a lentiviral construct that allowed for the use of transduced cells in long-term in vivo experiments to determine the effects of PAR-1 in melanoma. After injecting PAR-1 shRNA-transduced melanoma cells, both s.c. and i.v., we determined that PAR-1 expression in highly metastatic melanoma leads to increased experimental tumor growth and lung metastasis. Transduction of cells with PAR-1 shRNA was able to reduce these effects in vivo.

After determining that PAR-1 contributes to the acquisition of the metastatic phenotype, we used a liposomal system that allowed delivery of siRNA as a possible therapeutic modality for melanoma treatment. Lentiviral technology works well in the laboratory setting for stable silencing of genes. The use of viruses in clinical applications, however, is hindered by their induction of toxic immune responses and the possibility of gene expression changes following random integration into the host genome (42). Therefore, siRNA has the potential to become a safer and effective therapeutic alternative to viral therapy. However, the major limitations of siRNA systemic administration include degradation, poor bioavailability, and intracellular delivery. In fact, one of the main challenges that scientists have come on in using siRNAs for human therapeutics has been the development of suitable delivery agents (28, 43, 44). Potential carriers, such as cationic liposomes, have numerous limitations, such as toxicity, rapid elimination from blood, entrapment by the reticuloendothelial system, association with negatively charged serum proteins, colloidal instability, and failure to release incorporated materials (43). Furthermore, macrophages seem to preferentially take up charged liposomes (28, 45). Although addition of ligands to the cationic liposomes or inclusion of packaging elements within these liposomes has improved delivery and more specific targeting of the siRNA, undesired immune reactions have been reported (44).

In the present study, we used a more effective and less toxic neutral liposome that has previously been used effectively to deliver siRNA for the treatment of lymphoma and ovarian cancer as well as colorectal carcinoma (27–29). Liposomes, the first nanotechnology to benefit cancer patients, are continuing to evolve to deliver potentially useful therapies to tumors. Therefore, we wanted to determine whether systemic delivery of PAR-1 siRNA incorporated into DOPC liposomes was feasible and
whether this treatment could successfully affect melanoma growth and metastasis.

Our first challenge was to determine if systemic delivery of these neutral liposomes would reach the s.c. tumors as direct intratumor injections would cause an acute inflammatory response and alter the tumor microenvironment, thereby affecting the results. Furthermore, the use of DOPC liposomes had only been used effectively in vivo for i.p. tumors administered into the peritoneal cavity. After i.v. injecting a single dose of DOPC-incorporated Alexa Fluor 555–tagged siRNA, we determined that the siRNA was found within the s.c. tumor cells almost imperceptible to scavenging macrophages. This shows that delivery of siRNA would not be sequestered by macrophages and that sufficient levels of siRNA could reach the target tissues. Previously, Landen and colleagues (28) used these DOPC liposomes to treat ovarian cancer. They observed that the siRNA delivery in DOPC was not restricted to the vasculature and was efficiently delivered deep into the tumor parenchyma. Furthermore, they found that delivery of siRNA with DOPC liposomes did reach other organs, such as liver, lungs, and kidneys. However, they saw robust silencing of their target gene in i.p. tumors. These observations as well as our preliminary studies allowed us to conclude that using these neutral liposomes systemically to deliver siRNA was feasible.

We determined that twice weekly injections of 10 μg siRNA would be sufficient to silence PAR-1 in vivo. Indeed, the results of this experiment were dramatic as decreases in both melanoma growth and tumor weight were seen in mice treated with PAR-1 siRNA-DOPC compared with the NT control. These tumors were harvested and assayed for PAR-1 expression via immunohistochemistry and quantitative real-time PCR. Both assays showed a significant decrease in PAR-1 expression following treatment,

**Figure 5.** Inhibition of lung metastases by melanoma cells after twice weekly systemic administration of PAR-1 siRNA-DOPC. Nude mice were i.v. injected with $1 \times 10^6$ A375SM. Mice were subsequently treated i.v. with 10 μg of either liposome-incorporated PAR-1 siRNA or NT siRNA twice weekly for 5 wk. Lungs were harvested and fixed in Bouin’s solution, and individual tumor nodules were counted. There is a significant decrease in the number of lung colonies in PAR-1 siRNA–treated mice (median, 12) compared with those treated with NT siRNA (median, 62). One mouse died in the NT group 2 wk after treatment began. **, $P < 0.001$.

**Figure 6.** Effects of PAR-1 silencing on angiogenic and invasive factors. Immunohistochemical analysis was performed on tumors from systemically treated mice with PAR-1 siRNA-DOPC or NT siRNA-DOPC. Representative images show that silencing of PAR-1 affects angiogenic (IL-8 and VEGF) and invasive (MMP-2) factors. There is also a reduction in the number of blood vessels (CD31; microvessel density) in tumors treated with PAR-1 siRNA. As a negative control, the tumor samples were incubated without primary antibody. All images are at ×10 magnification.
which shows that the liposome-delivered PAR-1 siRNA reached its target and effectively decreased the expression levels of PAR-1 in vivo. Furthermore, experimental lung metastasis was also significantly decreased in PAR-1 siRNA-DOPC–treated mice, again attesting to the functionality of delivering siRNA in vivo with these neutral liposomes. The strong link between PAR-1 activation and angiogenesis led us to investigate whether silencing of PAR-1 would affect factors involved in angiogenesis, such as IL-8 and VEGF, as well as factors involved in invasion (MMPs). It was likely that the effects seen in vivo after PAR-1 silencing could be explained by decreases in angiogenesis and invasion, which minimized tumor growth and metastasis. Thus, we analyzed the tumors obtained from our in vivo studies by immunohistochemistry for MMP-2, IL-8, and VEGF expression and observed a decrease in all of these angiogenic factors in PAR-1 siRNA-DOPC–treated mice. Moreover, staining with CD31 clearly showed a reduction in the number of blood vessels within the tumors and, thus, inhibition of angiogenesis in the PAR-1 siRNA-DOPC–treated mice.

Taken together, we show that PAR-1 is a regulator of melanoma cell growth and metastasis. Furthermore, we show that delivery of PAR-1 siRNA via neutral liposomes in vivo is feasible and were able to reduce melanoma growth and metastasis by decreasing the expression of genes involved in angiogenesis and invasion. This further establishes the link between PAR-1 and the progression of melanoma. Our future experiments will focus on determining novel downstream genes affected by PAR-1 silencing in human melanoma. These might lead to genes that could be further targeted by siRNA-DOPC therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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