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Paclitaxel Tumor-Priming Enhances siRNA Delivery and Transfection in 3-Dimensional Tumor Cultures

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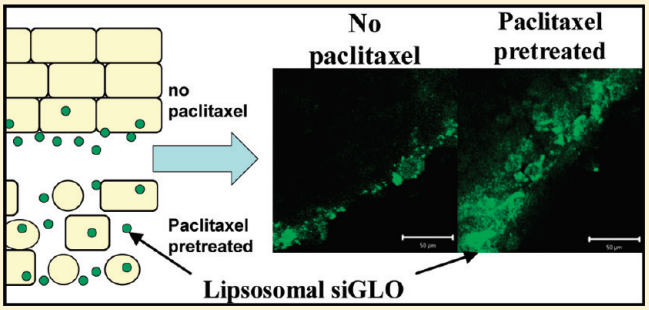
ABSTRACT: The clinical development of siRNA cancer therapeutics is limited by the poor interstitial transport and inefficient transfection in solid tumors. We have shown that paclitaxel pretreatment, by inducing apoptosis, causes expansion of the interstitial space and thereby improves nanoparticle delivery and transport in tumor interstitium (referred to as paclitaxel tumor priming) and efficacy of nanomedicines in tumor-bearing animals. The present study evaluated whether paclitaxel tumor priming improves the delivery and transfection of siRNA in 2- and 3-dimensional cultures of human oropharyngeal carcinoma FaDu cells. We used the fluorescent siGLO and confocal microcopy to monitor transport, and used survivin siRNA and immunostaining and immunoblotting to monitor transfection. Survivin is a chemoresistance gene/protein, inducible by chemotherapy. siRNA was loaded in cationic liposomes. The results showed that pretreatment with 50−200 nM paclitaxel (24 or 48 h before siRNA) enhanced the total uptake of siGLO into monolayers (∼15%, $p < 0.05$), and the depth of penetration into 3-dimensional spheroids and tumor fragment histocultures (2.1- to 2.5-times greater area under the penetration-depth curve). In both monolayer cells and histocultures, paclitaxel pretreatment induced survivin upregulation ($p < 0.05$). Survivin siRNA alone decreased the survivin levels in a dose-dependent manner, and applying survivin siRNA after paclitaxel pretreatment completely abolished the paclitaxel-induced survivin increases. These findings indicate that paclitaxel tumor priming did not compromise the siRNA functionality. In summary, paclitaxel tumor priming improved the penetration, transfection and functionality of siRNA in tumors, thus offering a promising and practical means to develop chemo-siRNA cancer gene therapy.



KEYWORDS: small interfering RNA, solid tumors, gene delivery, paclitaxel, survivin

INTRODUCTION

Small interfering RNAs (siRNAs) are short stretch (21−27 nucleotides) double-stranded RNA that degrade the complementary mRNA. siRNA is a naturally occurring endogenous regulatory process where dsRNA causes sequence-specific post-transcriptional gene silencing through induction of mRNA degradation.[1,2] siRNA represents a promising approach for producing gene-specific inhibition and knockdowns, manipulating tumor biology, interrogating consequences of genetic alterations, and designing new therapeutics. Due to their high potency, good selectivity and ease of construction, there has been a rapidly growing interest in their potential therapeutic applications, particularly for treating cancer through correcting faulty gene expression and functions.[3,4] There have been some successful applications of siRNA in cell line and animal studies, and the first proof-of-concept that siRNA can produce protein knockdown in humans was achieved with cyclodextrin-based polymeric nanoparticles.[5−9] Nonetheless, it is generally recognized that the major impediment to the research and use of siRNA therapeutics is inadequate delivery and transfection *in vivo*.

Over 85% of malignant diseases are solid tumors.[10] After intravenous administration, drug delivery to a solid tumor involves several processes including transport within a vessel, extravasation across the vessel wall into surrounding tissues, and transport through the interstitial space. Extravasation and interstitial transport (via diffusion and convection) are retarded by high interstitial pressure, high tumor cell density, hypovascularity, and/or a large fraction of stroma; these problems are more serious in larger, bulky tumors.[11] One approach to achieve adequate siRNA delivery in animals is hydrodynamic delivery, which involves using pressurized injection to disrupt the vascular and tumor tissue integrity.[12] This approach is associated with high animal morbidity and is not suitable for clinical use. A second major problem is the inability of siRNA to enter cells due to their unfavorable physicochemical properties (negative charges, large molecule weight and size).[13] This problem is partially overcome by using nanoscaled carriers such as cationic liposomes, but the larger size of carriers diminishes the interstitial transport, which may offset the benefits of using such carriers (see reviews[14,15]).

