RNA Interference

DOI: 10.1002/anie.201002867

Proof of RNA Interference in Humans after Systemic Delivery of siRNAs

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nanoparticles · RNA · RNA interference · small interfering RNAs

The rapid development of RNA interference (RNAi) in the last few years has fuelled hopes that this technology will soon offer new possibilities of treating diseases previously considered untreatable. For the discovery and 1998 publication of the mechanism of RNAi in the worm *Caenorhabditis elegans*[1] Andrew Fire and Craig Mello were honored with the 2006 Nobel Prize for Medicine or Physiology. RNAi involves an evolutionarily conserved mechanism through which the expression of genes is inhibited on the posttranscriptional level by double-stranded RNA molecules.[2,3] The use of RNAi for medical purposes became realistic after Thomas Tuschl and his co-workers were able to show in 2001 that short, 21 base pair long interfering RNAs (siRNAs) are capable of triggering RNAi in mammalian cells without simultaneously causing an interferon response.[4] Only some three years after this discovery the first clinical studies based on RNAi were begun.

Through this rapid progress, RNAi appeared to deliver on the promises that had never been fulfilled by the antisense and ribozyme-based approaches: with the aid of the anti-mRNA strategies the expression of any given detrimental gene could be blocked, so that cancer genes or viruses should be easy to inhibit. Similar to other fields of molecular medicine, for example gene therapy or the use of monoclonal antibodies, RNAi research was also stricken by serious setbacks. To begin with it was shown that the RNAi was not as specific as initially believed, and under certain circumstances it can trigger an interferon response;[5] later intracellularly highly expressed short hairpin RNAs (shRNAs) proved to be toxic to the liver.[6] A further blow was delivered by a publication that questioned whether clinically administered siRNAs are working by means of the RNAi mechanism or whether they may instead be triggering an unspecific response.[7] In the study in question, an siRNA directed against vascular endothelial growth factor (VEGF) or its receptor was injected directly into the eye. In animal experiments, these siRNAs were able to block the formation of new blood vessels (neovascularization) in the macula and thereby prevent vision loss. According to the study of Kleinmann et al.[7] the control siRNAs showed the same activity as the specific siRNAs, presumably because they activate Toll-Like Receptor 3 (TLR-3). Besides sometimes restricted specificity, one of the greatest challenges to the therapeutic use of RNAi is the transport into the target tissue and the cellular uptake of RNAis, referred to collectively as delivery.

In a recently published study in *Nature* a significant advance with respect to both delivery and proof of function of the RNAi mechanism by an siRNA in humans was achieved: In a clinical trial an siRNA was delivered to tumor cells with special nanoparticles, and PCR experiments showed that the siRNA caused the degradation of the target RNA through the RNAi mechanism.[8]

In the phase I trial, patients with melanomas were treated whose tumors had not responded to standard therapy. The siRNA used was directed against an established target for cancer therapy, the M2 subunit of ribonucleotide reductase (RRM2). The patients were given the siRNAs on days 1, 3, 8, and 10 of a 21 day cycle at different dosages.

What made this therapy special was the use of a targeted nanoparticle-based delivery system (Figure 1). These nanoparticles consist of a sphere made from a linear cyclodextrin-based polymer. Furthermore they contain molecules of polyethylene glycol (PEG) with adamantane on the ends which forms inclusion complexes with the cyclodextrin. The targeting of the nanoparticles was accomplished by adding transferrin proteins to the surface. Transferrin binds to its receptor, which is overexpressed on the surface of cancer cells, and this mediates entry of the nanoparticles into the cells. The siRNA was encapsulated in the core of the nanoparticle, designated CALAA-01.

In most clinical studies to date using RNAi, siRNAs were administered locally, for example directly into the eye, into the lungs, or on the skin. The clinical trial with CALAA-01 was the first to investigate administration by the intravenous route, in this case by means of a 30 min intravenous infusion. By means of the transferrin moieties the nanoparticles should find their way to tumor tissue through the bloodstream. Tumor biopsies revealed the presence of the nanoparticles inside the cells, and the amount measured correlated with the administered dose.

