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REVIEW ARTICLE

ADVANCES IN SYSTEMIC siRNA DELIVERY

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ABSTRACT

Sequence-specific gene silencing with small interfering RNA (siRNA) has transformed basic science research, and the efficacy of siRNA therapeutics in a variety of diseases is now being evaluated in preclinical and clinical trials. Despite its potential value, the highly negatively charged siRNA has the classic delivery problem of requiring transport across cell membranes to the cytosol. Consequently, carrier development for siRNA delivery is one of the most important problems to solve before siRNA can achieve widespread clinical use. An assortment of nonviral carriers, including liposomes, peptides, polymers and aptamers, are being evaluated for their ability to shepherd siRNA to the target tissue and cross the plasma membrane barrier into the cell. Several promising carriers with low toxicity and increased specificity for disease targets have emerged for siRNA-based therapeutics. This review will discuss nonviral approaches for siRNA therapeutics, with particular focus on synthetic carriers for in vivo systemic delivery of siRNA.

INTRODUCTION

The ability to downregulate target genes by using double-stranded RNA interference (RNAi) has revolutionized basic science research on signal transduction and gene function (1). RNAi also has tremendous therapeutic potential for treating diseases such as cancer or macular degeneration, in which an oncogene or angiogenic growth factor is overexpressed. The scientific community's commitment to RNAi technology is evidenced by the 2006 Nobel Prize and by high-profile startup biotech companies, as well as billion-dollar investments from established pharmaceutical companies.

Two approaches utilize RNAi to inhibit target genes: shRNA (short hairpin RNA) and siRNA (small interfering RNA) (2) (Fig. 1). Whereas the shRNA approach is usually promoter-dependent and can be delivered by both viral (e.g., lentivirus, adeno-associated virus) and nonviral (plasmid-based) methods, siRNA is a chemically synthesized RNA duplex and is generally delivered by nonviral delivery systems. In addition to siRNA, a 29-mer shRNA has also been chemically synthesized, and one report showed that the shRNA approach was more potent than the comparable siRNA (3).

Both shRNA and siRNA approaches harness the cellular machinery of microRNA for their activity and this provides the basis for efficacy and toxicity of RNAi. In contrast to chemically synthesized siRNA, the promoter-based shRNA approach requires multiple enzymatic and/or transport steps (e.g., transcription, nuclear export, Drosha and Dicer processing) before interaction with the RNA-induced silencing complex (RISC), which results in cleavage of the targeted mRNA. Saturation of enzymes or transport systems by viral vectors expressing high levels of shRNA may interfere with endogenous microRNA processing, leading to toxicity (4, 5).

To incorporate within RISC, double-stranded RNA longer than 23 nucleotides is cleaved by Dicer to form 19-23 siRNA duplexes with 5′-phosphorylated ends and 2-nucleotide unpaired and unphosphorylated 3′-ends. Notably, RNA duplexes larger than 30 nucleotides cause potent nonspecific gene silencing and an inflammatory interferon response. Thus, siRNA applications rely on synthetic 19-29 base pair double-stranded siRNA (6-8). Inside the cell, siRNA is incorporated into RISC, a protein–RNA complex that separates the strands of the RNA duplex and discards the sense strand (Fig. 1). The antisense RNA strand then guides the activated RISC to anneal and cleave the target mRNA (9). The endonuclease argonaute-2 plays a key role in unwinding the duplex (sense and antisense siRNA strands) and degrading the target mRNA (10). By recycling the target mRNA, the activated RISC complex may show a therapeutic effect for up to 7 days in dividing cells and for several weeks in nondividing cells. Furthermore, repeated administration of siRNA can result in stable silencing of its target (11).

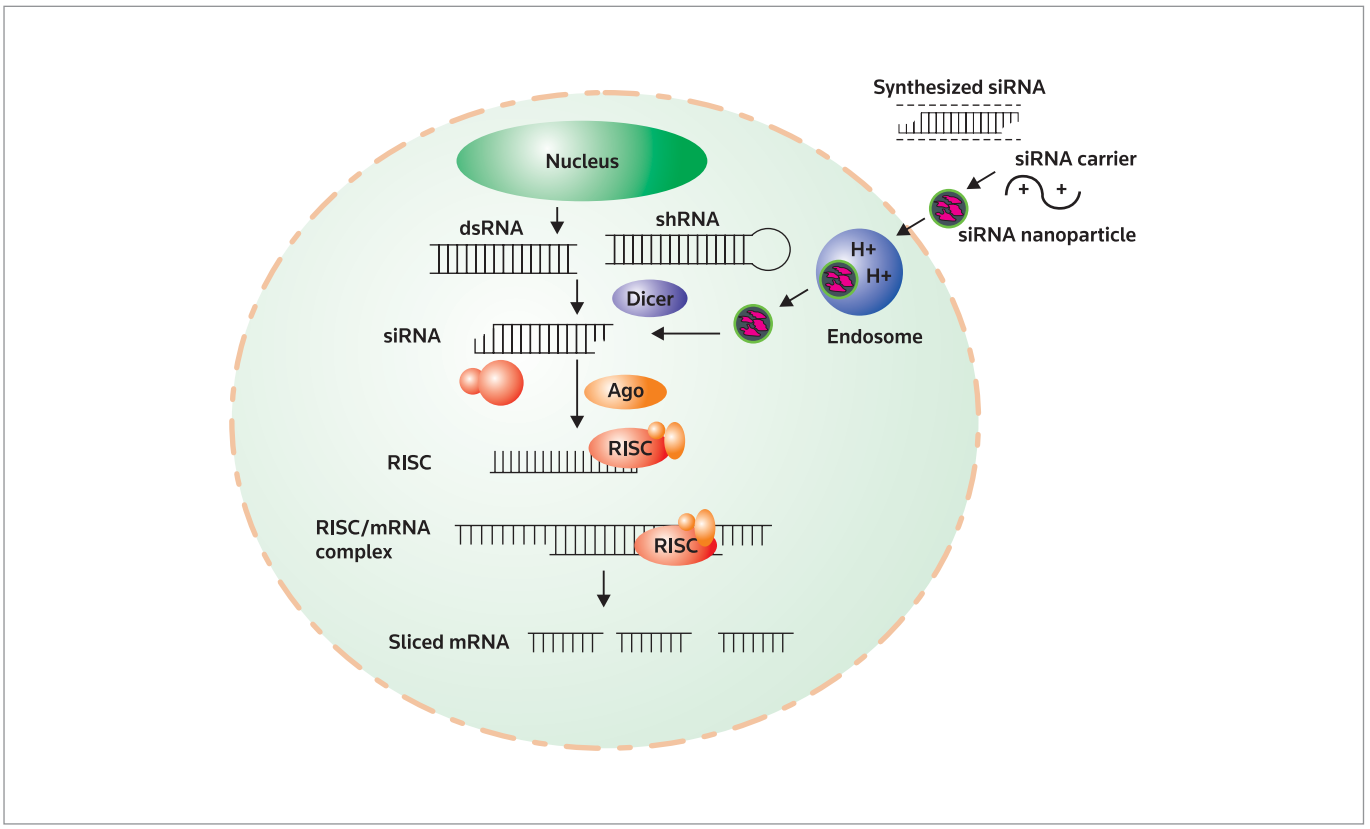

Figure 1. Mechanism of mRNA degradation by siRNA. The uptake of siRNA delivered by nanoparticles is by endocytosis. Once released by the nanoparticle into the cytosol, siRNA between 19 and 23 bases is incorporated into the RNA-induced silencing complex (RISC), a protein–RNA complex that separates the strands of the RNA duplex and discards the sense strand. The antisense RNA strand then guides the activated RISC to anneal and cleave the target mRNA. The endonuclease argonaute-2 plays a key role in unwinding the duplex and downregulation of the specific mRNA. Following mRNA cleavage, the activated RISC is capable of many rounds of mRNA cleavage. Promoter-based shRNA requires processing by nucleus and Dicer before incorporation into RISC.

Despite the promise of efficient and selective siRNA gene inhibition as a targeted therapeutic modality, it shares the classic delivery problem of antisense and gene therapies: nucleic acids are highly negatively charged and cannot easily be transported to the cytosol. The primary focus of this review will be to discuss recent in vivo advances and methods of systemic nonviral delivery of siRNA (Table I).

