Methodology Report
Efficient siRNA Delivery by the Cationic Liposome DOTAP in Human Hematopoietic Stem Cells Differentiating into Dendritic Cells

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Received 11 December 2008; Accepted 20 March 2009

Recommended by Bibekanand Mallick

RNA interference technology is an ideal strategy to elucidate the mechanisms associated with human CD34+ hematopoietic stem cell differentiation into dendritic cells. Simple manipulations in vitro can unequivocally yield alloreactive or tolerogenic populations, suggesting key implications of biochemical players that might emerge as therapeutic targets for cancer or graft-versus-host disease. To knockdown proteins typically involved in the biology of dendritic cells, we employed an siRNA delivery system based on the cationic liposome DOTAP as the carrier. Freshly-isolated CD34+ cells were transfected with siRNA for cathepsin S with negligible cytotoxicity and transfection rates (>60%) comparable to the efficiency shown by lentiviral vectors. Further, cathepsin S knockdown was performed during both cell commitment and throughout the entire 14-day differentiation process with repeated transfection rounds that had no effect per se on cell development. Tested in parallel, other commercially-available chemical reagents failed to meet acceptable standards. In addition to safe and practical handling, a direct advantage of DOTAP over viral-mediated techniques is that transient silencing effects can be dynamically appraised through the recovery of targeted proteins. Thus, our findings identify DOTAP as an excellent reagent for gene silencing in resting and differentiating CD34+ cells, suggesting a potential for applications in related preclinical models.

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1. Introduction

The discovery of siRNAs and subsequent advances in RNA interference (RNAi) technology has contributed to a major methodological shift in functional genomics [1]. Gene silencing, that can be successfully extended to high-throughput screening formats [2, 3], is a procedure that directly accelerates biological and biomedical research through the identification of molecular players involved in development, homeostasis, and disease. In the new millennium, the explosive growth of this field has also raised the prospect of RNAi as a therapeutic to treat a wide range of pathologies, in particular viral infections, cancer, and neurodegenerative disorders [4–7].

Synthetic siRNAs have emerged as a widespread means for gene silencing in mammalian cells [8], notwithstanding limitations associated with a nonrenewable supply, temporary silencing effect, and occurrence of off-target activity [9, 10]. Next-generation synthetic products were recently introduced to ensure the highest silencing power under a minimal occurrence of false positives (e.g., ON-TARGETplus [Dharmacon] and Silencer Select validated siRNAs [Ambion]), and provide the opportunity to transfet virtually any cell type in the absence of a carrier (Accell siRNAs [Dharmacon]). Further, shRNA expression vectors coupled to lentiviral transduction systems were developed to obtain unlimited resources, achieve stable integration into the genome in a widest range of both dividing and nondividing cells, produce sustained transgene expression, and comply with in vivo applications (e.g., MISSION [Sigma-Aldrich], BLOCK-iT [Invitrogen], SMARTvector [Dharmacon], GeneNet [System Biosciences], and Lenti-X...
[Clontech]) [11–13]. Finally, access to mimic and inhibitor miRNA libraries for use in either gain- or loss-of-function analyses, respectively, have enhanced the involvement of miRNAs (e.g., PremiR [Ambion], miRIDIAN [Dharmacon], and miScript [Qiagen]) in cytoplasmic posttranscriptional gene silencing [14], translational activation [15] and transcriptional control of gene expression in the nucleus [16].

However, the extraordinary benefits of these breakthroughs in RNAi technology may imply significant research investments and special requirements, such as biocontainment facilities dedicated to viral work. Chemical agents, instead, are relatively inexpensive, easy to handle and generally already optimized by the vendor for use with specific cell lines. Nonetheless, chemical transfections in finite cultures or freshly-isolated primary cells usually raise major challenges due to a combination of cytotoxic effects and poor transfection efficiencies. These problems become more obvious in dynamic systems such as stem cells subjected to differentiation.

Lentiviral vectors can successfully transduce hematopoietic stem cells (HSCs) [12, 17], but integrate stably in the genome and preclude the recovery of knocked-down proteins. Within nonviral transfection methods for HSCs, electroporation raises major survival issues due to apoptotic cell death [18], while nucleofactor technology was shown to deliver mRNA and plasmid DNA with inconsistent transfection and cell survival rates [19].

