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Hydrodynamic Delivery of siRNA in a Mouse Model of Sepsis

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Summary

The use of siRNA *in vitro* as well as in animal models has become more widespread in recent years, leading to further questions as to the best mode of delivery that will achieve optimal knockdown. While the exact mechanism of siRNA uptake at a cellular level has yet to be fully elucidated, various delivery techniques are being researched, including the use of viral vectors of shRNA, liposome encapsulations, and hydrodynamic delivery of naked siRNA. We describe the use of hydrodynamic administration as a technique to deliver, *in vivo*, naked siRNA constructs into experimental animals as a method of transient gene knockdown. This method may prove useful in situations where knockout animals do not exist, or to determine the effect of gene knockdown at specific time points during an experiment.

Key Words: siRNA; hydrodynamics; sepsis; mice.

1. Introduction

Hydrodynamic infusion was first described with plasmid DNA ***(1)*** in which plasmid DNA containing cDNA of luciferase and β-galactosidase as reporter genes was given as a large-volume, rapid tail vein injection. Among the organs expressing the transgene, including the lung, spleen, heart, kidney, and liver, the liver showed the highest level of gene expression (*see* **Note 1**). This study also demonstrated that volume and rate of injection were critical in the uptake of naked constructs, in that the plasmid had to be in a large fluid volume (i.e., equivalent of about one time the animal's blood volume) rapidly injected.

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One of the first studies that utilized this technique for siRNA showed that approximately 88% of hepatocytes take up siRNA after a hydrodynamic injection of 50 µg of Cy5-tagged siRNA in 2 mL ***(2)***. It should also be noted that fluorochrome-tagged siRNA is said to be not functional but can be useful for determining uptake of siRNA in specific cell types. Our own localization studies using GFP siRNA in GFP transgenic mice showed, in accordance with Liu et al., that siRNA was taken up in all major organs ***(3)***. In contrast to what has been suggested *in vitro* and with viral vectors ***(4–6)***, our lab and others have found no evidence of naked siRNA stimulating the interferon pathway *in vivo* ***(3,7,8)***. This latter point is not trivial, as the administration of any agent that can induce inflammation, can be a confounding variable in the examination of a given experimental pathology.

2. Materials

2.1. siRNA

1. Custom siRNA (19–21-mer duplexes) (Dharmacon, Lafayette, CO) 2′-deprotected, duplexed, desalted (ready to use). Predesigned siRNAs are also available using si*GENOME*™ (Dharmacon).
2. Control siRNA: GFP siRNA (Dharmacon) can be used, or a scrambled version of the experimental siRNA can be used. Scrambled sequences must undergo a BLAST search to rule out specificity against other possible sequences in the mouse genome, i.e., off-target effects.
3. Ice-cold sterile PBS.

2.2. Animal Groups and Injection Materials

1. The strain of mouse to be used, divided into at least three groups. Group 1: Saline control; group 2: nonsense siRNA control (GFP or scrambled siRNA); and group 3: siRNA-treated group.
2. Alcohol prep pads.
3. 25-gauge needles and 3-mL (cc) syringes.
4. A heat source: a heat lamp or a 55 °C water bath and gauze.
5. Rodent restraint.

2.3. Materials for Cecal Ligation and Puncture (CLP)

1. Anesthesia setup and isoflurane (Abbott Laboratories, North Chicago, IL).
2. Surgical instruments including forceps, surgical scissors, and a needle holder.
3. Lidocaine (Abbott Laboratories).
4. Lactated Ringer's solution (Baxter Healthcare Corp., Deerfield, IL).
5. Sterile surgical ties.
6. Electric razor to shave animal's abdomen fur (Wahl).

7. Betadine solution (Purdue Products, Stamford, CT).
8. 6-0 Ethilon suture material (ETHICON, Inc., Somerville, NJ).

3. Methods

The development of clinical as well as pharmaceutical interventions has proven to be a difficult task in treating sepsis. As human and animal sepsis is a complex condition, the timing of the therapy is critical (*see* **Note 2**). That said, as it is not known beforehand if an individual will become septic, possible treatments must be able to be given after the onset of sepsis, or as a "posttreatment." In this method, hydrodynamic delivery of siRNA is given 30 min post-CLP and as late as 12 h post-CLP. The idea of whether the injection can be delivered while the animal is under anesthesia is still of some controversy. While the protocol outlined here was performed in our laboratory without anesthesia, other labs have employed a light dosage of either isoflurane ***(9)*** or pentobarbital anesthesia ***(10)*** to carry out the hydrodynamic injection without morbidity or mortality (*see* **Note 3**).