OVERVIEW OF siRNA DELIVERY

Nonviral methods for in vivo siRNA delivery can be broadly classified into noncarrier and carrier approaches. Noncarrier siRNA delivery systems ("naked siRNA") usually depend on diseases or disease models in which local delivery may be effective. Such disorders that do not necessarily require a carrier include macular degeneration, wounds and infectious respiratory diseases (12, 13). In addition to localized delivery, systemic delivery of siRNA without a carrier may be possible using the hydrodynamic method and/or heavily modified siRNA. Systemic use of the hydrodynamic method is likely to be confined to animal models, but this approach may be useful in treating diseases localized to particular organs or limbs. The treatment of many diseases (in humans and animal models) will depend on systemic siRNA delivery, and success for this form of therapy requires the development of appropriate new carriers. Thus far, there have been no clinically proven effective systemic carriers for siRNA. Nonetheless, several vehicles for systemic delivery of siRNA are currently being tested for their efficacy in animal studies, including liposomes (14-19), cyclodextrin (20), polymers such as polyethylenimine (PEI) (21-23), peptides (24-31), micelles (32, 33), siRNA conjugates (18, 34-39), antibody–protamine fusion carriers (40, 41) and polyconjugates (42). Carriers of siRNA have targeted an array of diseases, including genetic disorders, infectious diseases of the liver, cancer and ocular diseases.

MODIFICATION OF siRNA FOR IMPROVED siRNA DELIVERY

Modifications of siRNA may greatly influence its activity and selection of the carrier (16-18). Many factors can affect the success of siRNA-mediated gene silencing, including its modification, the target sequence of siRNA and the type of carrier. The selection of the siRNA may also affect how it binds to the carrier and the overall stability and toxicity of the carrier. Thus, it is essential to consider the length of siRNA, the type of modification of siRNA and the siRNA carrier in terms of the carrier and the particular disease being treated.

Table I. *Systemic delivery of siRNA.*

Delivery method/vehicle	Route	Target organ	Target gene	Results	Ref.
Hydrodynamic	IV	Liver	*FAS*	Reduction of fulminant hepatitis	77
			CASP8	Reduction of fulminant hepatitis	76
			S gene of HBV	Inhibition of HBV replication	85
			S gene of HBV	Inhibition of HBV replication	17
		Lung	Influenza virus	Protection from lethal influenza challenge	74
		Kidney	*FAS*	Protection from renal ischemia–reperfusion injury	75
Modified siRNA targeting PTC	IV	Kidney	*TP53*	Protection from renal ischemic and nephrotoxic injury	90
Tf–PEG–CDP	IV	Neuro2A xenograft	*RRM2*	Inhibition of tumor growth	92
RVD-R9	IV	Brain	*GFP*	Inhibition of GFP in CNS	30
PEI	IV	Lung	*NP* and/or *PA* genes of influenza virus	Inhibition of virus replication	121
cRGD–PEG–PEI	IV	Xenograft	*KDR*	Inhibition of tumor growth	21
Atelocollagen	IV	Eye	*VEGFA, FLT1, KDR*	Inhibition of angiogenesis	124
	IV	PC-3 xenograft	*BCLX*	Inhibition of tumor growth	129
	IV	Bone	Enhancer of zeste homolog 2 (*EZH2*) siRNA	Inhibition of tumor bone metastasis	132
Branched histidine–lysine peptide	IV	Breast, squamous cell cancer xenografts	*RHBDF1*	Inhibition of tumor growth	29
HIV-specific Ab–protamine fusion	IV	HIV envelope-expressing melanoma xenografts	*MYC, MDM2, VEGF*	Inhibition of tumor growth	40
Anti-LFA-1 scFv–protamine fusion protein	IV	K-562 cells engrafted in lungs	*CCND1*	Inhibition of lymphocyte proliferation	41
Tf–HoKC DOTAP/DOPE liposome	IV	Breast cancer	*HER2*	Inhibition of tumor growth	170
Apo1-DOTAP liposome	IV	Liver	*X* gene of HBV	Reduction in HbsAg	154
Lactosylated DOTAP liposome	IV	Liver	Untranslated region (most effective targeted nucleotides 325-344)	Decrease in HCV replication	155
Cardiolipin/DOPE liposome	IV	Breast cancer xenograft	*RAF1*	Inhibition of tumor growth	15
SNALP liposome	IV	Ebola virus	Ebola virus polymerase gene	Protection against death	16
		Liver	*APOB*	Reduction in Apo B protein	18
DOPC liposome	IV	Ovarian carcinoma	*FAK*	Inhibition of tumor growth	158
	IV or IP	Ovarian carcinoma	*EPHA2*	Inhibition of tumor growth	159, 160
	IP	Ovarian carcinoma	*IL8*	Inhibition of tumor growth	161
	IV	Melanoma	*PAR1*	Inhibition of angiogenesis	162
Anti-β-7 Ab-conjugated DOPC liposome	IV	Colon	*CCND1*	Suppression of leukocyte proliferation	19
Cholesterol-siRNA conjugate	IV	Liver	*APOB*	Reduction in Apo B, cholesterol and LDL	35

Continued

Table I. *Cont. Systemic delivery of siRNA.*

Delivery method/vehicle	Route	Target organ	Target gene	Results	Ref.
Lipophile-siRNA conjugate	IV	Liver	*APOB*	Different conjugates, different target organ	163
α-Tocopherol-siRNA conjugate	IV	Liver	*APOB*	Downregulation of Apo B	36
siRNA polyconjugates	IV	Liver	*APOB, PPARA*	Downregulation of Apo B	42
PEG-siRNA conjugate micelles	IV	Prostate cancer xenograft	*VEGF*	Downregulation of VEGF PEI core-forming agent	33
Aptamer-siRNA conjugate	IV	Prostate cancer xenograft	*BCL2*	Inhibition of tumor growth	38

Ab, antibody; Apo B, apolipoprotein B; CDP, cyclodextrin-containing polycation; CNS, central nervous system; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; GFP, green fluorescent protein; HbsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IV, intravenous; LDL, low-density lipoprotein; PEG, pegylated; PEI, polyethylenimine; PTC, proximal tubule cells; RVG-R9, rabies virus glycoprotein peptide conjugated to a 9-mer polyarginine; SNALP, stable nucleic acid-lipid particle; Tf, transferrin; VEGF, vascular endothelial growth factor.

Length

Currently, 21-mer RNA duplexes, mirroring natural siRNAs, are the most commonly used for laboratory research or clinical development. Others include blunt 19-mer (43), blunt 25-mer (44), blunt 27-mer (7) and asymmetric 25/27-mer (8); siRNAs larger than 23-mers are enzymatically processed by the endonuclease Dicer into shorter species before loading into RISC. One of the more interesting alternative siRNAs is the asymmetric 25/27 duplex with 3′-DNA residues on the blunt end (called the 'R' duplex); these siRNAs are reported to be usually more potent than the more commonly used 21-mer duplexes (8). Moreover, these 'R' duplexes potentially have fewer off-target effects than the 21-mer duplex because of increased specificity in targeting the mRNA. Furthermore, Siolas et al. reported that synthetic 29-mer shRNAs were more potent inducers of RNAi than siRNAs (3). In addition to the biological differences in their siRNA efficacy, the length of the siRNA may have an essential role in stabilizing nanoparticles, particularly with peptide delivery systems.

siRNA chemical modification

siRNA in complex with cationic carriers generally activates proinflammatory cytokines significantly more than siRNA without carriers (45, 46). In complex with cationic carriers such as liposomes, specific sequences, such as 5′-UGUGU-3′, or less defined sequences within the siRNA duplex are immunostimulatory both in vitro and in vivo (45, 47, 48) (Fig. 2). In complex with cationic liposomes, siRNA activates cytokines by binding primarily to Toll-like receptor 7/8 (TLR7/8) in acidic endosomes. siRNA activates these receptors in a sequence-dependent manner and the pH-buffering agent chloroquine is known to suppress this activation (49). Indeed, carriers of siRNA with greater pH-buffering capacity may significantly decrease cytokine activation (unpublished results). Whether the nanoparticles provide an adjuvant template that activates the receptor or whether this is due to the mass action of a large number of siRNAs presented to the TLR within endosomes is not known. Nucleoside modification and nonactivating sequence selection are strategies currently available to avoid immunostimulation in siRNA technology. Nonetheless, with some therapeutic strategies, it may be desirable for the siRNA to induce cytokines to target viral infections or cancer (49, 50). For example, by inducing interferon alfa, siRNA may prove to be effective in reducing viral titers, including influenza (51) and hepatitis B virus (HBV) (17).

Chemical modification of siRNA can increase the stability of the RNA duplex to nucleases, minimize the possibility of immunostimulatory responses, decrease the possibility of off-target effects and improve its pharmacodynamic properties (52). Chiu and Rana analyzed the relationship between chemical modification and the efficiency of siRNA silencing by examining 30 different types of siRNA modifications (53). These modifications included replacement of the 2′-hydroxyl group of ribose with 2′-fluoro, 2′-*O*-methyl and 2′-hydrogen groups, or replacement of the phosphate backbone with phosphorothioate or boranophosphates. Compared to an unmodified GFP (green fluorescent protein) siRNA, the majority of siRNA modifications decreased the efficacy of silencing GFP. Limited siRNA modifications, however, with 2′-fluoro-, 2′-*O*-methyl and phosphorothioate may increase the half-life and stability of siRNA in cells without affecting their silencing efficacy (17, 53, 54). In addition, 2′-*O*-methyl modifications significantly reduced cytokine induction by antagonizing TLR7/8 receptors (17, 51, 55, 56). Initially, most groups used unmodified siRNA, but investigator awareness of off-target effects by unmodified siRNA duplexes has made selected modifications of siRNA with 2′-*O*-methyl and phosphorothioate linkages more common (57).