Lipofection has typically been considered inefficient for use with HSCs [20]. Nonetheless, based on the large number of products currently available from vendors and the advantages offered by a transient gene knockdown, we screened a variety of agents with different chemical formulations and found that the liposomal version of the monocationic lipid DOTAP is an excellent vector for the treatment of freshly isolated HSCs. Further, we observed that DOTAP is an efficient vehicle for gene knockdown in HSCs induced to differentiate into immunogenic dendritic cells in vitro through a conventional treatment based on Flt3L, GM-CSF, IL-4 and TNF-α [21–23].

2. Materials and Methods

2.1. Isolation of Human HSCs. Blood samples were collected from healthy volunteers who provided informed consent. Human peripheral blood mononuclear cells were isolated by density gradient centrifugation over Ficoll-Paque PLUS (GE Healthcare), after which CD34+ cells were purified by immunomagnetic selection using the minimagnetic-activated cell sorter (MACS) system (Miltenyi Biotec). Mean purity of CD34-enriched cells, determined by flow cytometry using a FACScan (BD Biosciences), was 94.8%, with a median value of 92 throughout an 86–98.5 range.

2.2. Differentiation of HSCs into Dendritic Cells. To generate immunogenic dendritic cells, CD34-enriched cells were transferred to 25 cm² flasks at a density of 10⁵ cells/mL and cultured for 14 days in RPMI-1640 medium (Euroclone) containing 10% fetal calf serum (FCS) (Euroclone) and a cytokine cocktail composed of human recombinant, Flt3L (50 ng/mL), GM-CSF (50 ng/mL), IL-4 (10 ng/mL), and TNF-α (2.5 ng/mL) (PeproTech EC). Every third day, half of the culture medium was replaced by fresh medium supplemented with the same cocktail. Cells were analyzed at day 3, 7, and 14 to evaluate the expression of specific markers of differentiation [23].

2.3. Evaluation of Different Reagents for siRNA Transfection of Untreated CD34+ Stem Cells. The following, commercially available reagents were tested to determine transfection rates and cytotoxicity effects: TransPass R1 and TransPass R2 (New England Biolabs), Lipofectamine 2000 and Lipofectamine LTX (Invitrogen), siPORT NeoFX and siPORT Amine (Ambion), and DOTAP (Roche). Tests were performed using a 96-well plate format. Freshly prepared CD34+ cells were seeded at a density of 2×10⁵/well. Fluorescein-labeled siRNA control (New England Biolabs), in a final concentration of 15 nM, was used to monitor the transfection procedures. Fluorescein-labeled siRNA was regularly diluted with RPMI-1640 without serum, except for the experiment performed with DOTAP, in which the diluent was HBS (Hepes-buffered saline: 20 mM HEPES, pH 7.4, containing 150 mM NaCl).

The transfection reagents were essentially employed as per each vendor's recommendations. TransPass R1 and TransPass R2 (0.2 µL) were diluted directly in the fluorescein-labeled siRNA solution (10 µL), incubated 30 minutes at room temperature and then added to the cultures (90 µL). Lipofectamine 2000 and Lipofectamine LTX (0.25 µL) were first diluted in 12.5 µL RPMI-1640 without serum and, after 5 minutes incubation, mixed with 12.5 µL of fluorescein-labeled siRNA. After 30 minutes incubation, the transfection complexes were added to the cells (75 µL).

siPORT NeoFX and siPORT Amine were first diluted (0.5 and 0.3 µL, resp.) in 10 µL RPMI-1640 without serum. After 10 minutes at room temperature, each carrier was mixed with 10 µL of fluorescein-labeled siRNA. The mixtures (20 µL) were incubated 10 minutes, then spotted in the assay wells. Within 5 minutes, cells were dispersed in a volume of 80 µL.

DOTAP (1.4 µL) was diluted in 7 µL HBS and then mixed, very slowly, with the same volume of fluorescein-labeled siRNA. After 30 minutes at room temperature, the mixture was added to cells (186 µL) resuspended in RPMI-1640 containing 20% FCS.

Except for the experiment performed with DOTAP, fresh medium was added to the assay wells 4 hours after each transfection process in order to achieve a final volume and a concentration of FCS of 200 µL and 10%, respectively.