3.1. Cecal Ligation and Puncture

1. The surgical procedure to generate sepsis, as previously described ***(11),*** can be carried out in any number of animal strains (i.e., outbred, inbred, knockout, etc.). We have commonly used C3H/HeN, Balb/C, or C57BL6 male mice.
2. The mice are lightly anesthetized using isoflurane. The abdomen is shaved and scrubbed with Betadine.
3. A midline incision (1.5–2 cm) is made below the diaphragm first in the skin and then in the muscle layer.
4. The cecum is isolated, ligated, and punctured twice with a 22-gauge needle. The cecum is then gently compressed to extrude a small amount of cecal material from each of the punctures.
5. The cecum is returned to the abdomen, and the muscle and skin incisions are then closed with 6-0 Ethilon suture material. Before suturing the skin, 2–3 drops of Lidocaine are administered to the wound for analgesia.
6. The mice are subsequently resuscitated with 1.0 mL of Lactated Ringer's solution subcutaneously. Sham controls are subjected to the same surgical laparotomy and cecal isolation, but the cecum is neither ligated nor punctured.

3.2. Hydrodynamic Delivery of siRNA

1. Prior to use, the siRNA must be reconstituted and aliquoted. By following the amount provided in the tube (in μg), add enough sterile PBS to yield a concentration of 5 μg/μL, or 50 μg/10 μL. Separate the reconstituted siRNA into 10-μL aliquots, and store in –20 °C. If siRNA is tagged with a fluorochrome, be sure to protect from light.

2. For each mouse to receive a hydrodynamic injection, add 1.5 mL of sterile PBS to an Eppendorf tube. Thaw a 10-µL aliquot of siRNA per mouse and add to the 1.5 mL of PBS. This will equate to 50 µg of siRNA per mouse.
3. Draw up the volume of siRNA into a 3-cc syringe capped with a 25-gauge needle. Make sure to purge the syringe and needle of air bubbles.
4. Restrain the mouse in the rodent restraint in such a way that the tail is completely exposed. For this aspect, short-term exposure to a volatile anesthetic like isoflurane can be utilized during restraint of the animal, but the anesthetic should be taken off just prior to the actual tail vein injection.
5. Clean the tail with an alcohol prep and place the heat source over the tail in order to dilate the veins, making them more visible. Three vascular structures will become visible, one in the middle and one on each side. Choose one of the "side" tail veins and proceed to inject the volume of siRNA within 5 sec. If the needle is placed correctly in the vein, the volume will inject in very easily. Upon completion of the injection, remove the needle and immediately hold a piece of gauze over the injection site until any bleeding has stopped and then return the mouse to its cage.

4. Notes

1. One way to determine the distribution of siRNA would be to inject GFP siRNA into GFP transgenic mice C57BL/6-TgN (ACTbEGFP)/Osb (Jackson Labs). In these mice, GFP constitutive transcription is under the control of a chicken beta-actin promoter and a cytomegalovirus enhancer, making all of the tissues, with the exception of erythrocytes and hair, appear green under ultraviolet excitation light ***(12)***. At the desired time point, mice are killed and tissues of interest are harvested and prepared for frozen sectioning. The tissues are then viewed using a fluorescent microscope and compared to that of a GFP mouse receiving a hydrodynamic injection of only PBS as a control. Our own localization studies using GFP siRNA in GFP transgenic mice showed that siRNA was taken up in all major organs ***(3)***.

   Localization of siRNA can also be determined using a fluorochrome-tagged siRNA, such as FITC or rhodamine ***(2)***. The siRNA manufacturer can add these tags to your custom siRNA. As with the GFP mice, wild-type mouse tissues that received a hydrodynamic injection of fluorochrome-tagged siRNA can also be viewed under fluorescence microscopy to look for the presence of the siRNA in the tissues. This approach is potentially also amenable to dual or three-color flow cytometric analysis, which might also allow potential assessment of cellular as well as tissue-specific targeting.
2. To assess the extent and duration of siRNA silencing *in vivo* at the mRNA level, RT-PCR or quantitative RT-PCR can be done on the tissues of interest using primers for the gene you are attempting to silence. For protocols concerning RNA isolation for primer production, RT-PCR, or quantitative RT-PCR, the reader is referred to the *Current Protocols in Immunology* series (John Wiley & Sons, Inc.).