DELIVERY OBSTACLES

The prospects for siRNA-based therapeutics to significantly improve metabolic, cancer or systemic infection treatment options have been limited by the inability to identify an effective carrier. Recent clinical studies indicate that carrier development is indeed one of the most important problems to be addressed, if not the most important. The in vivo obstacles for the siRNA nanoparticles are, not surprisingly, similar to those of gene therapy nanoparticles and depend on the route of administration, which can generally be divided into three categories. First, topical administration with siRNA includes the

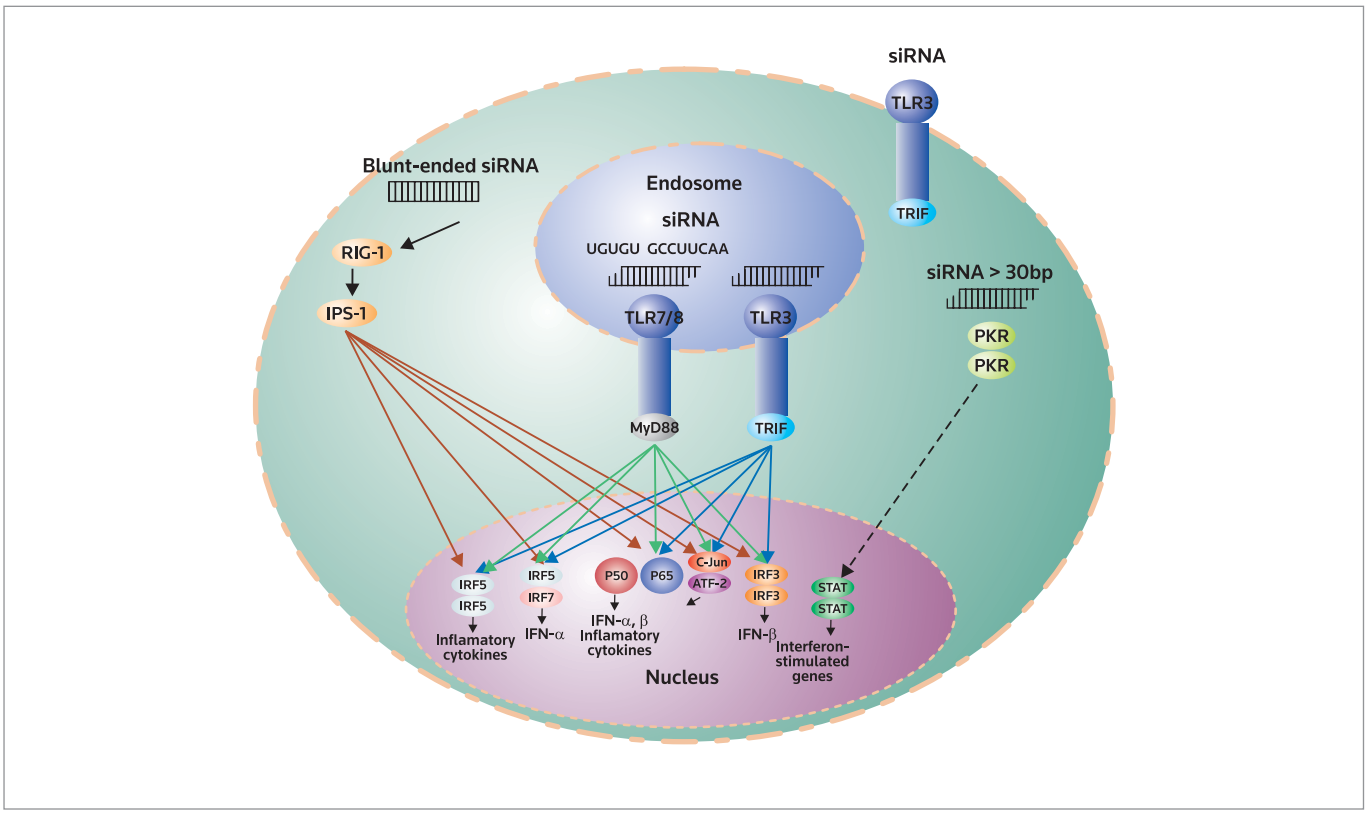

Figure 2. Induction of cytokines by siRNA. siRNAs without carriers do not activate or only poorly activate proinflammatory cytokines in vitro and in vivo. In complex with cationic carriers such as liposomes, specific sequences, such as 5'-UGUGU-3' and 5'-GCCUUCAA-3', or less defined sequences within the siRNA duplex are immunostimulatory both in vitro and in vivo. siRNA activates cytokines primarily by binding to TLR7/8 in acidic endosomes, but TLR3 located at the cell surface and within endosomes may also have a role in activating cytokines in selected cells. In addition, double-stranded RNA (including siRNA) greater than 30 bp, or blunt-ended siRNA may induce cytokines by binding to PKR and RIG-1, respectively. Whereas TLR7/8 receptors are activated by specific siRNA sequences, TLR3 and the cytosolic receptors PKR and RIG-1 are activated by siRNA independent of specific sequences. RIG-1, retinoic acid-inducible gene 1 protein; IPS-1, interferon-beta promoter stimulator 1; PKR, interferon-induced, double-stranded RNA-activated protein kinase; TLR, Toll-like receptor; IRF, interferon regulatory factor; STAT, signal transducer and activator of transcription.

treatment of diseases of the eye (stromal keratitis), skin (atopic dermatitis, wounds), vagina (herpes simplex virus) and rectum (inflammatory bowel disease). Second, local or direct administration of siRNA includes the treatment of diseases of the lungs (severe acute respiratory syndrome [SARS], influenza, respiratory syncytial virus [RSV]) or brain (Huntington's disease, gliomas). Third, systemic delivery may include treatment of diseases of the liver (hepatitis B, metabolic) or deep-seated localized or metastatic cancer. Each of these delivery methods has its own challenges, depending on the requirement of the carrier and the targeted disease. For example, with local intratumoral injections of siRNA, tissue specificity is not an issue, but widespread distribution within the tumor is a significant and challenging problem. Convection-enhanced delivery may be at least partially successful for intratumoral injections (58-67), but it is likely that additional advances will be required before this therapy will be successful.

Not surprisingly, there are significant obstacles for the systemic delivery of siRNA to its targets, including interaction with blood components, entrapment within capillaries, uptake by reticuloendothelial system (RES) cells, extravasation from blood vessels to target tissues and permeation within the tissue (Fig. 3). As long as siRNA remains within the nanoplex, filtration by the kidney glomeruli does not occur, but uncomplexed siRNA will be rapidly filtered by the glomeruli (40 kDa is the approximate molecular weight for filtration). As discussed below, glomerular filtration of the siRNA may be advantageous when the proximal tubule cells of the kidney are being targeted. For targeting hepatocytes, nanoparticles should be less than 100 nm to escape the fenestrations in vessels of the liver; this is based on the finding that chylomicrons > 100 nm cannot traverse the fenestrations and that the diameter of fenestrations varies between 50 and 150 nm in size in the human liver (68). For tumors, the vasculature may not be well formed, resulting in so-called "leaky" vessels (69-71), enabling large macromolecules to escape from the vessels into the tumors. In addition, accumulation of nanoparticles within tumors may be due to high microvessel density, increased vascular permeability due to vascular mediators (e.g., vascular endothelial growth factor [VEGF]) and impaired lymphatic clearance; collectively, these factors are known as Enhanced

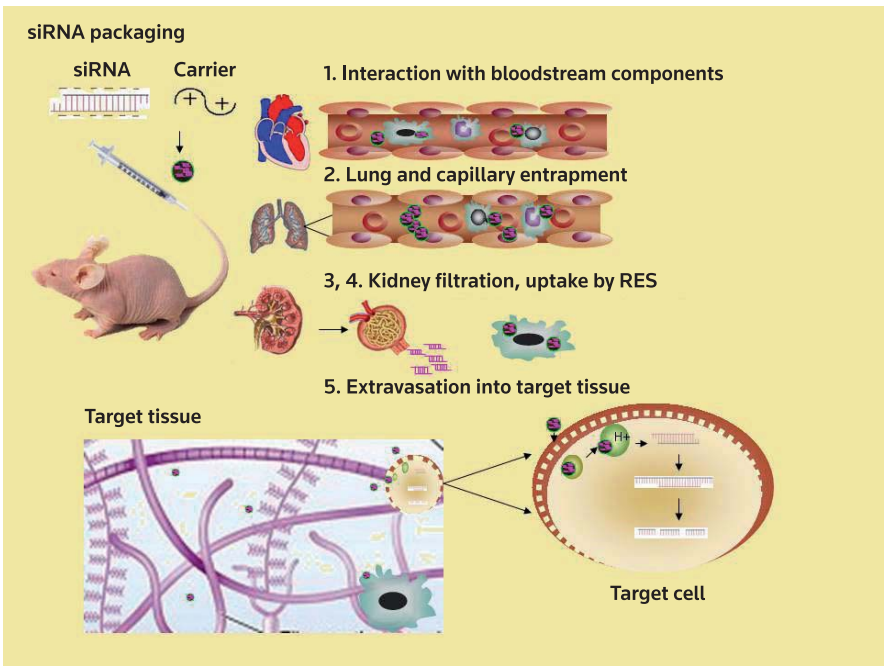

Figure 3. Barriers for systemic delivery of siRNA. There are five major extracellular barriers that nanoparticles delivering siRNA must overcome to reach the target cell. These obstacles are: 1) interaction with blood components; 2) entrapment within capillaries of lungs and other tissues; 3) uptake by reticulo-endothelial system (RES) cells and other phagocytic cells; 4) filtration by kidneys; and 5) extravasation from blood to target tissues.

