Research Focus

Antibody-directed cell-type-specific delivery of siRNA

Hans-Peter Vornlocher

Alnylam Europe AG, Fritz-Hornschuch-Strasse 9, 95326 Kulmbach, Germany

Over the past four years, chemically synthesized short interfering RNA (siRNA) has become the standard tool for specific silencing of gene expression *in vitro*. The most difficult task in transferring this technology to an *in vivo* setting is to develop appropriate delivery strategies. With this aim, Song *et al.* recently reported the development of antibody–protamine fusion proteins as vehicles for receptor-directed delivery of siRNA. When a mixture of siRNA targeting tumor-related genes was administered in this way, tumor growth was inhibited in an engineered melanoma model, demonstrating the therapeutic potential of this technology. However, several challenges remain to be overcome before targeted gene silencing can become a reality for patients.

siRNA: the problem of delivery

RNA interference (RNAi) is a powerful natural mechanism whereby double-stranded RNA molecules direct sequence-specific target RNA degradation. During RNAi, the enzyme dicer processes long double-stranded RNA into short interfering RNA (siRNA) of 21–23 nucleotides in length. siRNA then associates with multiple proteins to form an RNA-induced silencing complex (RISC) and triggers sequence-specific cleavage of the target mRNA, which is then further degraded [1]. Since the demonstration that chemically synthesized siRNA can bypass dicer and directly initiate RNAi, silencing by siRNA has revolutionized *in vitro* biology, becoming a simple tool for specific inhibition of gene expression [2]. siRNA-mediated silencing can also be a valuable tool for *in vivo* analysis of gene function by causing transient shutdown of protein production. In addition, siRNA is considered to have great therapeutic potential, having the major advantage of interfering with synthesis of disease-causing proteins at an early stage of gene expression.

In vitro, siRNA has been delivered to target cells by lipid-mediated transfection or via electroporation. However, these methods are not broadly applicable *in vivo*. So far the most serious hurdle for *in vivo* administration of siRNA has been the lack of efficient delivery of siRNA to target tissues and cell types. One way to deliver siRNA *in vivo* is the rapid injection of large volumes of siRNA-containing solution into the tail veins of mice [3]. Although such hydrodynamic delivery of siRNA has enabled siRNA-mediated protection from fulminant hepatitis [4] and inhibition of acute liver failure [5], this technique is not transferable to a clinical setting. In another method, chemical conjugation of a cholesterol moiety improved the stability and efficacy of siRNA targeting apolipoprotein B [6]. The levels of target mRNA and protein were reduced in a sequence-specific manner in the liver and jejunum after low-volume intravenous injection of the conjugated siRNA, and the expected mRNA cleavage products were detected in tissue samples. In addition, serum cholesterol was reduced significantly. Other approaches for *in vivo* delivery of RNAi-based therapeutics include formulation of siRNA in ligand-targeted nanoparticles [7] and intravenous administration of lipid-encapsulated siRNA in a Hepatitis B virus mouse model [8].

Recently, in an elegant set of experiments, Song and *et al.* from Judy Lieberman's group combined the nucleic-acid-binding properties of the small basic protein protamine (which is involved in sperm DNA stabilization) with the specific ligand-recognition properties of antibodies to achieve cell-type-specific siRNA delivery *in vitro* and *in vivo* [9]. Here, we will discuss the implications of this research and some of the challenges that remain to be resolved before antibody-directed delivery of siRNA-therapeutics can become a useful treatment in the clinic.

Fab-fragment–protamine–siRNA complexes for *in vitro* silencing

Receptor-mediated uptake has successfully been applied to the delivery of nucleic acids *in vitro* and *in vivo*. Targeting proteins such as antibodies or transferrin (an iron transporter, receptors for which are mainly found on erythroblasts) were conjugated to cationic polymers (e.g. polylysine [10] or polyethylenimine [11–13]) to mediate uptake of plasmid DNA and antisense oligonucleotides. The small cationic protein protamine has also been successfully used to link targeting proteins to effector nucleic acids. Transferrin was covalently linked to protamine to deliver functional plasmid DNA to erythroblasts [14]. Hormone receptors, such as the epidermal growth factor receptor family member ErbB2, expressed on the cell surface can also serve as target structures for nucleic acid delivery. A recombinant fusion protein of an ErbB2-specific single-chain antibody fused to protamine successfully transported plasmid DNA to ErbB2 antigen-expressing cells [15].