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Our group has recently developed the tumor-priming concept for enhancing the *in vivo* delivery of nanomedicines to solid tumors. Tumor priming uses apoptosis-inducing agents such as paclitaxel and doxorubicin to reduce the tumor cell density and expand the interstitial space, thereby enhancing blood perfusion, extravasation and interstitial transport of nanomedicines.[16,17] The time window for effective tumor priming is between 24 and 96 h when maximal expansion of interstitial space is attained. Our earlier studies using *in vitro* 3-dimensional models such as histocultures of xenografts and human patient tumors and *in vivo* models (tumor-bearing animals) have demonstrated tumor priming significantly improved the delivery and transport of protein-bound drugs and particulate systems including 200 nm latex beads and liposomal doxorubicin into solid tumors, in a tumor-selective manner such that tumor priming enhanced the efficacy of nanomedicines without enhancing the host toxicity.[17,18] Tumor priming therefore offers an opportunity to deliver siRNA cancer therapeutics. On the other hand, an earlier study showed that paclitaxel suppresses the endocytosis of liposomes in noncancer cell lines,[19] which may offset the benefits of greater delivery.

The present study evaluated the net effect of paclitaxel tumor priming on the delivery, transport, transfection and activity of siRNA. The experiments were conducted using monolayer cultures and 3-dimensional cultures (multicellular spheroids and tumor histocultures). We used the fluorescent siGLO and confocal microcopy to monitor spatial transport. The efficacy of transfection was monitored using survivin siRNA; the level of post-treatment survivin protein was measured by immunostaining or Western blot analysis. Survivin was selected as the target gene for the following reasons.[20] First, survivin is selectively expressed in elevated levels in a majority of human cancers compared with the undetectable expression in most differentiated adult normal tissues including liver.[20] Second, high expression of survivin correlates with chemo-/radio-resistance in multiple tumor types and with tumor progression and poor prognosis.[21,22] Third, inhibition of survivin by siRNA enhances the cell death induced by chemotherapy or irradiation including paclitaxel.[23−25]

EXPERIMENTAL SECTION

Chemicals and Reagents. siGLO and human survivin ON-TARGET duplex siRNA (5′-P-UCUGGCUCGUUCUCAGUGGUU) and nontargeting siControl (5′-AUGUAUUGGCCUGUAUUAG-3′) were purchased from Dharmacon (Chicago, IL). 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP), cholesterol and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-z*N*-(lissamine rhodamine B sulfonyl) (Rh-PE), were purchased from Avanti Polar Lipids (Alabaster, AL); paclitaxel was purchased from Handetech (Houston, TX); DRAQ5 was purchased from Biostatus (Leicestershire, U.K.); sterile pigskin collagen (Spongostan standard) was purchased from Health Designs Industries (Rochester, NY); antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Pierce m-PER Mammalian Protein Extraction Reagent was purchased from Thermo Sci. (Rockford, IL); and Lipofectamine-2000 and all other cell culture supplies were purchased from Invitrogen (Carlsbad, CA).

Cell Cultures. Human oropharyngeal squamous carcinoma FaDu cells, purchased from American Type Culture Collection (Manassas, VA), were maintained in colorless RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, penicillin G and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested from subconfluent cultures after a rinse with versene and 10 min incubation with 0.01% trypsin-EDTA, and resuspended in fresh medium for experiments.

Selection of Model siRNA. Two siRNA were used. The fluorescent siGLO (proprietary name of a siRNA transfection indicator available from Dharmacon) was used to monitor the interstitial and intracellular uptake and spatial distribution in monolayer and 3-dimensional cultures. siGLO comprises a 22 nucleotide double-stranded RNA that has no mRNA targets and does not interfere or compete with functional siRNA. siGLO also contains a nuclear transport peptide that causes cytoplasm-to-nucleus translocation of free siGLO released from the liposomes. Hence, appearance of red fluorescence in cells indicates internalization of liposomes and appearance of green fluorescence indicates internalization of siGLO. Appearance of colocalized, discrete red and green signals indicates that siGLO enters cells as intact liposomes and is retained in the liposomes, whereas separation of these signals indicates siGLO has been released from liposomes. Similarly, appearance of green fluorescence in the nucleus (stained with DRAQ5, represented as pseudoblue fluorescence in photomicrographs) indicates the release of siGLO from liposomes, endosomes and/or lysosomes; this is because only the free siGLO, with its nuclear transport peptide, can enter the nucleus. Because siGLO is not functional, a second siRNA, survivin-siRNA, was used to monitor transfection and protein knockdown.

Preparation of 3-Dimensional Multicellular Spheroids and Histocultures. Multicellular spheroids were prepared using the standard liquid overlay method.[26] Briefly, 2,000 FaDu cells in 200 μL of culture medium were seeded per well in a 96-well agarose-coated plate and incubated under standard cell culture conditions. The agarose coating limited the cell attachment to culture flasks and promoted cell−cell attachment. Multicellular spheroids of approximately 300 to 500 μm in diameter were formed 3−4 days after seeding. FaDu spheroids showed densely packed cells with extensive tight junction formation (unpublished data).