Permeability and Retention (EPR). Thus, unless the siRNA nanoparticle is only targeting the tumor blood vessels, it is important that the nanoparticle enter and pervade within the tumor. Our laboratory often targets tumors with siRNA that has dual inhibitory functions. For example, Raf-1 has an important role in tumor angiogenesis and tumor cell growth, and as a result, the *RAF1* siRNA nanoparticle will likely be more effective if it accumulates in both tumor blood vessels and perivascular tumor cells.

Although considerable overlap exists between the requirement for a carrier and different routes of delivery, it is frequently necessary for a carrier to deliver siRNA systemically rather than topically or locally. There are two notable exceptions that may successfully use siRNA injected systemically without a carrier: one is the hydrodynamic delivery method and the other utilizes a modified siRNA that takes advantage of its glomerular excretion. After discussing these two notable exceptions, we will review the current carriers used for siRNA therapy.

"NAKED" siRNA

Hydrodynamic delivery of siRNA

The hydrodynamic injection method involves rapid injection of a large volume of physiological solution (about 10% of body weight administered within 5-10 s) containing nucleic acid (72, 73). After injection of a relatively large volume in the tail vein of rodents, the liver is the primary target for this approach, although other tissues, including the lung (74) and kidney (75), have been targeted with lower efficiency. Originally, this approach was used for plasmids, but it has since been used successfully for the transport of siRNA (76-78), protein (79, 80) and synthetic compounds (80, 81) to the liver. The precise mechanism of entry of the siRNA is unclear, but volume overload, right ventricular overload, increased hydrodynamic pressure, hepatic congestion and enlargement of the fenestrae of the liver play an important role in their entry into hepatocytes. In addition, as a result of transient physical injury, siRNA may enter hepatocytes through macropinocytosis (82) or evanescent "pores" (83) in the cell membrane. In contrast, other cells of the liver, such as Kupffer cells and endothelial cells, do not have high uptake of nucleic acid (84).

There have been several applications for utilizing the hydrodynamic tail vein (HTV) method with siRNA. For example, by targeting apoptotic genes such as *Fas* (77) or *Casp8* (76), the incidence of fulminant hepatitis was reduced. Activation of FAS may occur as a result of viral infections or transplantation and caspase-8 has a critical role downstream from FAS and other death receptors. Codelivery of a plasmid expressing HBV with an siRNA targeting the *S* gene of the virus via the HTV approach resulted in reduction of serum hepatitis B surface and envelope antigens by approximately 80% on day 11

(85). With a similar approach targeting the *S* gene, modified and unmodified siRNA reduced HBV DNA levels by 3.7 and 2.2 log, respectively (56). Investigators have also used HTV to knock down peroxisome proliferator-activated receptor α (PPARα) in livers of mice and achieve metabolic phenotypes similar to those observed in PPARα$^{(-/-)}$ knockout mice (78).

Although 100-150 mL of DNA solution has been administered safely to pigs (86), we doubt that systemic delivery of nucleic acid by the hydrodynamic approach will find therapeutic application in humans. Nevertheless, the hydrodynamic approach with siRNA may gain acceptance for clinical applications when applied locally to diseases of the liver and other organs (87). With insertion of a balloon catheter into the hepatic vein, siRNA injection into a branch of the portal vein could be done repeatedly, avoiding the volume overload problems of HVT (88). Further control of the amount of fluid delivered and associated hydrodynamic pressure may be monitored in real time, reducing the side effects of this therapy (89).

Modified siRNA targeting proximal renal tubules

Systemic delivery of siRNA without carriers is primarily limited to the hydrodynamic approach. The exception is the treatment of acute renal injury. In a study by Molitoris and coworkers, acute renal injury was induced by the ischemia–reperfusion method or cisplatin in rats (90). Because a large amount of siRNA is excreted by the glomerulus and then reabsorbed in the proximal tubule, the kidney is an excellent organ to target with "naked" siRNA. Indeed, the accumulation of free siRNA in the kidney is 40 times higher than in any other organ. In addition, because the proximal tubule cell is most severely affected in acute renal injury, localization of high amounts of siRNA within this cell is ideal for therapy. On the basis of a previous report in which a chemical inhibitor of the proapoptotic p53 provided renoprotection (91), it was reasoned that an siRNA targeting p53 might also provide similar protection. With renal injury induced by ischemia–reperfusion, the investigators determined that a single systemic injection of a *Tp53* siRNA (12 mg/kg) 4 h after induction of acute renal injury provided significant biochemical and morphological protection. Similarly, multiple injections of *Tp53* siRNA administered at 4 h and on day 2 and day 3 after cisplatin treatment reduced renal injury compared to controls. The *Tp53* siRNA was modified by alternating 2-*O*-methyl modifications within its sequence (90), thereby prolonging its half-life in serum and within the cell. Currently, QP-1002 is being developed by Quark Pharmaceuticals for systemic delivery of an siRNA targeting p53 in acute renal injury and delayed graft function.

CATIONIC POLYMERS

Cyclodextrins

Considerable effort has been directed toward the development of siRNA nanoparticles targeting tumors, and with its entry into clinical trials, the cyclodextrin-containing polycation (CDP) nanoparticle has advanced further than other carriers. As a result, much can be learned from the preclinical literature on this carrier and perhaps be applied to other carriers. The CDP-siRNA nanoparticle has been used primarily to target tumors and is comprised of three components: the cationic CDP polymer which binds siRNA, an adamantine–polyethylene glycol (AD–PEG) stabilizing agent and an adamantine–polyethylene glycol–transferrin (AD–PEG–Tf) targeting component. Because there is an overabundance of transferrin receptors on many tumors, uptake within tumor cells of the CDP labeled with the Tf targeting ligand was increased. Notably, since adamantine has a very high binding affinity toward cyclodextrins (10^4-10^5 M^{-1}), it provides a simple method to attach stabilizing and targeting components to cyclodextrin. In addition, imidazoles are conjugated to the backbone of cyclodextrins to enhance disruption of endosomes and cellular trafficking of the nanoparticle. Interestingly, cyclodextrin binds to siRNA even when these two components are injected separately into the bloodstream of mice. Initially, investigators demonstrated that the CDP-siRNA nanoparticle targeting the *EWS-FLi1* fusion gene inhibited Ewing's sarcoma xenografts, and more recently, the investigators determined that the CDP carrier of an siRNA targeting ribonucleoside-diphosphate reductase subunit M2 (*Rrm2*) had potent antitumor activity (92). Although an excess of cyclodextrin is required to form the nanoparticle with free polymers, the nanoparticle is stable in the bloodstream. In contrast to cationic liposomal carriers, Tf–PEG–CDP in complex with siRNA does not elicit an immunostimulatory response in mouse models. Moreover, the Tf–PEG–CDP *Rrm2* siRNA can be administered safely to nonhuman primates. Tf–PEG–CDP in complex with *Rrm2* siRNA (CALAA-01) has been approved for clinical trials.

Cationic cell-penetrating peptides

Cationic cell-penetrating peptides (CPPs) have been used to carry macromolecules, including plasmids, proteins, peptides and, more recently, siRNA, across membranes into cells in vitro and in vivo (93-97). CPPs are small arginine-rich peptides that include HIV-1 Tat (98-100), Penetratin™ from the antennapedia protein (101), transportan (a hybrid derived from galanin and mastoparan) (102) and polyarginine-synthetic peptides (103-108). These arginine-rich peptides range from 8 to 30 amino acids in length and interact with negatively charged glycosaminoglycans on the cell surface (109, 110).

