6-2011

Transfection of human platelets with short interfering RNA.

Wei Hong
Thomas Jefferson University

Altaf A Kondkar
Thomas Jefferson University

Srikanth Nagalla
Department of Medicine, Division of Hematology, Thomas Jefferson University

Wolfgang Bergmeier
Thomas Jefferson University

Ying Jin
Thomas Jefferson University

See next page for additional authors

Published in final edited form as:
Clin Transl Sci. 2011 June ; 4(3): 180–182. doi:10.1111/j.1752-8062.2011.00279.x.

Transfection of Human Platelets with Short Interfering RNA

Wei Hong, M.D., **Altaf A Kondkar, Ph.D.**, **Srikanth Nagalla, M.D.**, **Wolfgang Bergmeier, Ph.D.**, **Ying Jin, M.D.**, **Jay H. Herman, M.D.**, and **Paul F. Bray, M.D.**
The Cardeza Foundation for Hematologic Research and the Department of Medicine, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA

Abstract

Platelets contain mRNAs and are capable of translating mRNA into protein and it has been previously demonstrated that platelets increase their levels of integrin β3 over time while in Blood Bank storage conditions. We are unaware of prior attempts to introduce nucleic acids into platelets. Considering the potential clinical and research utility of manipulating platelet gene expression, we tested whether small inhibitory RNAs (siRNAs) could be transfected into normal human platelets. Multiple conditions were tested, including lipofectamine vs. electroporation, different amounts of siRNA, the effect of different buffers and the presence of plasma during transfection, and the time for optimal siRNA incorporation after transfection. Using flow cytometry to assess transfection efficiency, we found that optimal transfection was obtained using Lipofectamine, washed platelets and 400 pmoles siRNA. Cell-sorting of transfected platelets suggested the incorporated siRNA was able to knockdown the level of a targeted mRNA. This is the first ever demonstration that nucleic acids can be introduced directly into platelets, and offers proof-of-concept for manipulating gene expression in platelets by non-viral methods. Future technical improvements may permit improving the quality and/or lifespan of stored human platelets.

Introduction

Platelet reactivity plays an important role in the pathogenesis of thrombotic and hemorrhagic disorders. Antiplatelet agents are effective for modulating platelet prothrombotic activity. No agents are known to enhance platelet function as might be desired in the bleeding patient with or without thrombocytopenia, and often the only option is platelet transfusion. But the well-characterized platelet storage lesion limits both platelet availability and function after storage.[1] Modulating inherent or acquired platelet reactivity and platelet lifespan could be of potential benefit to patients with hemorrhagic disorders.

Because platelets are anucleate and lack the ability to transcribe DNA, there has been little reason to attempt to modify gene expression in the platelet itself. But elegant studies have established that platelets contain megakayocyte-transcribed mRNAs that are translated into protein in response to physiological stimuli.[2] Human platelets have been shown to synthesize proteins like actin, thrombospondin, fibrinogen, von Willebrand factor and membrane glycoprotiens (GPIb, integrin αIIb and integrin β3).[2–4] And it is especially noteworthy that platelets in Blood Bank storage conditions increase their levels of integrin

Corresponding Author: Paul F. Bray, 1015 Walnut Street, 324 Curtis building, Philadelphia, PA, 19107. Phone: 215-955-8544 Fax: 215-955-9170. paul.bray@jefferson.edu.
Reprint request: Paul F. Bray, 1015 Walnut Street, 705 Curtis building, Philadelphia, PA, 19107. Phone: 215-955-8544 Fax: 215-955-9170. paul.bray@jefferson.edu

Conflict of Interest. The authors have no conflicts of interest to disclose.

β3 (the β subunit of the platelet fibrinogen receptor) over time and that the half-life of this mRNA is longer in platelets than in several different nucleated cells.[5–7]

Small interfering RNA (siRNA) and microRNA (miRNA) modulate protein expression by regulating RNA levels and/or translation. Others and we have shown that platelets express high levels of miRNAs.[8–10] Because platelet mRNA translation may be of substantial importance and considering the potential clinical and research utility of manipulating platelet RNA expression, we asked a simple question: can normal human platelets be transfected with siRNA?

Methods

Platelet preparation

Blood (40 ml) was collected in 3.8% sodium citrate (1:9 citrate to blood vol/vol) from healthy individuals under a protocol approved by the Institutional Review Board for Human Subject Research at Thomas Jefferson University. Blood was centrifuged at 200 × g for 15 minutes to isolate platelet-rich plasma (PRP). In most experiments, platelets were washed in modified Tyrode's buffer (containing 138 mM NaCl, 5.5 mM dextrose, 12 mM NaHCO3, 0.8 mM CaCl2, 0.4 mM MgCl2, 2.9 mM KCl2, 0.36 mM Na_2HPO4 and 20 mM Hepes, pH 7.4) in presence of 1 μM prostaglandin I_2, and resuspended in the same buffer.