Song *et al.* tested the functionality of this approach for siRNA delivery [4]. At the genetic level, protamine was fused to F105, the heavy chain of a Fab fragment specific for the HIV envelope protein gp160, and the resulting Fab fragment was expressed in COS cells. Fluorescently labeled siRNA bound to the recombinant F105–protamine fusion with a stoichiometry of 1:6.

Corresponding author: Vornlocher, H.-P. (hpvornlocher@alnylam.de).
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Delivery of the Fab–protamine-complexed siRNA to Jurkat cells depended on HIV infection (and therefore on gp160 expression) in addition to the presence of F105 and protamine in the siRNA complex. In subsequent experiments, siRNA-mediated silencing of green fluorescent protein (GFP) expression in HeLa cells also depended on HIV infection and siRNA delivery by a functional F105–protamine–siRNA complex [4]. Even more exciting, Song *et al.* used their strategy to silence expression of the major HIV core protein p24 in cultured HIV-1-infected T cells, which are otherwise difficult to transfect with nucleic acids.

To take one step closer to a therapeutically relevant system, Song *et al.* generated B16 melanoma cells expressing gp160, thereby making these cells targets for the same delivery approach [4]. siRNA targeting mRNA for the oncogenes *c-myc* and *MDM2* and for the vascular endothelial growth factor *VEGF* efficiently reduced proliferation only in the gp160-expressing B16 melanoma cells when delivered using the F105–protamine fusion. Expression analysis of interferon-β and two interferon-responsive genes, 2′-5′ oligoadenylate synthetase (*OAS1*) and signal transducer and activator of transcription 1 (*Stat1*), did not indicate that any stimulation had been triggered by F105–protamine-delivered siRNA in the gp160-expressing B16 cells. Activation of interferon pathways has been described for expressed short hairpin RNA (shRNA) and siRNA [16,17].

Song *et al.* also demonstrated the transferability of this approach using another antibody. At the genetic level, a protamine fragment was fused to a single-chain antibody specific for ErbB2 and the construct was expressed in insect cells. siRNA directed against Ku70 (a protein involved in DNA repair) and formulated with this fusion protein efficiently silenced the target in ErbB2-expressing breast cancer cells. Again, delivery depended on the presence of antibody and protamine in addition to ErbB2 expression.

In vivo silencing

The most important step in developing a delivery system for therapeutic siRNA is demonstration of efficient delivery and specific siRNA-mediated silencing *in vivo*. For this purpose, Song *et al.* intravenously or intratumorally injected mice harboring a subcutaneous B16-gp160 xenograft, using fluorescently-labeled siRNA in phosphate-buffered saline (PBS), formulated with the lipid-based transfection reagent Oligofectamin, or complexed with F105–protamine. Although no siRNA was detectable in the mice when delivered in PBS, Oligofectamin-formulated siRNA was detected in tumor cells and in adjacent tissue. However, only F105–protamine-complexed siRNA was restricted to the grafted B16 cells, demonstrating the specificity of delivery. In the same system, a mixture of siRNA (80 μg) targeting *c-myc*, *MDM2* and *VEGF* was injected three times intratumorally or intravenously into mice carrying the B16-gp160 tumor. Nine days after implantation, the tumor size in animals that received the F105–protamine–siRNA complex was a third of that in animals that had been treated with unformulated siRNA. Intratumoral injection of siRNA was slightly more efficient than systemic intravenous delivery. Together, these exciting results demonstrate the therapeutic potential of siRNA delivered using the antibody–protamine fusion system.