Although CPPs, along with their cargos, were initially considered to enter cells through a fusogenic mechanism, more recent reports have determined that CPPs enter cells primarily by macropinocytosis, a type of endocytotic pathway (111-113). siRNA has been delivered by CPPs by two methods: 1) by conjugating siRNA to the CPP (114-116); and 2) via a noncovalent CPP-siRNA polyplex (96). Conjugation of siRNA with CPP has shown conflicting results. Whereas in vitro results have not clearly demonstrated efficacy, animal models using the CPP conjugates have demonstrated efficacy in downregulation of the target gene (114-116). Although investigators suggested that these CPP-siRNA conjugates were soluble, more recent results indicate that their efficacy may have been due to their forming nanoparticles (96). When larger nanoparticles were removed, the remaining soluble conjugates ineffectively suppressed gene expression. Thus, it appears that soluble CPP-siRNA conjugates may not effectively downregulate their targets.

As a potential solution to this problem with conjugates, Eguchi et al. developed a CPP containing a duplex siRNA-binding domain (DRBD) (95). CPP-DRBD-delivered siRNA induced rapid RNAi in a

large percentage of primary and transformed cells. At least with in vitro cell culture studies, this appears to be an effective carrier for siRNA, but this approach has not been tested in animal models.

In contrast to low-molecular-weight soluble CPP-siRNA conjugates, noncovalent CPP-siRNA polyplexes effectively reduce their target in vitro. Of note, two groups have shown that polyarginine fusions are effective carriers in vivo (117, 118). When a cholesterol–polyarginine (a 9-mer arginine, R9)/*Vegf* siRNA nanoparticle was prepared and added to CT-26 cells, VEGF levels in the medium were reduced by 40% compared to untreated or polyarginine/*VEGF* siRNA controls (117). This approach was further validated when this cholesterol–polyarginine carrier of *Vegf* siRNA administered intratumorally decreased CT-26 xenografts by approximately 7-fold over a 17-day period. The second group created a fusion product between a 9-mer arginine-rich peptide and a short peptide derived from rabies virus glycoprotein (RVG), a protein that recognizes the nicotinic acetylcholine receptor on neuronal cells (30). Interestingly, using a GFP transgenic mouse model this group found that RVG-R9/GFP siRNA injected i.v. crossed the blood–brain barrier and decreased GFP expression in the brain, while not affecting its expression elsewhere. This is the first report demonstrating that targeted CPPs can transport siRNA across the blood–brain barrier.

Cationic synthetic polymers

Of the synthetic polymers, such as the dendrimer polyamidoamine (PAMAM) and PEI, that have shown efficacy as carriers of siRNA (119, 120), only PEI has been extensively explored as a carrier of siRNA for in vitro and in vivo studies. PEI is a synthetic cationic polymer with a linear or branched structure. Because PEI effectively binds and condenses nucleic acids into stabilized nanoparticles, it has been widely used as a carrier for oligonucleotides, plasmids and siRNA. In addition to its ability to condense nucleic acids, the pH-buffering property of PEI disrupts endosomes, thereby enabling nucleic acids to reach the cytosol. Although plasmids must still reach the nucleus, siRNA only needs to reach the cytosol where RISC is formed to degrade mRNA. The commonly used PEI in complex with siRNA has been administered locally and systemically (either i.p. or i.v.). Although unmodified branched PEI nanoparticles have frequently been used for cell culture transfection experiments, a few studies have used unmodified PEI nanoparticles in animal models.

For example, Ge et al. found that systemically delivered PEI-siRNA nanoparticles inhibited influenza virus in mouse lungs (121). When 60 µg of siRNA was injected i.v., there was a 10-fold reduction in virus titers in the lungs, and when 120 µg of siRNA was used, a > 1,000-fold reduction in lung virus titers was observed in some mice. Despite this apparent success, branched 25-kDa PEI in complex with nucleic acids is known to be toxic to the lungs, and it is likely that part of the reduction in viral titers in the lungs may have been due to toxicity of the PEI polyplex. Moreover, the commonly used branched PEI in cell culture transfection experiments and/or siRNA delivery is generally known to be toxic to most cells. Thus, there is significant concern regarding toxicity of nanoparticles formed from siRNA and unmodified PEI with high molecular masses (e.g., 25 kDa) and doses, and the clinical use of high-molecular-weight unmodified PEI will likely be quite limited (122, 123).

Strategies to modify the structure of PEI to reduce toxicity while retaining its potent ability to deliver siRNA are being developed. One strategy to reduce the toxicity of PEI-siRNA nanoparticles is to develop particles that would increase the target specificity of the particle. An example of this approach was reported by Schiffelers et al., who developed self-assembling PEI-siRNA nanoparticles targeting tumor angiogenesis. The investigators targeted a critical receptor in tumor angiogenesis, VEGFR-2, which is upregulated in mitogenic endothelial cells. When injected i.v. through the tail vein of tumor-bearing mice, PEGylated PEI with an RGD peptide ligand in complex with *Kdr* siRNA inhibited the growth of neuroblastoma xenografts by about 90%. Biological activity of the siRNA asssociated with PEGylated PEI was found to be sequence-specific and the specificity of the nanoplex for tumor vessels was dependent on the presence of peptide ligand and could be competed by free peptide (21). Although the investigators did not perform toxicity studies, reduced toxicity to nontumor tissues (e.g., liver, lung) is expected because of the greater specificity.

Using these targeted pegylated PEI carriers, siRNA polyplexes targeting *Vegfa*, *Flt1* and/or *Kdr* also reduced angiogenesis in two models of ocular diseases (124). These PEI polyplexes were effective when given locally and/or systemically, and interestingly, the mixture of three siRNAs reduced angiogenesis more than a single siRNA inhibitor. By pinpointing several targets in the VEGF pathway in a nontumor disease, the mixture of siRNAs may be particularly effective in those systems in which compensatory mechanisms are limited. Furthermore, the ability to direct PEI-siRNA nanoparticles with the RGD ligand specifically to diseases with increased angiogenesis makes it likely that other ligands attached to PEI will show equal or greater specificity. In experiments with KB epidermal carcinoma cells, which have high levels of folate receptors, a folate-modified PEI (125) in complex with GFP-siRNA reduced GFP expression by 80%, while unmodified PEI-GFP-siRNA reduced GFP expression by 10% (N/P ratio, 16:1). Moreover, the type of PEG and PEGylation pattern should be considered. It is generally accepted that addition of PEG to PEI is required for greater specificity, longer half-life and reduced immunogenicity. Less appreciated is that the degree and pattern of PEGylation of PEI can reduce toxicity, such as erythrocyte aggregation and hemorrhage (126).

Other groups have been successful in minimizing toxicity by using low-molecular-weight (LMW) PEI or biodegradable PEI. LMW PEI (4-10 kDa) in complex with plasmids and/or siRNA has minimal toxicity when compared to the 25-kDa PEI (23, 127). Furthermore LMW PEI fully protects siRNA against enzymatic degradation and delivers siRNA into cells, where it efficiently induces RNAi. Although the LMW form of PEI is an effective carrier of siRNA in cell culture experiments, the utility of the LMW PEI nanoplexes for systemic delivery will likely depend on their stability to blood components.

A third approach to develop PEI polyplexes with low toxicity was to synthesize a ketalized PEI carrier. These ketalized carriers were found to be significantly less toxic than unketalized PEI. Branches of PEI were modified with acid-degradable amine groups via ketal linkages. Because the ketal linkages are sensitive to mildly acidic conditions around pH 5, this bond rapidly breaks apart in acidic endosomes. Once the ketalized amine-containing groups of PEI are degraded, siRNA efficiently dissociates from the polymer. Notably,

only the ketal groups of PEI are biodegradable, while the PEI template is not. Whereas LMW PEI favors plasmid delivery, higher molecular weight increases siRNA import. These three approaches or a hybrid of these approaches suggest the possibility of developing a nontoxic targeted PEI delivery system for siRNA.

Atelocollagen

The cationic atelocollagen, prepared from calf dermal collagen, shows low antigenicity, and through ionic interactions it forms a macromolecular complex with DNA or RNA. Two groups from Japan have shown the efficacy of atelocollagen as a carrier of siRNA in several animal models targeting bioluminescent bone metastases, antiapoptotic factors in tumor xenografts and monocyte chemoattractant protein 1 (MCP-1) in inflammatory diseases (128-133). For example, i.v. administration of the atelocollagen *Bcl-x* siRNA (100 µg of the siRNA) reduced tumor growth by about 40%, and combined therapy with the *Bcl-x* siRNA particle and cisplatin showed synergy, reducing tumor growth by about 75% (129). For systemic injections, the complex was first prepared by mixing soluble atelocollagen (0.05%) with siRNA at 4 °C, and then upon warming to room temperature, a macromolecular complex between 100 and 300 nm was formed. Initial studies showed that atelocollagen particles can be administered safely without the induction of cytokines or tissue toxicity. Although these complexes have not been modified to target tumors or inflammatory tissues, the particles accumulate selectively within these diseased tissues because of the enhanced permeability and retention (EPR) effect.