To assess siRNA silencing at the protein level, a Western blot can be performed using protein from the tissues of interest. Assuming one has a relatively specific poly- and/or monoclonal antibody, which can be utilized for Western blotting, the reader is again referred to *Current Protocols in Immunology* for details on tissue/cell lysis, gel electrophoresis, protein transfer, and detection.

The extent of siRNA-induced silencing on a cellular level can also be determined by flow cytometry if antibodies for the siRNA target gene product of interest and given cell phenotype exist as direct fluorochrome conjugates. This may also be possible by immuno-histochemical or whole-tissue section approaches but requires more controls and blinded assessment or a method for clearly quantitating image probe intensity, as siRNA treatment typically produces gene knockdown (partial suppression) and not a *complete* knockout.

3. As mentioned in the introduction to this chapter, inflammation may be of some concern in that the *in vivo* infusion of siRNA (being a double-stranded RNA) might induce an inflammatory response through the activation of the interferon or the classic NF-κB pathway ***(5)***. We have shown, however, that animals treated solely with siRNA did not show an increase in plasma IFN-α levels as did animals treated with poly I:C ***(3)***. IFN-α is well documented to rise in response to TLR3, PKR, and/or IRF-STAT1 activation by double-stranded RNA ***(13,14)***. This would suggest that hydrodynamic delivery of siRNA at this concentration differs distinctly from alternative forms of double- or single-stranded RNAs typically derived from viruses or poly (I:C), as a stimulant of interferon activation. In addition, naked siRNA constructs given locally in the form of an intratracheal instillation did not induce local tissue inflammation ***(7)***.
4. Off-target effects can occur if your siRNA sequence has homology to mRNA transcripts other than your gene of interest ***(15)***. In this case, transcripts that are not related to your gene of interest may be silenced as well. Off-target effects can be significantly reduced by careful selection of your target sequence, thorough sequence validation, and adequate experimental controls. To optimize gene silencing, it is highly recommended that you test more than one siRNA target sequence per gene (particularly if your target gene has not been published or tested by the company that produces the siRNA). If the results are consistent from at least two different siRNA target sequences, it is more likely that any biological effects observed in your experiments are specifically due to loss of the gene of interest.
5. Due to the nature of the hydrodynamic injection, there are obvious limitations in that an injection of this volume and rate cannot be given clinically. However, the primary objective of such an approach is, first, to document the significance, i.e., contribution, a given gene makes to the pathology seen in a given experimental model. Second, as is the case in our studies of sepsis, it is also possible to document that siRNA can be used in a posttreatment scenario ***(3)***. In those studies we also found that hydrodynamic fluid delivery had no marked deleterious effects when administered at 30 min, 1 h, or 12 h post-CLP. If anything, there was a transient survival benefit at 36 h post-CLP, which was subsequently lost

(3). It is also important to appreciate that all arms of a study such as this should receive hydrodynamic fluid administration, so that the differences seen remain independent of those effects simply attributable to large volume fluid administration alone.

With respect to the duration of target gene suppression, we found that the suppression of both message and protein production in the liver lasts up to 10 days in our model *(3)*, but we have not explored the nature of this persistence. At present, we can only speculate that cells in the liver under *in vivo* conditions, which do not divide at the frequency of cell lines in culture (to which comparisons are made), lead to less dilution of the functional siRNA levels present and thus may allow the silencing effect to persist much longer *(2)*. While this is only a prolonged but not permanent suppression, toxicity has not been a concern thus far. This, unfortunately, is not the case with vectored short hairpin (sh) RNA constructs. Recent studies suggest cumulative toxicity may be observed with such vectored systems over time *(5)*. In this case, however, prolonged suppression allows for further time points to be studied in which to analyze the effect of gene target knockdown. The duration of knockdown is most likely also cell- or tissue type-specific, so this needs to be redetermined based on the tissue of interest.

In conclusion, hydrodynamic delivery of naked siRNA constructs, while not necessarily a clinically significant modality, represents a potentially valuable tool for the *in vivo* transient silencing of select target genes in any number of experimental settings in mice. Here we have provided a basic protocol for this approach and have attempted to outline some of the controls as well as concerns the investigator should keep in mind. Finally, as more is understood about the mechanism of RNAi action, its delivery, and the uptake of siRNA into cells, it is likely this method will be modified or an alternative developed for better use in the clinical setting.

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