Brief Report

Tumor-Targeting Nanoimmunoliposome Complex for Short Interfering RNA Delivery

KATHLEEN F. PIROLLO,¹ GERALD ZON,² ANTONINA RAIT,¹ QI ZHOU,¹ WEI YU,¹
RICHARD HOGREFE,² and ESTHER H. CHANG¹

ABSTRACT

The potential of short interfering RNA (siRNA) to be developed for therapeutic use against cancer depends on the availability of an efficient tumor-specific delivery vehicle. We have previously shown that a nanoscale non-viral liposome-based complex that includes an anti-transferrin receptor single-chain antibody fragment as the targeting moiety can, when systemically administered, specifically and efficiently target primary and metastatic tumors and deliver molecules useful in gene medicine, including plasmid DNA and antisense oligonucleotides. Here we explore the ability of this complex to deliver a fluorescein-labeled siRNA to tumor cells *in vivo* and examine the intracellular localization *in vitro* by confocal microscopy. We show that the immunoliposome–siRNA complex maintains its nanoscale size and, using three separate tumor models, can efficiently and specifically deliver siRNA to both primary and metastatic disease after systemic delivery, thus increasing the possibility for translating the potent effects of siRNA observed *in vitro* into clinically useful therapeutics.

INTRODUCTION

RNA INTERFERENCE (RNAi) is induced by short interfering RNAs (siRNAs), which are 21- to 25-nucleotide duplexes with a characteristic structure of a symmetric 2- to 3-nucleotide 3'-end overhang, as well as a 5'-phosphate and free 3'-hydroxy terminus. They become incorporated into the RNA-induced silencing complex (RISC) and once paired, act as a guide for endonucleolytic cleavage of complementary targeted mRNA, which occurs near the center of the target–siRNA duplex (reviewed in Elbashir *et al.*, 2001; Hannon, 2002; Martinez *et al.*, 2002; Caplen and Mousses, 2003; Lu *et al.*, 2003; Caplen, 2004; Ryther *et al.*, 2005).

The application of these potent sequence-selective siRNAs to suppress expression of specific transcripts has become a useful technique for probing gene function *in vitro* and has led to the promise of their use for *in vivo* applications. However, low transfection efficiency, poor tissue penetration, and nonspecific immune stimulation by siRNAs administered *in vivo* have hindered their therapeutic applications. Efficient *in vivo* use of

siRNAs as therapeutic molecules against diseases such as cancer hinges on the availability of a delivery vehicle that is tumor specific and can be delivered systemically, safely, and repeatedly. Methods for *in vivo* delivery of siRNA have been the focus of several publications using viral vectors (reviewed in Lu *et al.*, 2003), liposomes (Sioud and Sorensen, 2003; Morrissey *et al.*, 2005), and polyethyleneimine (PEI) (Urban-Klein *et al.*, 2005). However, most of these approaches were not specifically targeted to tumor. Schiffelers *et al.* (2004) linked siRNA against vascular endothelial growth factor (VEGF) receptor-2 to PEI that was PEGylated with an RGD peptide ligand at the distal end, as a means to target tumor neovasculature. They reported inhibition of both tumor angiogenesis and growth rate. Song *et al.* (2005) used a protamine–antibody fusion protein to deliver siRNA specifically to human immunodeficiency virus (HIV)-infected or envelope-transfected cells *in vitro* and *in vivo*.

We have developed a systemically administered tumor-targeting immunoliposome complex in the nanometer size range for use in gene medicine (Xu *et al.*, 1999a,b, 2001, 2002; Rait

¹Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, D.C. 20057.

²TriLink BioTechnologies, San Diego, CA 92121.

et al., 2002, 2003; Pirollo *et al.*, 2005). This nanoimmunodelivery system (NDS) is composed of a cationic liposome encapsulating the payload. Decorating the surface of the liposome is a targeting moiety that can be a ligand (e.g., folate or transferrin) or an antibody fragment directed against a cell surface receptor, which facilitates entry of the complex into cells via receptor-mediated endocytosis (Cheng, 1996; Cristiano and Curiel, 1996). This NDS has been successfully employed to systemically deliver genes (Xu *et al.*, 1999a,b, 2001, 2002), antisense oligonucleotides (Rait *et al.*, 2002, 2003), and even magnetic resonance imaging (MRI) contrast agent (Pirollo *et al.*, 2005) specifically to tumor cells, both primary and metastatic.