Polylysine and lysine-rich polymers

Polylysine was one of the earliest carriers of nucleic acids. Hanson et al. demonstrated that galactosylated polylysine effectively delivered plasmids to hepatocytes in vivo (134). By titrating the ionic strength of the solution, polylysine–plasmid polyplexes formed nanoparticles that were between 10 and 20 nm (134, 135). Although these initial studies were promising, the use of polylysine as a carrier was associated with cytotoxicity, including complement activation (136-139) and red blood cell lysis (140). Modification of polylysine with PEG reduced the side effects (136) and PEG–polylysine–plasmid nanoparticles are currently in phase I/II clinical trials. As yet, unmodified polylysine carriers have not been effective carriers of siRNA (28).

In contrast, various modifications of the polylysine carriers have yielded effective delivery systems for siRNA. One such modification that increases the ability of lysine-rich peptides to transport nucleic acids is the addition (or incorporation) of histidines into the lysine peptide (for a recent review of histidine–lysine-containing peptides, see Ref. 26). When a reducible oligolysine was compared to a reducible oligolysine–histidine peptide, the lysine–histidine peptide was a more effective carrier of siRNA (28). While lysine is important for binding DNA/RNA, histidine has an important role in buffering acidic endosomes, thereby leading to endosomal disruption and release of nucleic acid (141). Histidines may also have a role in stabilizing the nanoparticle (142). Furthermore, specific ratios and patterns of histidine and lysine have been found to enhance siRNA delivery (143). With a peptide synthesizer, the specific patterns of histidines and lysines can be varied to optimize the histine–lysine peptide carrier for a particular form of nucleic acid. Histidine–lysine peptides with higher lysine content are usually more effective carriers of plasmids, but are not effective carriers of siRNA. In contrast, carriers with a higher ratio of histidine to lysine content are more effective carriers of siRNA. With branched histine–lysine peptides carrying *Raf1* siRNA, there was an approximately 60% inhibition of the growth of human breast carcinoma MDA-MB-435 xenografts, without toxicity. In a second study that validated the histidine–lysine polymer as a useful systemic carrier of siRNA, histidine–lysine polymer in complex with human rhomboid family member 1 (*Rhbdf1*) siRNA significantly reduced target expression and tumor growth in a mouse xenograft model (29). Recently, Stevenson and colleagues have demonstrated that reducible histidine–lysine peptides of lower molecular weight are effective carriers of siRNA (28). These studies suggest that binding and release of the siRNA (or plasmids) from the carrier are critical and the design of polymers for siRNA and plasmids may differ.

In place of the endosomolytic histidine-rich peptides, Meyers et al. coupled the endosomolytic agent mellitin to pegylated polylysine (144), and also conjugated siRNA with peptide through a disulfide linkage to prevent extracellular dissociation. Because of the high reducing intracellular potential (about 5 mM levels of glutathione), however, siRNA would be released from the polymer within the cytosol. For cell culture experiments, the polymer-siRNA conjugate formed by disulfide bonds showed superior results when compared to polymer-siRNA nanoplexes formed by ionic interactions. For animal studies, however, these nanoparticles proved to be toxic to the liver and lung when administered i.v.

Protamine

Protamines are low-molecular-weight proteins (50-110 amino acids) that can contain up to 70% arginine (145). Over 40 years ago, protamine was recognized to stimulate the uptake of nucleic acids (146-148). More recently, protamine was noted to interact with plasmid DNA to form nanoparticles for transfecting cells in vitro and in vivo. To augment transfection, protamine has often been added to plasmids or antisense oligodeoxynucleotides to increase the stability of the nanoparticles, and/or neutralize and condense the nucleic acid to enable encapsulation (149). Similar approaches have been used for siRNA delivery. By neutralizing and condensing siRNA, protamine has been useful in encapsulating siRNA within liposomes (19, 150) (see Liposome section below for further discussion).

Antibody–protamine fusion carriers for siRNA have been used in several animal models mirroring human diseases. To inhibit tumor growth, investigators implanted a melanoma cell line expressing the HIV envelop protein. A single-chain antibody–protamine construct in which an Fab fragment targeting the HIV envelope was fused with protamine in complex with an siRNA cocktail (*Myc, Vegf* and *Mdm2* siRNA) reduced melanoma growth. While intratumoral injection of the cocktail siRNA inhibited tumor growth by approximately 80% compared to untreated controls, i.v. injection of the nanoparticle inhibited tumor growth by approximately 60%. This inhibition was specific in that tumors not expressing the HIV envelope were not inhibited. A more clinically relevant erbB-2–protamine fusion protein in complex with siRNA specifically reduced the growth of breast cancer cells (40).

In addition to these in vivo experiments, Song et al. showed that an HIV-specific antibody–protamine siRNA nanoparticle targeting the HIV capsid gene *gag* inhibited HIV replication in difficult-to-transfect T lymphocytes in vitro (40); these data coupled with the in vivo data for tumors expressing the HIV envelope suggest that this carrier might be effectively used in HIV. In another application, an anti-LFA-1 antibody–protamine fusion product carrying cyclin D1 (*Ccnd1*) siRNA was able to suppress gene expression and cell proliferation in activated lymphocytes, which are usually difficult to transfect with nonviral carriers. One anti-LFA-1 antibody, AL-57, has high affinity for human LFA-1 integrin, which undergoes a conformational change in activated lymphocytes (41). In a mouse model in which human myelogenous leukemia K-562 cells expressing activated LFA-1 were engrafted in the lungs, the AL-57 antibody–protamine carrier of siRNA injected i.v. specifically delivered fluorescently labeled siRNA to the cells. Notably, there was no evidence of cytokine induction or toxicity with these fusion siRNA products. Moreover, with six siRNA in complex with the antibody–protamine fusion construct, the molecular weight of the nanoparticle would be about 100 kDa, significantly greater than the 40-kDa cut-off for renal filtration. These experiments using different targets and animal models demonstrate the safety, flexibility and utility of the antibody–protamine fusion carrier.

LIPOSOMAL DELIVERY OF siRNA

Cationic liposomes

Liposomes have been used for the delivery of nucleic acids for over 25 years, as first demonstrated by their ability to transport the preproinsulin gene to the liver (151). Because of initial technical challenges encountered with the incorporation of negatively charged DNA within neutral liposomes, this methodology was soon supplanted once cationic liposomes were developed in 1989 (152). Cationic liposomes can combine quickly with negatively charged nucleic acids to form lipoplexes, and the ease of preparation of these lipoplexes has enabled many researchers to study their favorite gene in vitro and in vivo. Indeed, many cationic liposome products developed initially for gene delivery have been modified for siRNA delivery, e.g., Lipofectamine 2000 (Invitrogen), DMRIE-C (Invitrogen), Oligofectamine, (Invitrogen), DOTAP (Roche Applied Science), X-tremeGene (Roche), siPORT NeoFx (Ambion) , RNAifect (Qiagen) and GeneSilencer (Genlantis).

In addition to being the most commonly used delivery agent in vitro, cationic liposome carriers have often been used for in vivo studies with siRNA. Several studies have shown that they are effective carriers for the systemic delivery of siRNA. Pirollo and coworkers targeted several different xenografts with an *Erbb2*-specific siRNA in complex with 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) liposomes (153). These liposomes contain histidine–lysine peptides on their surface to facilitate their escape from endosomes, as well as a single-chain antibody fragment targeting transferrin receptors, which are elevated on the membranes of tumor cells. The immunoliposome-*Erbb2* siRNA significantly inhibited the growth of pancreatic xenografts, and combining gemcitabine with these nanoparticles resulted in a synergistic interaction and almost completely inhibited tumor growth.

In addition to tumors, cationic siRNA lipoplexes have also been used to target diseases of the liver. DOTAP/cholesterol liposomes in complex with HBV siRNA successfully reduced viral protein expression of hepatitis B. Eight days after a single i.v. administration of the HBV siRNA nanoparticles (2 mg/kg), HBV surface antigen was reduced by over 70% compared to in the control siRNA nanoparticle group (154).

Watanabe and colleagues used lactosylated cationic siRNA lipoplexes and also showed marked reduction of hepatitis C expression in the liver in a transgenic mouse model (155). Importantly, these lactosylated DOTAP-siRNA lipoplexes did not induce interferon alfa.

Although the above studies with DOTAP lipoplexes show considerable promise, many lipoplexes induce a strong cytokine response (51, 156). Perhaps because these lipoplexes were targeted and administered at low doses, these results showed strong efficacy with a lack of toxicity. In addition to these encouraging results, there are three types of cationic liposomes that merit further discussion.