An important criterion demonstrating the success of an RNAi experiment is showing the knockdown of the target gene. Using quantitative reverse transcription polymerase

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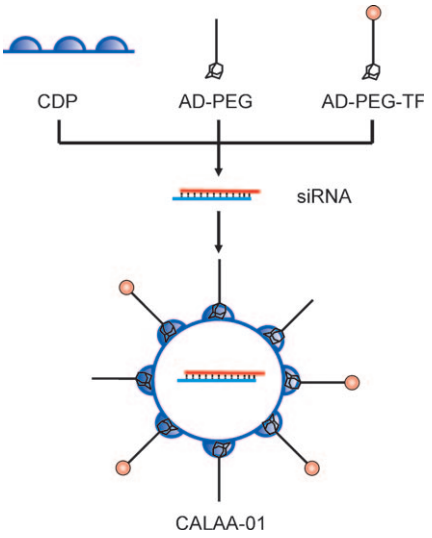

Figure 1. Structure of the nanoparticle CALAA-01 for the targeted delivery of siRNAs. The nanoparticle is composed of a linear cyclodextrin-based polymer, polyethylene glycol decorated with adamantane on the ends (AD-PEG), and other PEG molecules bearing transferrin moieties (AD-PEG-TF), as well as the enclosed siRNA.

chain reaction (RT-PCR), the authors were able to show that the RRM2 mRNA was decreased relative to that before the treatment. The amount of protein, which was investigated using Western blots and immunohistochemistry, was also decreased by treatment. This is, however, not definitive proof that the siRNAs worked by the RNAi mechanism. It is also possible that an unspecific effect is involved. In addition, for ethical reasons, in human trials truly complete data sets with all desirable controls are not possible.

Owing to this uncertainty, in further experiments Davis et al. verified that an RNAi effect had been observed. They did this by employing a special PCR method that could directly demonstrate the cleavage of the target RNA. In the RNAi pathway the siRNA is incorporated in a multimeric protein complex, the RNA-induced silencing complex (RISC), in the process of which one of the two strands of RNA is lost. The remaining strand hybridizes with the target RNA, bringing it into proximity of RISC. The Argonaut protein 2 in RISC cleaves the target RNA between the tenth and eleventh nucleotide from the 5′-end of the siRNA.

To verify the cleavage site, a special PCR technique was employed, which begins by ligating an adapter to the 5′-end of the RNA. The RNA is then reverse-transcribed and amplified by PCR, and the PCR product is directly sequenced. This procedure does not provide quantitative measurements of the amount of the mRNA fragment, but it does allow the cleavage site to be determined with precision. This demonstrated that the cleavage of the mRNA does not result from an unspecific process but instead occurs because of the RNAi-mediated activity of the siRNA.

Interestingly mRNA fragments could be demonstrated in one patient at the beginning of the second round of treatment. This means that the RNAi mechanism must have been active over the course of several weeks between the two rounds of treatment. The duration of RNAi activity depends on the rate of cell doubling. Since the patient's condition remained stable between the two rounds of siRNA infusions, it is reasonable to assume that the cells divided only slowly and therefore a long-lasting effect was observed. It is also not known how long the nanoparticles release siRNAs within the cell.

Owing to the euphoria concerning the RNAi method, the publications mentioned above which criticize the RNAi method are sometimes used as undifferentiated global arguments against the technology. As a result, it is, for example, often overlooked that the work of Kleinmann et al.,[7] in which the blockage of neovascularization was possibly a result of TLR-3 activation rather than a specific RNAi effect, pertains to only naked siRNAs. There is, however, general agreement that efficient application of RNAi requires a means of delivery. The nanoparticle-based approach described above represents a great advance. No information about the outcome of the clinical results has been provided, but this is due to be published separately on conclusion of the study. It is also vitally important that the results are confirmed with a larger number of patients. In the existing study only three patients were examined, and the RNA-cleavage products could be detected in only one of the patients, the one in the highest dose regimen. Nevertheless, it is certain that this proof of concept that a specific RNAi effect can be produced in humans will further inspire the medical development of RNAi.

Received: May 12, 2010
Published online: July 20, 2010

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