Histocultures were prepared as previously described.[27] Briefly, FaDu cells were implanted subcutaneously in immunodeficient mice, which were cared for in accordance with institutional guidelines and had access to food and water *ad libitum*. Tumors of about 1 cm were excised and cut into 1 mm^3 fragments. Four to 6 tumor fragments were placed on 1 cm^3 blocks of collagen sponge presoaked in culture medium, and cultured in six-well plates under standard cell culture conditions for 48 h before experiments.

Liposomal siRNA Preparation. Cationic liposomes comprising 50:50 DOTAP:cholesterol were used as siRNA carriers (referred to as DC-liposomal siRNA). Fluorescence-labeled liposomes were obtained by adding Rh-PE to DC-liposomes at a DOTAP:cholesterol:Rh-PE molar ratio of 50:50:1.5. Liposomes were prepared using the dry film hydration plus extrusion method.[28] Thin lipid coatings in glass tubes were rehydrated with RNase-free HEPES buffer (pH 7.5, Dharmacon) at 60 °C with occasional vortexing. The resulting lipid emulsion was forced through a miniextruder (Avanti Polar Lipids, Alabaster, AL) containing a polycarbonate filter (100 nm pore size), back and forth 19 times to form cationic liposomes. DC-liposomal siRNA was prepared by incubating the liposomes with siRNA at an 88:1

molar ratio of DOTAP (1 positive charge per molecule) to siRNA (~44 negative phosphate charges per molecule) with an overall ± charge ratio of ~2:1. The lipid/siRNA mixture was incubated at room temperature for 20 min, and diluted with antibiotic-free culture medium to the desired concentrations. The mean diameter and zeta potential of liposomal siRNA were 355 nm (polydispersity index = 0.241) and +40.4 mV, respectively, as measured using Brookhaven 90Plus particle size analyzer (Holtsville, NY) and Malvern Zetasizer NanoZS90 (Worcestershire, U.K.) at 25 °C (based on 3 independently prepared samples).

Effects of Paclitaxel Pretreatment on Uptake and Transport of Liposomal siRNA on a Cellular Level. Monolayer cultures (40−60% confluent) grown in 96-well plates (for uptake study) or poly-L-lysine coated coverslips (for imaging) were treated with paclitaxel (50, 100, 200 nM) for 8 h, washed twice with drug- and antibiotic-free medium (30 min intervals between washes), incubated with drug-free medium for 24 or 48 h, and then treated with DC-liposomal siGLO (100 nM) for 5 h. Cells were then processed, as follows. Note that similar treatment schedules were used in studies with 3-dimensional cultures.

For measuring total uptake, cells were washed three times with ice-cold PBS and then lysed with 1% TritonX-100 in PBS. The fluorescence intensities of siGLO and Rh-PE in the cell lysates were measured using a Bio-Tek FL500 microplate reader (Winooski, VT). The excitation/emission wavelengths were 494 nm/520 nm for siGLO (green fluorescence) and 550 nm/590 nm for Rh-PE (red fluorescence), respectively. Results were normalized for cell numbers.

For intracellular distribution of siGLO, cells were stained with the nuclear dye DRAQ5 (5 μM for 10 min), washed twice with ice-cold PBS, then fixed with 10% buffered formalin and examined using confocal microscopy (Zeiss 510 META; Carl Zeiss, Thornwood, NY). siGLO, Rh-PE and DRAQ5 were viewed using Ch 3 (green, 488 nm/520 nm excitation/emission), Ch 5 (red, 543 nm/590 nm) and Ch 6 (far red, shown as pseudoblue, 633 nm/670 nm), respectively. The thickness of the optical sections was 3 μm.

Effects of Paclitaxel on the Penetration of Liposomal siRNA in 3-Dimensional Tumor Models. Spheroids and histocultures were treated with paclitaxel and DC-liposomal siGLO as described for monolayer cultures, with the exception that the paclitaxel treatment was extended to 48 h. The latter was selected to mimic the conditions *in vivo*, e.g., paclitaxel is usually given as 3 h infusion and has a terminal half-life of 3 to 53 h in humans.[29] The spatial distributions of siGLO and Rh-PE were examined using confocal microscopy. The thickness of the optical sections was 5 μm. Fluorescence intensities were quantified using LSM-5 Image Examiner software. Ten evenly spaced profiles from the spheroid border to center were recorded per image, and at least 3 samples were analyzed per treatment group. Data were pooled, and the average values were used to construct the intensity−distance profiles, and the area-under-the-curve (AUC) values were calculated using the trapezoid rule.

Effects of Paclitaxel on the Transfection and Functionality of Liposomal siRNA in Monolayers and 3-Dimensional Histocultures. This was studied using survivin siRNA. Monolayers or histocultures were treated with paclitaxel and/or survivin siRNA, allowed to grow in drug- or siRNA-free medium for 48 h, and then analyzed for the survivin protein levels. As comparison, cells were also treated with survivin siRNA loaded in the conventional transfection carrier Lipofectamine-2000. Lipofectamine-siRNA was freshly prepared before use by incubating 50 pmol of siRNA with 2.25 μL of Lipofectamine-2000 at room temperature for 20 min, and the mixture adjusted to 50 nM siRNA concentration with RPMI medium.