For in vivo studies, DOTAP/cholesterol liposomes were found to be effective for the delivery of nucleic acids, and only a few alternative lipids have been found to be equivalent or more effective. Interestingly, a liposome comprised of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cardiolipin in complex with a luciferase reporter plasmid administered i.v. resulted in tumors expressing luciferase at seven times higher levels than the DOTAP/cholesterol lipoplex. The cardiolipin analogue of the liposome was significantly less toxic than the DOTAP/cholesterol liposome; whereas two-thirds of the mice died when injected with 100 mg/kg of DOTAP/cholesterol liposomes, no mice died at a similar dose of the cardiolipin-containing liposome. In addition, *Raf1* siRNA in complex with the cardiolipid-containing liposome resulted in about 50% greater inhibition of breast cancer xenografts compared to the liposome control mismatch siRNA group. Unfortunately, there was no direct comparison with the DOTAP/cholesterol siRNA lipoplex, and the cardiolipin siRNA mismatch lipoplex inhibited tumor growth about 25% more than the free siRNA control. Despite drawbacks to this study, the significant inhibition of tumor growth with *Raf1* siRNA, the lower toxicity of the lipid and the greater in vivo transfection efficiency for the cardiolipin-containing liposomes suggest that further studies of these liposomes are needed (15).

Unlike the lipoplexes discussed in previous paragraphs, Li et al. prepared nanoparticles in which the siRNA (targeting luciferase) was internalized within liposomes (157). Minimizing direct ionic interactions between siRNA and cationic lipids may reduce cytokine induction. siRNA was incorporated within liposomes by first mixing siRNA with carrier thymus DNA and neutralizing the negatively charged nucleic acids with the highly basic protamine. The protamine/nucleic acid complex was then entrapped within DOTAP/cholesterol (1:1 molar ratio) liposomes to obtain liposome–polycation–DNA (LPD) nanoparticles. The positive charge on liposomes from the DOTAP lipids may promote interaction with the negatively charged cell membranes, thereby increasing endocytosis. For increased tumor specificity and stability of the LPD, the preformed nanoparticles were modified with PEG and anisamide. The anisamide ligand has high affinity for the sigma factor receptor that is expressed on the cell surface of several types of cancers. After a single i.v. injection, the

targeted luciferase siRNA nanoparticle downregulated luciferase levels by 70-80% in a lung cancer metastasis model compared to the targeted control siRNA nanoparticle. Interestingly, cytokine induction was minimal with this targeted LPD formulation, particularly when protamine/siRNA or DOTAP/siRNA complexes would likely induce strong cytokine responses.

Another extensively studied form of liposomes for siRNA delivery is the stable nucleic acid–lipid particle (SNALP). SNALP nanoparticles are pegylated liposomes with low cationic lipid content that incorporate nucleic acids, including siRNA, within the lipid envelope. Although SNALP contain a very low cationic lipid content for plasmid DNA delivery (molar percent 5-10%), these vehicles still contain a relatively low amount of cationic lipids for siRNA delivery (molar percent 30%). Morrissey et al. showed that in vivo delivery of siRNA-SNALP complexes that targeted HBV RNA could inhibit HBV replication (17). Three daily i.v. injections of 3 mg/kg reduced HBV levels by about 10-fold for up to 7 days. Moreover, Zimmerman et al. targeted apolipoprotein B (Apo B) with siRNA-SNALP nanoparticles in nonhuman primates (18). Apo B, found in the liver and jejunum, is associated with serum lipid abnormalities, elevated low-density lipoprotein (LDL) and a high incidence of atherosclerotic heart disease. With a single siRNA-SNALP injection, there was maximal silencing of 90% for *APOB* mRNA expression in the liver of nonhuman primates. At the dose of 2.5 mg/kg the SNALP-*APOB* siRNA nanoparticle induced significant reductions in Apo B protein, serum cholesterol and LDL for 11 days. Nevertheless, at this dose the SNALP-*APOB* siRNA particle was associated with transient elevations in liver enzymes. Another study showed that SNALP formulation targeting the polymerase gene of the Zaire strain can protect guinea pigs from lethal challenge with Ebola virus (16). Whereas polyethylenimine-siRNA reduced viremia and partially protected guinea pigs from death, the SNALP-siRNA nanoparticles completely protected them from death. In contrast to a previous study (18), SNALP-siRNA nanoparticles induced interferon alfa and beta (16), but it is unlikely that this was the primary factor in their efficacy. The most effective of the siRNAs (*Kdr*) in reducing viremia and preventing death induced the lowest levels of interferon alfa and beta (16). Although a cytokine response may act in concert with the siRNA, in other cases, high cytokine levels may be deleterious with some therapies, in that they can mask the efficacy of the therapeutic gene. Indeed, there has been controversy as to whether the effects claimed in several studies for therapeutic siRNA might really be due instead to cytokine induction (51, 156). Although these cationic liposomal siRNA studies appear promising, there are no FDA-approved products of cationic liposomes as carriers of nucleic acids, despite their long history of development.

Neutral liposomes

Initially, neutral liposomes were used for in vitro and in vivo delivery of nucleic acids, but their use as carriers of nucleic acids was limited after the development of cationic liposomes (151). Nevertheless, with the realization that significant toxicities were associated with cationic liposomes, neutral liposomes have re-emerged as promising carriers of siRNA. Recently, several studies from M.D. Anderson have shown the utility of neutral liposomes composed of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) as siRNA carriers in vivo (158-162). Tumor growth in several different tumor xenograft models was inhibited with nonligand, nonpeglyated liposomes with siRNA targeting the thrombin receptor (melanoma), interleukin-8 (ovarian), EPHA2 (ovarian) and focal adhesion kinase (ovarian). Maximal inhibition of xenograft growth with DOPC-siRNA liposomes was observed for melanoma. Compared to the control siRNA nanoparticles, the DOPC-siRNA nanoparticles targeting thrombin receptor inhibited the growth of melanoma xenografts and the number of lung metastases by about 80%. A marked reduction in thrombin receptor and angiogenic (VEGF, IL-8) and invasive (matrix metalloproteinase-2, MMP-2) factors that it regulates was also observed with therapy. Similarly, although to a lesser extent, the neutral DOPC-siRNA nanoparticles reduced the growth of ovarian tumor xenografts; compared to empty liposomes, DOPC-siRNA targeting the *Epha2* oncogene reduced the growth of HeyAP xenografts by 35%, and in combination with paclitaxel, DOPC-*Epha2* siRNA nanoparticles had an additive effect and reduced tumor weight by 75% (159).

Although incorporation of DNA in neutral liposomes and stability within the bloodstream were initially problematic, many of these barriers have been overcome with the development of new strategies. Freeze-thawing allows a high incorporation of siRNA within liposomes, and coating the surface of these liposomes with hyaluronic acid stabilizes the nanoparticles in the bloodstream. These significant advances were highlighted in a recent *Science* article. To define the role of cyclin D in a mouse model of colitis, Peer et al. utilized β-7 antibody conjugated with targeted stabilized liposomal nanoparticles (19). Unlike cationic liposomal carriers of siRNA, the investigators used neutral liposomes in which cyclin D siRNA in complex with protamine was incorporated inside the liposomes. Although β-7 integrins are ubiquitously present on leukocytes, the β-7 antibody had significantly higher affinity toward the activated integrin receptor on leukocytes; activation of the receptor occurs in inflammatory states such as colitis and malignancies. Recognition of the activated integrin receptor by the β-7 antibody nanoparticles enabled high specificity and uptake by endocytosis. Notably, the liposomal nanoparticles were stabilized with hyaluronic acid, which significantly increased their serum half-life. Systemic injection of cyclin D1 siRNA nanoparticles targeted activated leukocytes and reversed experimentally induced colitis in mice by suppressing leukocyte proliferation and T helper cell cytokine expression. This study revealed cyclin D1 to be a potential anti-inflammatory target for siRNA therapy, and suggested that this therapy may not only be applicable to colitis, but also certain malignancies. At least for this therapeutic application, targeted neutral liposomes may be more effective than antibody–protamine fusions as carriers for cyclin D siRNA. The authors point out that the immunoliposomal carrier (4000 siRNA/carrier) has a much greater capacity to entrap siRNA compared to the antibody–protamine fusion carrier (6 siRNA/carrier). Nevertheless, no comparisons of their efficacy to deliver siRNA to their targets in vivo were done, and entrapment (or carrying) capacity is only one of many important factors that determine efficacy of the siRNA nanoparticle.

siRNA CONJUGATES

Lipophiles conjugated to siRNA

Lipophiles conjugated to siRNA can improve their stability, pharmacokinetics and biodistribution. Cholesterol-*Apob* siRNA had a half-life of

45 min and widespread tissue distribution, whereas unconjugated siRNA had a half-life of 6 min and was not detected in tissues. When 50 mg/kg of a modified *Apob* siRNA conjugated to cholesterol was administered i.v., *Apob* mRNA was decreased 57% and 73%, respectively, in the liver and jejunum. Consequently, Apo B protein was reduced by 68%, cholesterol by 37% and LDL by 44% (35). No off-target effects were observed despite the high dose of the conjugate administered. The transport and efficient uptake of the cholesterol-siRNA conjugates by the liver and jejunum depend on their interaction with lipoproteins (163). The cholesterol-siRNA conjugate targets the liver when it combines with LDL, while the conjugate is directed toward the jejunum if it interacts with very low density lipoprotein (VLDL).