In all instances, transfection and cell survival were evaluated 24 hours after the transfection method. Transfection rates were expressed as the ratio between transfected (fluorescein-labeled) and total number of viable cells measured via DAPI nuclear staining and trypan blue exclusion test. Signals were captured by a Nikon Eclipse TE2000-S fluorescence microscope equipped with a Cell-F video camera (Olympus) and processed via the Cell-F software.
2.4. Silencing of Cathepsin S Gene via the DOTAP Reagent in HSCs Subjected to Differentiation. Freshly isolated CD34+ cells were resuspended in RPMI-1640 supplemented with the agents used to generate immunogenic dendritic cells. Immediately after treatment, cells were subjected to transfection using a predesigned siRNA targeting the CatS gene (CTSS: ID-113084-113085, Ambion).

Cells subjected to mock- and scrambled-siRNA transfection (ID-46183G, Ambion) under the same experimental conditions were employed for control purposes. The procedure, run in a 24-well plate format via triplicate tests, was implemented using a 25 nM final siRNA concentration. Each well-contained 2 × 10^5 cells in a volume of 500 μL. The siRNAs were combined with DOTAP and maintained for 30 minutes at room temperature to form complexes. The mixture (50 μL) was then overlaid dropwise on the cell cultures. Following 4-hour incubation, 1.2 mL of RPMI-1640 containing 10% FCS and differentiating agents was added to each well. To ensure silencing of genes for the entire 14-day differentiation process, cells were centrifuged, resuspended in 500 μL of cytokine-enriched culture medium and exposed to a second (day 3) and third (day 9) transfection round under the same experimental conditions.

2.5. Western Blotting. Preparation of cell extracts, electrophoresis (12% gel) under reducing conditions and Western blotting procedures were performed as previously described [24].

Precursors and mature forms of cathepsin S were analyzed using polyclonal antibodies from Santa Cruz Biotechnology as the primary antibodies. Immunodetection was carried out by employing the enhanced, chemiluminescent Amersham ECL Plus kit. For each blot, several time exposures were performed to confirm that the results were within the linear response range of the film. Densitometric scans via an MCID system (InterFocus Imaging) were used to demonstrate that the intensity of the bands was proportional to protein content.

3. Results and Discussion

Several transfection reagents were employed to evaluate cytotoxic effects and the capacity to deliver a fluorescein-labeled siRNA control in freshly isolated, untreated HSCs. Commercially-available agents were chosen to explore a variety of structural diversities and chemical formulations as per each vendor's product description. The screen included a cationic lipid (TransPass R1), a nonlipid cationic polymer (TransPass R2), a lipid-based formulation designed for reverse transfection protocols (siPORT NeoFX), a polycation mixture (siPORT Amine), and different versions of cationic liposomes (Lipofectamine 2000, Lipofectamine LTX), an animal origin-free product, and DOTAP, obtained from N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate.

Four chemicals (i.e., Lipofectamine LTX, siPORT NeoFX, siPORT Amine, and DOTAP) were relatively well tolerated by HSCs (≥85% viability) (Figure 1(a)), however only DOTAP was found to combine a lack of cell cytotoxicity with efficient (60%) siRNA transfer (Figure 1(b)). TransPass R2 showed...
repeated transfections of a scrambled siRNA had no impact on the temporal expression of antigens typically modulated by silencing, we implemented the DOTAP protocol for three knockdowns did not affect cell viability (Figure 2). Furthermore, overall metabolic activity and cell proliferation were measured during HSCs differentiation subjected to two and three transfection rounds compared to untrasfected cells (Figure 3).

These results were found to be consistent throughout the screen of >20 different populations of freshly isolated HSCs and therefore demonstrate a reliable and flexible experimental approach. This system was therefore explored to study the biochemical implication of cathepsin S in the molecular mechanisms associated with HSC differentiation into dendritic cells along the immunogenic or immunosuppressive pathway (Martino et al., submitted manuscript).

Together, our findings point to DOTAP as a chemical suitable for siRNA delivery into HSCs, in light of a transfection efficiency (~60%) comparable to the transduction rate recently achieved in the same human cells through lentiviral vectors [17], and the excellent tolerance to repeated exposures. The ability to dramatically enhance antisense oligodeoxyribonucleotide uptake had, in previous studies, been taken as evidence for DOTAP’s biocompatibility with HSCs [28]. However, absence of any disruption during HSC differentiation further suggests that the DOTAP protocol herein described may be successfully extended to other HSC-related systems, including models aimed at addressing existing controversial perspectives on HSC transdifferentiation and overall plasticity [29, 30].