Perspectives

The work by Song and colleagues describes an intriguing delivery strategy for siRNA, combining the specificity of monoclonal antibodies with the powerful method of RNAi-mediated gene silencing. Besides the efficient shuttling of siRNA through the cell membrane, the F105–protamine system also provides the ability to target siRNA specifically to selected cell types. A theoretical benefit of a tissue-type and cell-type specific delivery is the prevention of silencing in cells that are not involved in the particular disease. This should expand the number of targets that are suitable for an siRNA therapeutic approach to genes that are essential in non-target cells. In addition, the restriction of delivery to target structures alone (e.g. tumor cells) might prevent the potential activation of the interferon pathway, which has been reported for siRNA delivery to plasmacytoid dendritic cells [18]. Overall, we should look forward to further reports on the utilization of this promising technology for siRNA delivery in the near future.

Outstanding questions

Several questions remain to be addressed before the mechanisms underlying this delivery system can be fully understood. The route by which siRNA is delivered to the cytoplasm remains to be determined. Song *et al.* suggested an endocytotic uptake starting from clathrin-coated pits. Another option is siRNA uptake via a specific transporter system that is triggered by the high concentration of siRNA on the target cell surface.

Additional experiments are needed to investigate the breadth of applicability of this technology using other antibody–receptor combinations to demonstrate broad applicability for multiple target cell types with therapeutic relevance *in vivo*.

Whether production of a drug that consists of an siRNA and a recombinant antibody fusion protein is feasible from a commercial viewpoint remains to be determined. All challenges associated with the development of antibody-based drugs will also relate to this delivery technology. Among others, this includes potential immunogenicity of the antibody or of the antibody–siRNA complex.

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Friend or foe? Antimicrobial peptides trigger pathogen virulence

Jennifer L. Bishop and B. Brett Finlay

Room 301, 2185 East Mall, Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada, V6T 1Z4

In an age of antibiotic-resistant pathogens, antimicrobial peptides have emerged as novel therapeutics hailed for their bactericidal and immunomodulatory properties. However, a recent paper by Bader *et al.* demonstrates that these molecules also trigger bacteria to arm themselves against host immune responses. The authors show that the two-component regulatory system PhoP–PhoQ of *Salmonella* is activated not only in cation-deficient environments as previously thought, but also by binding to antimicrobial peptides, thus promoting gene transcription necessary for *Salmonella* survival within the host. Thus, the antimicrobial peptide might be a double-edged sword, promoting antibacterial immunity while simultaneously triggering pathogen virulence.

Antimicrobial peptides: the way of the future?

Antimicrobial peptides (AMPs) are small cationic molecules that are part of the nonspecific defenses that kill bacteria, control bacterial infections and coordinate host responses to infection in many animal cells, such as those of amphibians, crustaceans, fish, birds and mammals, including humans. These molecules are crucial for the clearance of bacterial, fungal, viral and parasitic infections in various model systems and thus are integral to animal immune responses [1]. As their name suggests, AMPs are bactericidal, controlling infections by physical disruption of bacterial membranes. The net positive charge of most AMPs, combined with their amphipathic conformation, enables these molecules to bind selectively to negatively charged bacterial membranes, creating pores or leaks that increase cell permeability and lead to cell lysis [2]. In addition, AMPs such as the human cathelicidin LL-37 are crucial signaling molecules within the innate immune system, being involved in (i) mast-cell degranulation, (ii) chemotaxis of neutrophils, T cells and monocytes, (iii) inhibition of proinflammatory responses of host cells to bacterial components, (iv) macrophage cytokine production and (v) angiogenesis [3]. AMPs can also modulate adaptive immune responses, acting as adjuvants to stimulate humoral and even antigen-specific cytotoxic-T-cell responses [3]. In an era of antibiotic-resistant pathogens, these immunomodulatory properties of AMPs have made them top targets in the search for novel antibacterial therapeutics to boost the immune system rather than attack the pathogen [4]. Commercialization of AMP immunomodulatory therapies is now being extensively explored; biotechnology companies throughout the USA and Canada are developing topical peptide-based treatments, many of which are in Phase II clinical trials, for infectious diseases and inflammatory conditions [5]. However, because many of the immunomodulatory effects of AMPs have been shown only *in vitro*, design of AMP-based therapies is challenging and must take into account whether these immunomodulatory properties are maintained in physiologically relevant animal models [3]. Other obstacles, including high cost

Corresponding author: Finlay, B.B. (bfinlay@interchange.ubc.ca).
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