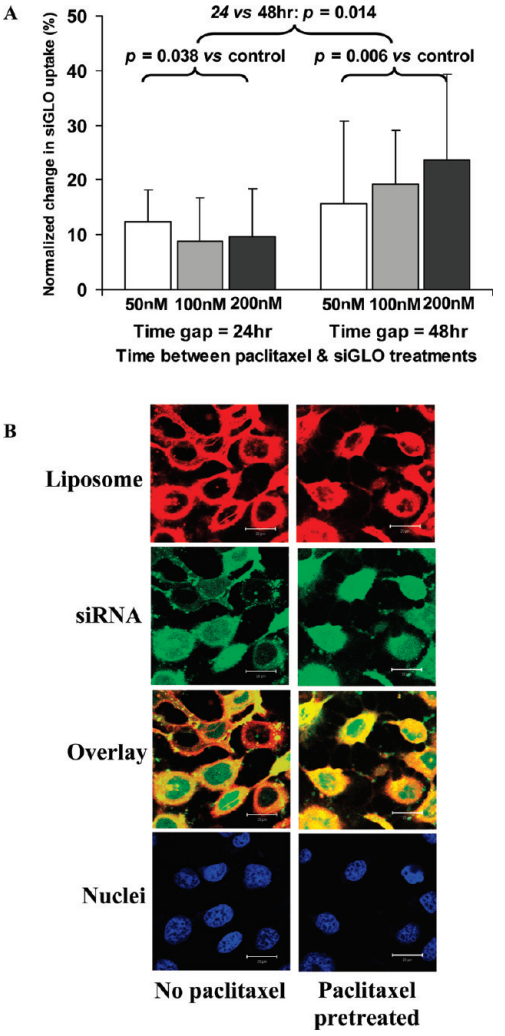

Figure 1. Effects of paclitaxel on cellular uptake and intracellular distribution of liposomal siRNA. Monolayer FaDu cells were pretreated with paclitaxel (50, 100, or 200 nM) or drug-free medium for 8 h, allowed to recover in drug-free medium for 24 or 48 h, and then treated with Rh-PE-labeled DC-liposomal siGLO (100 nM) for 5 h. (A) Total amount of cell associated siRNA. Results were expressed as % increase relative to the control groups without paclitaxel. Mean + one SD (3 experiments, 4 replicates per group in each experiment). Statistical analyses comparing the 24 and 48 h groups, and comparing these two groups with the drug-free control group used two-tailed Student *t* tests. The *p* values are shown. (B) Confocal microscopic images of cellular distribution of siGLO (green), Rh-PE-labeled lipids (red), and DRAQ5 (blue). The paclitaxel concentration was 100 nM and the recovery period was 24 h. Cells were stained with the nuclear dye DRAQ5 after the siRNA treatment. 1000× magnification.

Different methods were used to measure the protein levels in monolayers and histocultures. Monolayers were analyzed using Western immunoblotting. Cells were harvested and extracted for proteins using Pierce m-PER supplemented with 0.1% (v/v) protease inhibitor cocktail. Aliquots of cell lysate were resolved on a 15% SDS−polyacrylamide gel (25 μg protein loading per lane) and electrotransferred onto a polyvinylidene difluoride