It is possible that other chemical agents, not included in our screen, may exhibit a performance comparable, if not better, to that produced by DOTAP. In this regard, the cationic lipophosphoramide KLN-5 was shown to be a nontoxic, highly efficient vehicle for transgene delivery into HSCs [31]; however, this chemical has not yet been tested in repeated siRNA transfection procedures and, to our knowledge, is not widely available through vendors. It must be noted that while a large number of lipid formulations could be designed to introduce nucleic acids in cells, a complex combination of chemical-physical and biological factors may severely limit the selection of chemicals capable of transfecting synthetic siRNAs with high transfection scores in HSCs. Indeed, cationic liposomes such as DOTAP, Lipofectamine 2000 and Lipofectamine LTX...
Figure 3: Expression of phenotypic markers throughout HSC differentiation into immunogenic dendritic cells in presence of multiple, DOTAP-mediated transfection rounds. HSCs were subjected to differentiation agents, and then immediately split into two parallel cultures, one of which was maintained in the absence of manipulations, while the other was subjected to DOTAP-based, scrambled siRNA transfection rounds. Phenotypic antigens typically modulated during HSC differentiation into immunogenic dendritic cells were analyzed in HSCs at day 0 (white bars), day 3 (grey bars), and day 14 (black bars). In all instances, analyses were performed through a FACScan flow cytometer using monoclonal antibodies labeled with fluorescein isothiocyanate or phycoerythrin. Data shown are representative of triplicate determinations.

Figure 4: Silencing of cathepsin S during HSC differentiation. (a) Levels of cathepsin S were assessed by Western blotting in dendritic cells generated from the differentiation in vitro of HSCs. During the 14-day differentiation process, cells were subjected to DOTAP-based transfection rounds on day 0, 3, and 9 using a CTSS-targeting siRNA as described in the Materials and Methods. Untreated and scrambled siRNA-transfected cells were employed for control purposes. Levels of precursor (66 KDa) and mature (26 KDa) forms of cathepsin S at different time points (i.e., days 7 and 14) are indicated. β-actin was used to normalize the signals. (b) Shown is the same analysis as in panel A, except for the number of transfections, that were performed on day 0 and 3 only. Graphs are representative of experiments consistently reproduced using at least 20 different populations of HSCs.
display a positively-charged headgroup that engages electrostatic interactions with the sugar-phosphate backbone, a spacer that may or may not be designed to facilitate such interactions, and 1–3 hydrocarbon chains that may vary in length, extent of saturation and distribution of cis and trans configurations [32]. Thus, cationic liposomes can be subject to a high degree of structural permutations, likely correlated with a capacity to transfer nucleic acids in specific cell types. Despite structural similarities and a shared clathrin-mediated endocytosis process for DNA delivery [33, 34], these reagents exhibited a very wide performance range when tested in the HSC system. Marked differences in vector efficiencies may also arise from different mechanisms of internalization linked to subsequent, intracellular trafficking routes associated with macromolecular fates [33].

4. Conclusions

Our findings clearly point to DOTAP as an ideal vehicle for in vitro studies that require gene expression modulation in HSCs. It will, therefore, be intriguing to investigate whether this reagent can also be adapted to in vivo applications, for example, through combination with low-molecular-weight polyethylenimines. These may indeed synergize with liposomes for DNA uptake [35] and have already been proven to be effective in preclinical models [36, 37]. This approach, if successful, would obviate the safety concerns associated with viral-mediated insertional mutagenesis.

Acknowledgments

The authors would like to thank Anna C. Berardi from IRCCS-Bambino Gesù Roma, Italy, for useful discussions and comments on the manuscript. This work was supported by Grants from MIUR (FIRB- Idea Progettuale n.RBIP06FH7J002), Fondazione Cassa Risparmio di Perugia (Project 2007.0149.02), Ministero della Salute (Project n, RF-UMB-2006-339457), and Consorzio INNB to A.O.

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Journal of Biomedicine and Biotechnology