In addition to cholesterol, α-tocopherol and bile acids have been conjugated to siRNA (34, 36, 163). Similar to the cholesterol-siRNA conjugate, the α-tocopherol-*Apob* siRNA conjugate reduced hepatic *Apob* mRNA and serum cholesterol levels. The dose required to achieve maximum inhibition with α-tocopherol, however, was significantly lower at 2 mg/kg (36).

siRNA polyconjugates

Rozema and coworkers have developed an siRNA polyconjugate nanoparticle that targets liver hepatocytes to silence *Apob* and *Ppara* genes (42). The siRNA polyconjugate was synthesized by first conjugating siRNA to the endosomolytic PBAVE polymer through a disulfide bond and then attaching PEG and *N*-acetylgalactosamine (a liver-targeting ligand) to the polymer. The hepatocyte-specific nanoparticle, with a size of 10 nm, significantly smaller than SNALP liposomes, reduced *Apob* and *Ppara* mRNA by 76% and 64%, respectively, in a mouse model. As expected, serum cholesterol levels were reduced by 30% in the *Apob* siRNA-treated mice relative to control mice. Notably, this study showed no evidence of significant toxicity, with minimal and transient elevations in serum levels of liver enzymes and cytokines.

siRNA-PEC MICELLES

Kim and coworkers constructed polyelectrolyte complex micelle nanoparticles (PEC micelles) to target VEGF, a growth factor essential for tumor angiogenesis (32, 164). The PEC micelles were composed of the PEG-*Vegf* siRNA conjugate and core-forming PEI, which interacted with siRNA to stabilize the nanoparticles. Using atomic force microscopy, the PEC micelles were shown to range in size between 50 and 80 nm. The siRNA was conjugated to PEG through disulfide linkages, and in the highly reducing environment of the cytosol the siRNA was released to associate with RISC. In prostate cancer PC-3 cells, PEC silenced VEGF expression by more than 95%. Interestingly, these PEC micelles were significantly more effective than PEI/*Vegf* siRNA polyplexes in silencing VEGF (32). Building on these in vitro results, the growth of PC-3 tumor xenografts was reduced by about 85% by PEC administered by intratumoral or systemic injection (33). Concomitantly, VEGF levels and microvessel density were significantly inhibited in PEC-treated xenografts. Although these systemically delivered nontargeted micelles accumulated in several organs, PEC micelles showed low toxicity and induced low levels of interferon alfa.

APTAMER-siRNA

Aptamers are small (< 15 KDa), highly structured, single-stranded RNA or DNA molecules, isolated from combinatorial libraries by a method known as SELEX (Systematic Evolution of Ligands by EXponential enrichment). Aptamers can be readily synthesized in large quantities and can be chemically modified to avoid degradation by nucleases. By binding with high affinity to target molecules, aptamers have a number of biological applications, including target validation, as inhibitors of receptors or enzymes, and as carriers for nucleic acids. An aptamer targeting VEGF (pegaptanib) is a clinically approved agent for neovascular age-related macular degeneration. As carriers of siRNA, aptamers have not been used for systemic delivery, but they offer great promise due to their low-nanomolar binding affinities toward their targets and low immunogenicity.

Chu et al. developed an aptamer that targeted prostate-specific antigen (PSA), which is highly expressed on LNCaP prostate cancer cells (37). Through a strepavidin–biotin linkage, a laminin A/C siRNA was conjugated to the aptamer. The aptamer had high affinity for prostate-specific membrane antigen (PSMA; K_d = 2 nM), with rapid internalization of the aptamer conjugate by clathrin-mediated endocytosis. The siRNA-dependent inhibition of laminin A/C with the aptamer carrier was equivalent to that of Oligofectamine liposomal carriers; inhibition of laminin A/C gene expression was about 70%. Notably, the growth of LNCaP tumor xenografts was inhibited by more than 90% with intratumoral injections of anti-PMSA aptamer conjugated to *Bcl2* siRNA (38).

In contrast to the previously mentioned anti-PSMA aptamer studies, Wullner and coworkers used an anti-PSMA bivalent aptamer conjugated to an siRNA targeting eukaryotic elongation factor 2 (EF-2) (165). The bivalent aptamer-*Eef2* siRNA conjugate inhibited its target mRNA and protein significantly more than the monovalent aptamer conjugate; perhaps more important, these larger bivalent aptamer conjugates are less likely to be filtered by the glomeruli. These aptamers induced siRNA sequence-specific apoptosis only in PSMA-expressing cells and no interferon alfa was induced in any of the tested cells.

In addition, the aptamer-siRNA approach has also been used to inhibit HIV replication (39). This was the first study in which there was a dual inhibitory function of the aptamer-siRNA conjugate on the disease target: both the anti-gp120 aptamer and the *tat/rev* siRNA have potent anti-HIV activities in T cells. Furthermore, the aptamer-siRNA conjugate specifically entered CHO cells expressing the cell-surface protein gp160. To increase stability and prevent degradation by nucleases, the sense strand of the aptamer had its pyrimidine bases modified by 2′-fluoro ribose bases; aptamers have also been modified by replacing phosphate linkages with boranophosphates (166). Furthermore, the investigators corroborated their earlier study in demonstrating that the 27-base siRNA duplexes were more effective than the 21-base siRNA duplexes. From these tumor and HIV studies, it is difficult to predict whether systemic therapy with aptamer conjugates will be effective. The small size of aptamers increases the likelihood that they would be secreted by the kidney. Moreover, the highly negatively charged RNA may interact with serum proteins and significantly affect biodistribution and specificity of the aptamers. Nevertheless, multimerization of

aptamers, as done by Wullner and colleagues (165), is expected to reduce renal excretion, while selective pegylation will inhibit protein absorption and also reduce renal excretion.

CONCLUSIONS

Great advances have been made in developing carriers for siRNA thanks to the efforts of many researchers working to improve delivery methods for antisense oligonucleotides and plasmids. Much of the toxicity of siRNA therapeutics, including off-target effects or cytokine induction, has been circumvented by modification of the bases. Careful evaluation of selected modifications of the siRNA duplex commonly yields an siRNA that is as effective as the natural siRNA at cleaving the mRNA substrate. These modifications can greatly reduce cytokine induction and off-target effects, and significantly increase intracellular and extracellular stability to nucleases. As we have reported in this review, there are many nonviral carriers or approaches that offer exciting opportunities to deliver siRNA to their disease targets. To date, 12 clinical studies are ongoing. Most of these siRNA clinical trials target ocular diseases, but two clinical trials are treating renal disease and one phase I clinical trial is targeting cancer. The clinical trial for cancer patients utilizes a targeted carrier for systemic delivery of therapeutic siRNA. In addition to the ongoing clinical studies, there are many promising preclinical carriers that have demonstrated efficacy for siRNA delivery.

New strategies such as the siRNA cocktail approach for silencing multiple upregulated genes are being developed for the treatment of many human diseases. Nonetheless, a greater understanding of the interactions of the nanoparticles and the biological milieu is needed to further improve siRNA delivery systems. The physicochemical properties formed by modified or unmodified siRNA with their varied carriers are essential to enable tissue targeting and prevent entrapment of the nanoparticles by other nontargeted tissues. The size, surface charge and three-dimensional morphology can greatly affect biodistribution and pharmacokinetics after systemic injection of the nanocomplexes. Many factors may affect the formation and stability of the nanocomplexes, such as changes in siRNA length and siRNA modifications, use of different carriers, ratios of siRNA and carriers, and buffers used in their preparation, which can further decide the fate of siRNA complexes in vivo. Although biophysical properties (size, charge, etc.) of nanoparticles are frequently reported, these particles are prepared in nonphysiological solutions. Few studies have examined the biophysical properties of the nanoparticles in the presence of high levels of serum and their interacting proteins (167-169). Furthermore, we are not aware of any study that has examined the biophysical properties of nanoparticles once they have been exposed to whole blood and the dynamic shear forces of travel through the vasculature. Although half-life studies and incorporation of hydrophilic shields on the surface of nanoparticles partially address the issues of in vivo stability, much remains to be learned about interactions between whole blood and the siRNA delivery system in order to develop a more stable nanoparticle that is clinically relevant. Despite the technical challenges of such experiments, the isolation and determination of the structure and associated proteins of systemically delivered nanoparticles would no doubt provide insight into the development of an improved carrier. With greater knowledge of the interactions of biological systems and nanoparticles and the resulting effects on their stability and cellular entry, the utility of systemic siRNA in human subjects is expected to become a reality.

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