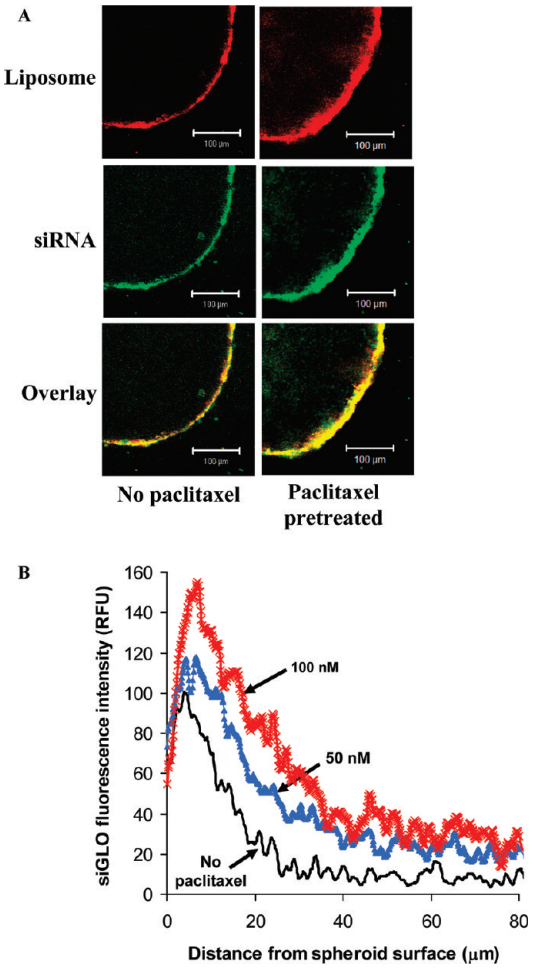

Figure 2. Effects of paclitaxel pretreatment on the penetration of liposomal siRNA into multicellular spheroids. Spheroids were pretreated with paclitaxel (50 or 100 nM) for 48 h, followed by incubation in drug-free medium for 24 h and then with Rh-PE-labeled liposomal siGLO (100 nM) for 5 h. (A) Representative confocal microscopic images of spheroids showing the distribution of siGLO (green) and Rh-PE (red). 100× magnification. (B) Quantification of siGLO penetration in spheroids. Fluorescence signals corresponding to siGLO were quantified and plotted against the distance from the outer-edge of spheroids. Each curve represents the average value of three or more samples per treatment group (obtained from three separate experiments, ten measurements per sample). RFU is the relative fluorescence units. The respective AUC values are 1930, 3598, and 4748 RFU-μm for no paclitaxel treatment, and pretreatment with 50 nM and 100 nM paclitaxel ($p < 0.01$, one-way ANOVA).

membrane. The membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% dry skim milk powder. Survivin and actin were detected with rabbit survivin monoclonal antibody (1:1000) and antiactin antibody AC-40 (1:1500 dilution), respectively, followed by treatment with the horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (1:4000 for survivin, 1:5000 for β-actin) and Amersham ECL Plus Western Blotting Reagents (GE Healthcare, Pittsburgh, PA). Proteins were visualized using enhanced chemiluminescence.

As the substantial intratumoral heterogeneity in tumor histocultures will result in highly variable Western blotting results, the survivin protein levels were measured using immunohistochemical staining and quantitative image analysis. Briefly, histocultures were fixed in formalin, embedded in paraffin wax, and cut into 5 μm thick sections. The sections were immunostained using goat polyclonal IgG against survivin (C-19) as primary antibody (1:1000 dilution) and horseradish peroxidase conjugated anti-goat IgG (1:1000 dilution) as secondary antibody, counterstained with hematoxylin, and examined under a microscope. For each image, the areas with the highest staining intensity (located on the periphery) were marked and the staining intensity in a rectangle-shaped region of interest (1/2 width × 1/2 height of the image) was recorded and analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Six or more images were analyzed per treatment group.

Statistical Analysis. Comparison of values between groups was performed using two-tailed Student's *t*-tests and ANOVA for repetitive measurements.

RESULTS

Effects of Paclitaxel Pretreatment on Cellular Uptake and Intracellular Distribution of Liposomal siRNA. Figure 1A shows the results on the total cellular uptake of DC-liposomal siGLO in monolayer culture. Cells received 8 h pretreatment of paclitaxel at 50−200 nM, followed by siGLO treatment initiated 24 or 48 h later. These paclitaxel treatments were cytotoxic and reduced the cell numbers by 19−29% (measured before cell lysis for fluorescence measurement). Interestingly, paclitaxel did not reduce but instead increased the total siGLO accumulation, measured as the total amount of cell-associated siGLO ($p < 0.05$). SiGLO accumulation was higher when the treatment time gap was increased from 24 to 48 h ($p < 0.05$).

Figure 1A measures the quantitative uptake of fluorescent siRNA. However, the siRNA may not be properly distributed inside the cells (e.g., trapped in endosomes or adsorbed on cell surface). Figure 1B shows the confocal microscopy results to address this issue. Without paclitaxel, the red fluorescence corresponding to the Rh-PE lipid was localized in the cytoplasm, whereas the green fluorescence corresponding to siGLO was dispersed in the cytoplasm and the nucleus. Pretreatment with 100 nM paclitaxel 24 h prior to siGLO did not alter the intracellular distribution of DC-liposomal siGLO. These imaging results further confirm that the above quantitative data obtained using a fluorescence microplate reader (Figure 1A) were due to internalization of siGLO/liposome in cells and not a result of cell surface adsorption.

Effects of Paclitaxel on the Penetration of Liposomal siRNA in 3-Dimensional Tumor Models. Figure 2A shows the confocal microscopic results on the penetration and spatial distribution of DC-liposomal siGLO in spheroids, and Figure 2B shows the quantitative imaging results. The location of the first above-background fluorescence signal was considered the outer edge of the spheroids. Due to the unavoidable uneven edge, the average signal usually initially increased with depth to reach the maximal level, followed by a decline due to diminished penetration. Without paclitaxel, the red and green fluorescence signals corresponding to liposomes and siGLO were colocalized and were restricted to the periphery of the spheroid. Pretreatment with 50 or 100 nM paclitaxel 24 h prior to siGLO increased the penetration depth of DC-liposomal siGLO, such that the AUC under the siGLO penetration-depth curve at 100 nM paclitaxel was 2.5-times the paclitaxel-free control value ($p < 0.01$ one-way ANOVA).