Here we demonstrate the ability of our immunoliposome NDS to target and deliver a modified siRNA specifically and efficiently to tumor cells (both primary and metastatic) *in vivo* after intravenous administration. In addition, confocal microscopy shows strong, diffuse (not punctate) cytoplasmic localization of the siRNA fluorescently labeled with 6-carboxy-fluorescein (6-FAM) on transfection by means of the NDS. The high tumor selectivity and deep tumor penetration of this complex suggest that this NDS has the potential to overcome one of the major obstacles to the effective use of siRNA as a therapeutic modality.

MATERIALS AND METHODS

Cell lines

Human pancreatic cancer cell lines (PANC-1 and Capan-1) and a human prostate cancer cell line (PC-3) were obtained from the Tissue Culture Shared Resource of the Lombardi Comprehensive Cancer Center (Washington, D.C.). Capan-1 cells were maintained in Iscove's modified DMEM (Biofluids Division of Biosource International, Rockville, MD) containing 4 mM L-glutamine, sodium bicarbonate and supplemented with 2 mM L-glutamine, 50 μ g/ml each of penicillin, streptomycin, and neomycin, with 20% non-heat-inactivated fetal bovine serum (FBS; Quality Biological, Gaithersburg, MD). PC-3 cells were maintained in Ham's F12 medium supplemented with 10% heat-inactivated FBS plus L-glutamine and antibiotics as described above. The MDA435/LCC6 cell line was a gift from R. Clarke (Lombardi Comprehensive Cancer Center). MDA435/LCC6 is a tumorigenic cell line isolated from spontaneous ascites in mice inoculated in the mammary fat pad with the invasive and metastatic human breast cancer cell line MDA-MB-435 (Leonessa *et al.*, 1996). Both MDA435/LCC6 and PANC-1 cells were maintained in improved minimum essential medium (IMEM; Biofluids Division of Biosource International) supplemented with L-glutamine and antibiotics as described above, plus 5 or 10%, respectively, heat-inactivated FBS.

Complex formation

Cationic liposome (DOTAP:DOPE [Avanti Polar Lipids, Alabaster, AL]) (Lip) was prepared by the ethanol injection method as previously described (Xu *et al.*, 2002). The targeting moiety used in these studies is an anti-transferrin receptor single-chain antibody fragment (TfRscFv) (Haynes *et al.*, 1981; Batra *et al.*, 1991; Xu *et al.*, 2001, 2002). TfRscFv contains the

complete antibody-binding site for the epitope of the TfR recognized by the monoclonal antibody 5E9 (Batra *et al.*, 1991). The TfRscFv-Lip-siRNA (scL-siRNA) complex was formed in a manner identical to that previously described (Yu *et al.*, 2004). The size of the complex was determined by dynamic light scattering on a Malvern Zetasizer 3000HS (Malvern Instruments, Malvern, UK).

siRNA

The siRNA employed in these studies was synthesized and purified by TriLink BioTechnologies (San Diego, CA) [personal communication (G. Zon and R. Hogrefe); and R. Hogrefe, A. Lebedev, K. Pirollo, A. Rait, E. Chang, and G. Zon, unpublished data]. A phosphoramidite reagent was used to label the sense strand of the siRNA with the 6-carboxyl isomer of fluorescein (6-FAM-labeled siRNA).

Confocal microscopy

6-FAM-labeled control (non-sequence-specific) modified hybrid siRNA was synthesized and purified by polyacrylamide gel electrophoresis by TriLink BioTechnologies. PANC-1 or MDA435/LCC6 cells were seeded at 5.5×10^4 cells per chamber of a 4-chamber Falcon culture slide (BD Biosciences Discovery Labware, Bedford, MA), or on coverslips in a 24-well tissue culture plate. Twenty-four hours later they were transfected (Xu *et al.*, 2001) for 6 hr in serum-free medium with either scL carrying 1 μ M 6-FAM-labeled siRNA, or a 1 μ M concentration of the uncomplexed (free) 6-FAM-labeled siRNA. For the 6-hr confocal time point, cells were washed immediately after the 6-hr transfection and fixed with 3.7% paraformaldehyde, and the slide or coverslips were mounted using a ProLong antifade kit (Molecular Probes/Invitrogen, Eugene, OR). For the 24-hr time points, after the 6-hr transfection, serum

FIG. 1. Western blot analysis of transferrin receptor (TfR) protein levels in human pancreatic cancer cell lines. Total cellular protein (20 μ g) from a series of human pancreatic cancer cell lines and normal human fibroblast cell line H500 were separated by 8% discontinuous SDS-polyacrylamide gel electrophoresis, transferred to nylon membrane, and hybridized with anti-human TfR (Zymed Laboratories/Invitrogen) antibody. Actin levels were determined as an internal control for protein loading.