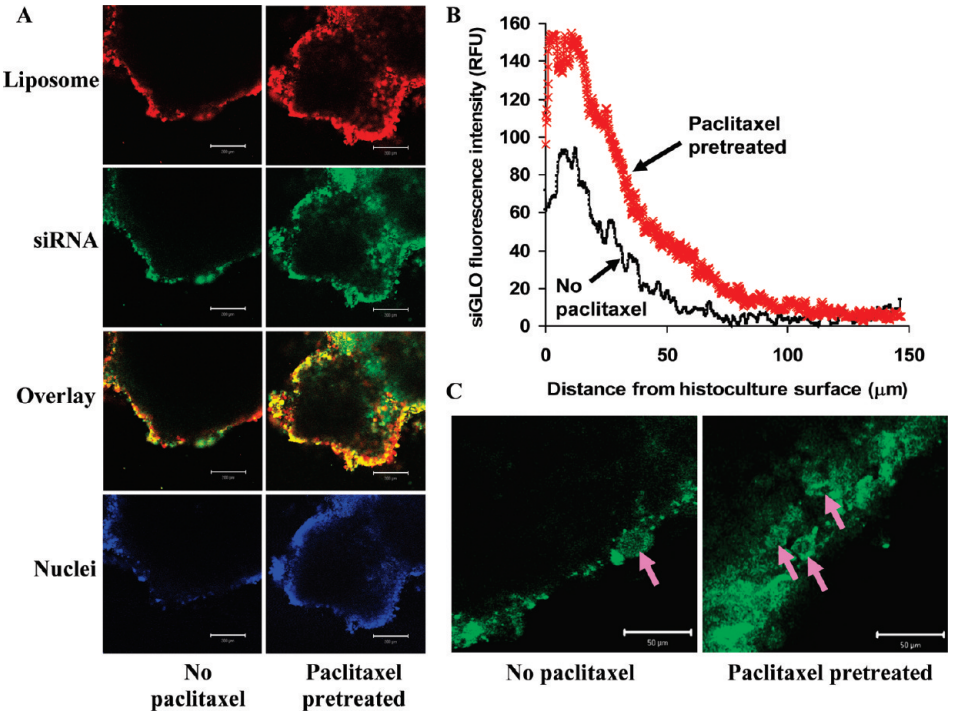

Figure 3. Effects of paclitaxel on penetration of liposomal siRNA into tumor histocultures. Histocultures were pretreated with 100 nM paclitaxel for 48 h, followed by incubation in drug-free medium for 24 h and then with Rh-PE-labeled liposomal siGLO (100 nM) for 5 h. (A) Representative confocal microscopic images of histocultures showing the distribution of siGLO (green) and Rh-PE (red). 100× magnification. (B) Quantification of siGLO penetration in histocultures. Each curve represents the average value of measurements in three or more samples per treatment group (obtained from three separate experiments, 10 measurements per sample). RFU is the relative fluorescence units. The respective AUC values are 3100, 2719, and 6490 RFU-μm for no paclitaxel, concurrent treatment with paclitaxel, and pretreatment with paclitaxel. (C) Distribution of siGLO into cells in histocultures. 400× magnification. Arrows indicate the siGLO inside cells.

Qualitatively similar results were found in histocultures (Figure 3A). Without paclitaxel, the fluorescence signals corresponding to liposomes and siGLO were colocalized, confined to the tumor periphery, and coincided with the locations of the nuclear dye (indicated by the psuedo-blue fluorescence). Pretreatment with 100 nM paclitaxel enhanced the penetration distance, such that the AUC under the siGLO penetration-depth curve was 2.1-times the control value without paclitaxel (Figure 3B). Of interest are the paclitaxel-induced changes in the dispersion of the siGLO fluorescence from being punctated to being diffused, with evidence of intracellular localization (Figure 3C).

Effects of Paclitaxel on Transfection and Functionality of Liposomal siRNA. Figure 4 shows the Western immunoblotting results in monolayer cells. Without pretreatment, transfection with DC-liposomal survivin siRNA reduced the protein level, in a siRNA-dose dependent manner, such that the protein level at the 50 nM siRNA dose was <10% of the baseline level. Paclitaxel pretreatment increased the survivin level by about 350% ($p < 0.01$); this finding is consistent with the literature reports that chemotherapy generally enhances survivin expression.[24] Adding DC-liposomal survivin siRNA to paclitaxel reversed the survivin upregulation, again in a siRNA-dose dependent manner, such that the survivin level returned to the baseline level at a 50–75 nM siRNA dose. Survivin expression after DC-liposomal siRNA was further compared with that after conventional Lipofectamine carrier; we found similar extends of protein knockdown for both transfection techniques. This was the case for cells treated with or without paclitaxel pretreatment.

Figure 5 shows the immunohistochemical staining and image analysis results in tumor histocultures. In the absence of paclitaxel pretreatment, DC-liposomal survivin siRNA (100 nM) had no effect on the survivin expression. Paclitaxel pretreatment significantly increased the survivin expression ($p < 0.05$). As was observed in monolayer culture, paclitaxel pretreatment induced an increase in survivin, albeit to a quantitatively lower extent (1.6-fold versus 2.8-fold). This paclitaxel-induced survivin increase was completely reversed by the addition of DC-liposomal survivin siRNA, indicating effective transfection and functionality of the siRNA.

DISCUSSION

This study addressed several questions on using paclitaxel tumor priming to promote the activity of siRNA therapeutics. The first question was whether the microtubule-stabilizing activity and cytotoxicity of paclitaxel compromises the uptake of liposomal siRNA in monolayer cells. Our results indicate paclitaxel pretreatment, 24–48 h prior to siRNA, yielded significant, albeit relatively modest, increases in the total siRNA uptake. The 24–48 h time window is within the 24–96 h window for effective *in vivo* tumor priming and maximally expanding the interstitial space.[16,30]

We previously showed that paclitaxel tumor priming mitigated the barriers for delivering small molecule drugs and nanoparticles (Doxil and latex beads which have respective diameters of 85 and 100 nm) to solid tumors.[16,17] The present study addressed the question whether the same principle applies to cationic liposomal

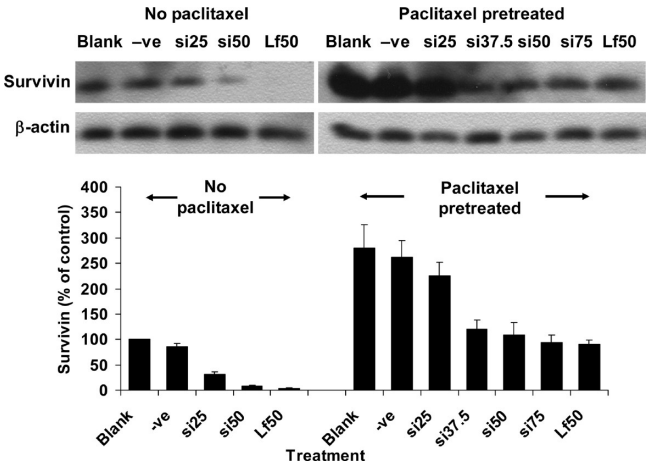

Figure 4. Effects of paclitaxel on transfection and functionality of liposomal survivin siRNA in monolayers. Monolayer cultures of FaDu cells were pretreated with 100 nM paclitaxel or drug-free culture medium for 24 h, followed by DC-liposomal survivin siRNA (0, 25, 37.5, 50, and/or 75 nM or si25-75) or the biologically inactive Nontargeting siControl #3. For comparison, cells were also treated with 50 nM survivin siRNA loaded in the conventional Lipofectamine-2000 carrier (Lf50). Cells were extracted and processed for Western blotting. The survivin levels were normalized for the actin levels. Data are expressed as % of the blank control. Mean + one SD (3 experiments). Blank = liposome without siRNA; −ve = liposome loading Nontargeting siControl.

siRNA that has a larger particle size and positive surface charge. The results demonstrate that paclitaxel tumor priming significantly enhanced the penetration of DC-liposomal siRNA in 3-dimensional spheroids and histocultures. It is noted that nanoparticle transport in spheroid and histoculture systems, as they are maintained *in vitro* and devoid of blood perfusion, reflects only the diffusive transport (i.e., without the convective transport).

We have shown that paclitaxel pretreatment can improve the uptake of liposomal siRNA at both cellular (Figure 1) and tumor levels (Figures 2 and 3). In view of the finding that paclitaxel caused less than 30% increase in cellular uptake but more than 2-fold increase in tumor uptake, the improvement of the interstitial liposomal-siRNA transport in the tumor is more likely the predominant mechanism by which paclitaxel enhances tumor siRNA uptake, at least in the tightly packed FaDu models used in the present studies. Paclitaxel may show greater benefits in tumors with less tightly packed cells. Additional studies are warranted.

The third question was whether the paclitaxel tumor priming-induced improvement of siRNA delivery and cellular internalization leads to improvement in the transfection and functionality of siRNA agents. This question was addressed in two ways: by monitoring the intracellular trafficking of siGLO and by monitoring the levels of the targeted protein survivin.

Our results indicate paclitaxel tumor priming enhanced the total siGLO uptake in cells in monolayers and in 3-dimensional spheroids and histocultures. The increase in total siGLO uptake may derive from reducing the degradation of siGLO, as reported for DNA-lipoplexes,[31] or from improving the liposome endocytosis. Additional studies to quantify the relative distribution of siGLO and liposomal lipid in the cytoplasm and nucleus are needed to address the mechanisms by which paclitaxel enhances