FIG. 2. Localization of fluorescently labeled siRNA in human pancreatic cancer cells. PANC-1 cells were transfected *in vitro* with either scL-complexed 6-FAM-labeled siRNA or free (uncomplexed) 6-FAM-labeled siRNA. After 6 hr of incubation in serum-free medium the cells were fixed immediately (6 hr) or serum was added and the cells were incubated for an additional 18 hr before fixing (24 hr). The samples were imaged by confocal microscopy at an original magnification of $\times 20$ or $\times 60$. *Top*: scL-complexed siRNA (1 μM). *Bottom*: Uncomplexed siRNA (1 μM). DIC, differential interference contrast.

was added to the chamber (or wells) and the cells were incubated for an additional 18 hr before being fixed as described above. The cells were observed directly with an Olympus 1X-70 laser confocal scanning microscope imaging system equipped with an upright confocal microscope and a $\times 20$ or $\times 60$ oil immersion objective.

In vivo targeting studies

Orthotopic tumors of human pancreatic cell line Capan-1 were induced as previously described (Alisauskus *et al.*, 1995; Pirollo *et al.*, 2005). In general, 1×10^7 cells in Matrigel basement membrane matrix were subcutaneously inoculated into 6- to 8-week old female athymic nude (NCR-*nulnu*) mice. When the tumor reached approximately 1 cm^3 , the tumor was harvested and a single-cell suspension of 1×10^7 cells in Matrigel was surgically implanted into the pancreas of the mice. PC-3 human prostate cancer orthotopic tumors were induced by surgical implantation of $<1\text{-mm}^3$ sections of subcutaneously induced tumors into the capsule of the dorsal lobe of the prostate of 6- to 8-week old male nude (NCR-*nulnu*) mice (Yang *et al.*, 1999). Lung metastases were induced by the intravenous inoculation of 8×10^6 MDA435/LCC6 cells through the tail vein of female nude mice. Six to 15 weeks postsurgery (PC-3/Capan-1) or 8 weeks postinjection (MDA435/LCC6), depending on the tumor model, the complex (scL) carrying 6-FAM-labeled siRNA (9 mg/kg) was intravenously injected into the mice. At the appropriate time, the animals were killed and tumor and other organs were excised, photographed, and examined for fluorescence with a Nikon epifluorescence stereoscope. In some cases, the organs were subsequently formalin fixed, embedded, and stained with hematoxylin-eosin. All animal studies were performed in accordance with institutional guidelines by approved protocols.

RESULTS AND DISCUSSION

The disruption of uncontrolled or abnormal cellular signaling is a rational approach to cancer therapy. Unfortunately, the lack of specificity of traditional small molecule-based drugs can result in reduced efficacy and unwanted side effects. Although approaches that target the encoding RNA by means of antisense oligonucleotides or ribozymes are being tried, a newer strategy that inhibits gene expression at the posttranscriptional level via a naturally occurring gene-silencing mechanism (RNAi) has also been developed. However, successful application of the RNAi approach has also been limited by a lack of efficient, systemically administered tumor-specific delivery. The development of a delivery system that could reach and efficiently transfect metastatic, as well as primary, tumor cells would greatly enhance the applicability of siRNA technology as both a research tool and a therapeutic modality. Although various *in vivo* delivery methods are being explored as alternatives to free siRNA, most notably viruses and liposomes (reviewed in Caplen and Mousses, 2003; Caplen, 2004; Ryther *et al.*, 2005), the vast majority still lack tumor-specific targeting capability, and the high penetrance required for potential clinical efficacy. As our nanoimmunocomplex delivery system (NDS) has been successfully employed to efficiently and preferentially deliver

antisense oligonucleotides as well as plasmid DNA to tumor cells, we examined its use for the systemic delivery of siRNA. For these targeting and confocal experiments, a control modified hybrid siRNA molecule, one that does not have homology to any sequences in GenBank, was used. This modified hybrid is composed of sense DNA-antisense RNA strands, where 2'-*O*-methyl (2'-*O*-Me) substituents have been incorporated in the central region of the sense strand with DNA flanks. This 19-mer construct is also blunt ended, as opposed to the commonly employed 21-mer construct with 2-base pair overhangs. The sequence is as follows:

Control sense: 5'-TIC Icc gaa cgu guC ICI T-3'
Control antisense: 5'-ACG UGA CAC GUU CGG AGA A-3'

where I represents 2'-deoxyinosine and lowercase letters indicate 2'-*O*-Me RNA.