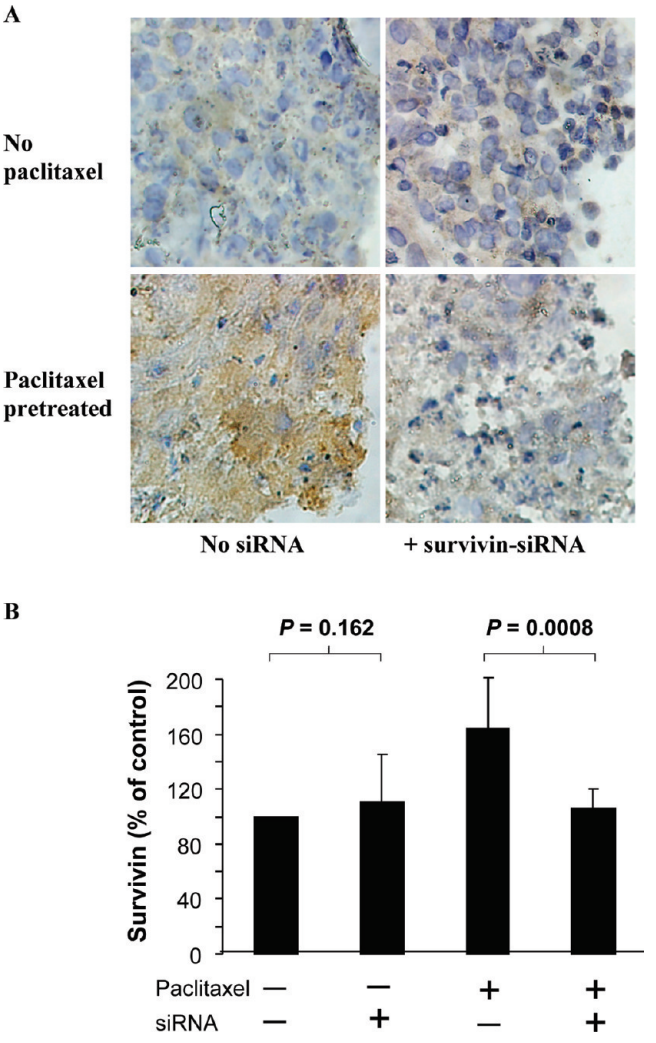

Figure 5. Effects of paclitaxel on transfection and functionality of liposomal survivin siRNA in tumor histocultures. FaDu histocultures were pretreated with 100 nM paclitaxel for 48 h, followed by incubation in drug-free medium for 24 h and then with DC-liposomal survivin siRNA (100 nM). Negative controls were similarly processed except without paclitaxel and/or siRNA treatments. Areas of histocultures showing the greatest immunohistochemical staining were analyzed. (A) Representative staining of histocultures after different treatments. Brown color indicates survivin protein. Cell nuclei were stained with hemotoxylin (blue). 400X magnification. (B) Survivin levels. Quantification was performed using ImageJ image analysis program. Data are expressed as % of untreated controls. Mean + one SD (2 experiments, total of 6 or more samples per treatment group).

the siGLO uptake. With respect to protein knockdown by DC-liposomal survivin siRNA, the results in monolayers confirm that paclitaxel pretreatment did not reduce or enhance the functionality of siRNA, whereas the results in histocultures indicate paclitaxel pretreatment promoted the activity of siRNA such that protein knockdown was evident only after the pretreatment and not in the untreated controls. The difference in the results in monolayers and histocultures indicate paclitaxel improved the activity of DC-liposomal siRNA by improving its delivery into histocultures.

The final question was whether DC-liposomal survivin siRNA can reverse the paclitaxel-induced upregulation of this apparently

universal chemoresistance protein. The results indicate complete reversal. This finding, in view of the earlier reports that survivin knockdown promotes apoptosis[23] and sensitizes cancer cells to paclitaxel[23,24] and in view of the high tumor selectivity of survivin expression, suggests potential therapeutic utility of combining paclitaxel with survivin siRNA.

Overall, paclitaxel exhibits "dual effect" on how a solid tumor handles liposomal siRNA: by increasing the depth and amount of their penetration as a result of improved interstitial transport and by improving the cellular siRNA uptake and activity. The relative contribution of these two components possibly varies from one tumor type to another one and requires further investigation.

CONCLUSIONS

The present study demonstrated that paclitaxel tumor priming improved the uptake/penetration, transfection and functionality of siRNA loaded in cationic liposomes in 2-dimensional monolayers and 3-dimensional tumor spheroids and histocultures. The finding that the increased siRNA uptake was achieved at cytotoxic paclitaxel concentrations indicates that the mechanism for the paclitaxel-enhanced cellular accumulation of siRNA is independent of its cytotoxic action. Furthermore, because cells surviving cytotoxic treatment are more likely to be drug-resistant, the finding of greater siRNA accumulation in paclitaxel-pretreated cells supports the use of siRNA to target chemoresistance genes such as survivin. As paclitaxel is a widely used chemotherapeutic agent in multiple types of cancer,[32] this strategy offers a promising and practical means to pursue siRNA-based chemogene therapy of solid tumors.

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ABBREVIATIONS USED

AUC, area-under-the-curve; DC-liposomes, DOTAP:cholesterol (50:50) liposomes; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; Rh-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl); siRNA, small interfering RNA

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