Complex size

The size of the TfRscFv-liposome-siRNA (scL-siRNA) complex was measured by dynamic laser light scattering. The size of the complex, given as cumulative mean, was found to be $211 \pm 18 \text{ nm}$. The polydispersity, representing the reproducibility of the values during repeat scans, was 0.18 ± 0.05 . These results demonstrate the uniformly small size of the complex containing the modified hybrid siRNA molecule. This small size is critical for the complex to be able to pass through the small capillaries and penetrate throughout the tumor.

Intracellular localization

Transferrin receptor (TfR) levels are elevated in various types of cancer cells (Ponka and Lok, 1999). These include breast cancer (Elliott *et al.*, 1993) and pancreatic cancer, as shown in Fig. 1. Using Western analysis, we evaluated the TfR levels in a number of pancreatic cancer cell lines. The antibody used was a commercially available (Zymed Laboratories/Invitrogen, South San Francisco, CA) mouse anti-human TfR monoclonal antibody. As shown in Fig. 1, all four pancreatic cancer cell lines (Capan-1, Colo-357, PANC-1, and AsPC-1) possess elevated TfR levels as compared with H500, a normal human fibroblast cell line. Analysis of actin levels demonstrated that equal amounts of protein had been loaded in each lane. Once bound to the TfR, entry of the complex into the cell is facilitated by receptor-mediated endocytosis, a highly efficient internalization pathway present in eukaryotic cells (Cheng, 1996). To compare the transfection efficiency and the intracellular localization of the scL-delivered molecule, a fluorescein-labeled (6-FAM) control (non-sequence-specific) modified hybrid siRNA was used, either complexed to scL or as free (uncomplexed) siRNA. An unliganded (no TfRscFv) version of the liposome-siRNA was not used as a control in these studies because we have previously demonstrated the requirement for the targeting molecule to achieve tumor specificity (Xu *et al.*, 1999a,b, 2001, 2002; Rait *et al.*, 2002; Yu *et al.*, 2004). Human pancreatic cancer cell line PANC-1 was transfected with either the scL complex carrying $1 \mu\text{M}$ 6-FAM-labeled siRNA, or $1 \mu\text{M}$ free 6-FAM-labeled siRNA and examined by confocal microscopy. As shown in Fig. 2, after 6 hr virtually all cells transfected with the scL-siRNA complex exhibited intense, dif-

fuse cytoplasmic staining. In contrast, cells treated with siRNA without the benefit of complexing with the NDS exhibited weak fluorescence 6 hr, and even 24 hr, after transfection. Similar results were observed in a second tumor cell line, MDA435/LCC6, a metastatic line derived from ascites fluid in a mouse bearing an MDA-MB-435 human breast cancer tumor (data not shown). These results demonstrate the high transfection efficiency of the scL-siRNA NDS versus free siRNA. In addition, as a control siRNA was used in these studies the lack of cell death seems to indicate the absence of nonspecific cytotoxic effects by the modified hybrid type of siRNA molecule.

Tumor-specific targeting by scL-siRNA

The ability of the systemically administered scL NDS to deliver siRNA specifically to tumors was assessed with the 6-FAM-

labeled control modified hybrid siRNA molecule. PC-3 human prostate cancer and Capan-1 human pancreatic cancer orthotopic tumors were induced as described in Materials and Methods. Six to 12 weeks postsurgery, mice implanted with the PC-3 tumor had large palpable tumors. The animals were intravenously injected once with the scL-siRNA complex at an siRNA dose of 9 mg/kg. Twenty minutes postinjection, the mice were killed and the tumor and liver were examined for fluorescence, using a Nikon epifluorescence stereoscope. In Fig. 3A the tumor mass is engulfing the prostate. The left seminal vesicle is involved with the tumor. Under fluorescence, the entire tumor and the left seminal vesicle exhibit strong fluorescence. In contrast, only minimal signal is evident in the normal liver. The ability of the scL NDS to specifically target and transfect tumor cells is further supported by the fact that the blood vessels visible on the surface of the tumor in Fig. 3 appear dark in the fluorescence image, indi-

FIG. 3. scL NDS-mediated targeting of siRNA to primary and metastatic tumors in orthotopic mouse tumor models. Orthotopic tumors of human prostate (PC-3) or pancreatic (Capan-1) cancer cell lines were induced as described in Materials and Methods. The scL complex carrying the 6-FAM-labeled siRNA at 9 mg/kg was intravenously injected via the tail vein and the animals were killed 20 min later. (A) PC-3 orthotopic prostate tumor and seminal vesicles. (B) A separate, larger orthotopic prostate tumor showing the intact tumor and a central cross-section. The arrow indicates a reference point between the photograph and fluorescence image. (C) Liver with metastasis derived from a Capan-1 orthotopic pancreatic tumor. The identical field is shown in bright-field and fluorescence views with the arrows indicating the metastasis.

FIG. 4. Comparison of free and scL-mediated siRNA targeting to metastasis. Lung metastases were induced by intravenous injection of MDA435/LCC6 cells as described in Materials and Methods. 6-FAM-labeled siRNA at 9 mg/kg, either free or complexed with the scL NDS, was injected via the tail vein. Three hours later the animals were killed. (A) A small tumor nodule in the lung of the mouse that received scL-siRNA. The arrow indicates this nodule in the photograph and the bright-field view. (B) A larger nodule in the lung from the mouse that received free (uncomplexed) siRNA. The arrow indicates the nodule in the photograph and the bright-field view.

cating that, although the complex passes through, the endothelial cells of the blood vessels are not transfected. In Fig. 3B a cross-section through the center of a separate larger orthotopic prostate tumor exhibits high fluorescence, showing the deep penetration of the complex into the tumor.

The ability to target metastases was assessed with the Capan-1 orthotopic and MDA435/LCC6 lung metastasis models. Approximately 15 weeks after implantation of cells into the pancreas, to allow for development of metastases, each mouse was intravenously injected as described above with scL-siRNA (at 9 mg/kg) and killed 20 min postinjection. A small metastasis was visible between two lobes of the liver (Fig. 3C, bright field). This area exhibited strong fluorescence compared with the surrounding normal liver. Histological examination of the area demonstrated the presence of tumor cells between the liver lobes (Fig. 3C, histology). The enhanced tumor delivery and specificity for metastatic disease shown by the scL-siRNA NDS, as compared with intravenously administered free siRNA, were also demonstrated in the MDA435/LCC6 mouse model. Cells were intravenously injected as described in Materials and Methods. Approximately 8 weeks later 6-FAM-labeled siRNA (9 mg/kg), either complexed with the scL NDS or as free (uncomplexed) 6-FAM-labeled siRNA, was administered once via tail vein injection. Three hours postinjection, the animals were

killed and the lungs and liver were examined. Visible metastases were present in the lungs of both mice. However, there was a significant difference in the level of fluorescence (Fig. 4). In Fig. 4A, in the animal that received scL-siRNA, a small lung metastasis is clearly visible in the bright-field image and is indicated by the arrow in the photograph of the lung. This small nodule displayed intense fluorescence, whereas the background of normal lung and liver remained dark. In contrast, a larger tumor nodule in the lung from the mouse injected with free siRNA exhibits a much lower level of fluorescence (Fig. 4B). Moreover, the normal tissue from this animal, particularly the liver, has a higher background level of fluorescence. The metastasis in Fig. 4B is larger than that present in the mouse in Fig. 4A. Thus, this metastasis should have displayed even stronger, rather than weaker, fluorescence compared with that seen in the tumor in Fig. 4A. This, along with the higher level of nonspecific fluorescence in the normal liver, demonstrates that delivery of siRNA by the scL NDS enhances tumor-specific uptake. The bright-field and fluorescence views in both Fig. 4A and B are of the same field. All of the fluorescence images were taken with identical settings, exposure time, and magnification.

Taken together, these studies indicate that our established TfRscFv-Lip nanoimmunocomplex can also be used to deliver siRNA. The NDS will maintain its small size when encapsu-

lating siRNA and, when administered systemically, can specifically and efficiently target both primary and metastatic cancers of various types including prostate, pancreatic, and breast. Because increased transferrin is required for rapid cellular growth, the transferrin receptor is elevated on most, if not all, tumor types (Ponka and Lok, 1999). Thus, use of this TfRscFv NDS targeted for siRNA is applicable to a broad range of cancers. Moreover, as a nonviral modality, it is free of the concerns regarding side effects associated with viral delivery methods (Scanlon, 2004). Thus, we have developed a way to systemically and efficiently deliver siRNA to tumor cells wherever they reside in the body, a system with the potential to facilitate the translation of siRNA from the bench to the bedside.

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Address reprint requests to:

Dr. Esther H. Chang

Department of Oncology

Lombardi Comprehensive Cancer Center

Georgetown University Medical Center

3970 Reservoir Road, NW

The Research Building, E420

Washington, D.C. 20057-1460

E-mail: change@georgetown.edu

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