Platelet Transfection and mRNA Quantification

Fluorescently-labeled siRNAs for glyceraldehyde 3-phosphate dehydrogenase (FAM-GAPDH siRNA) and a scrambled negative control (FAM-scrambled) (Ambion Inc., TX) were used for transfection of platelets. Platelets (2×10^8 in one mL) were transfected with siRNA using either a cationic-lipid mediated delivery or electroporation, subjected to gentle constant shaking in a 12-well plate at room temperature and harvested at 4–48 hours. Electroporation was performed in a Nucleofactor II® (Amaxa Inc. Walkersville, MD) using the proprietary conditions of program X-005 (recommended for the Meg-01 cell line) and 3 different proprietary buffers. Cationic-lipid mediated delivery[11] was performed using different amounts of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in a 12-well plate. Transfection efficiency was assessed by flow cytometry (FACScan, Becton Dickinson). In some experiments, transfected platelets were isolated using a fluorescence activated MoFlo High Performance Cell Sorter (Dako, Denmark) and platelet RNA was extracted using TRIzol® (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 100 ng of total RNA using Super-Script III reverse transcriptase (Invitrogen), and 10 ng of cDNA was subjected to real-time PCR. Changes in GAPDH mRNA levels were determined by TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA). The relative GAPDH mRNA expression in the different experimental conditions were compared using the $2^{-\Delta\Delta Ct}$ method (ABI PRISM 7900 HT Sequence Detection System). 18S RNA was used as an endogenous control to normalize for differences in starting cDNA levels between samples.

Results and Discussion

The major purpose of this study was to determine whether normal human platelets could be transfected with fluorescently-labeled small inhibitory RNA (siRNA). Two methods of transfection, electroporation and cationic-lipid mediated delivery (Lipofectamine), were tested. All transfections utilized 2×10^8 platelets in a final volume of one mL. We initially tested the different electroporation solutions (proprietary solutions C, V and L) provided by the manufacturer (Amaxa Inc. Walkersville, MD). Cell counting 4 hours post transfection revealed a ten-fold reduction in platelet number in cells transfected with solutions L and V, suggesting these conditions induced platelet agglutination or aggregation. Electroporation

solution C and transfection with lipofectamine produced no loss in platelet number. However, initial flow cytometric analysis showed no platelet fluorescence in the electroporated platelets, and a low level of fluorescence with the lipofectamine treated platelets (not shown). Next, we tested the effect of using different amounts of the lipofectamine reagent and the effect of transfection of washed platelets or platelets in plasma (PRP). Flow cytometric analysis revealed a higher transfection efficiency using washed platelets, and 6 μL lipofectamine produced more platelet fluorescence than 0, 1 or 20 μL of lipofectamine. In addition, more fluorescence was observed 48 hours after transfection than 4 hours after transfection. Subsequent transfections utilized 6 μL lipofectamine and washed platelets. The last set of pilot studies tested the effects of using different amounts of siRNA and of analyzing platelet fluorescence at 24 or 48 hours after transfection. As shown in Fig. 1A, 400 nmole siRNA produced more fluorescence than 25, 100 or 200 nmole. The 24 hour time point was slightly better than 48 hours, and post-transfection addition of 50% RPMI with 10% fetal bovine serum was of no clear benefit.

The above studies identified the optimal transfection conditions as: 6 μL lipofectamine, washed platelets, 400 nmol siRNA and harvest platelets for analysis at 24 hours post transfection. Using these conditions, we observed a 70-fold higher transfection as compared to no lipofectamine (Fig. 1B, 8.4% vs. 0.12%, shown in 2nd and 3rd panels, respectively), indicating the effect of transfection and membrane permeabilization. Other experiments yielded transfection efficiencies of 14% (not shown). Our experimental conditions exclude the possibility of non-specific association of siRNA with the platelets because there was no difference in platelet fluorescence with or without the FAM-siRNA (Fig. 1B, upper 2 panels). Thus, although the transfection efficiency was relatively low, these data demonstrate that indeed, normal human platelets could be transfected with siRNA.

We also performed preliminary experiments to test the ability of transfected siRNA to knock down platelet mRNA levels. Twenty-four hours after transfection, approximately 7×10^7 platelets were subjected to 4 hours of sorting on a FACS, yielding $2–5 \times 10^6$ platelets per transfection, which were sorted directly into Trizol®. This limited yield of transfected platelets did not permit protein assessment, but was sufficient for qPCR quantification of mRNA. Compared to non-transfected platelets, platelets transfected with an siRNA for GAPDH showed a 33% reduction in GAPDH mRNA (Fig. 1C, left panel). Lastly, compared to platelets transfected with a scrambled siRNA, we observed a 26% reduction of GAPDH mRNA in those platelets incorporating the siRNA that targets GAPDH (Fig. 1C, right panel). We cannot exclude the unlikely possibility that the scrambled siRNA but not the GAPDH siRNA enhanced GADPDH translation. Because we started with small blood volumes, too few platelets remained after cell sorting to determine GAPDH protein levels.

These results offer proof-of-concept that nucleic acids can be introduced into platelets by non-viral methods. Clearly, future efforts are needed to increase transfection efficiency and to determine to what extent this approach can be scaled-up for research studies or clinical use. Although our preliminary studies suggest siRNAs have the potential for modifying platelet gene expression, future work can test knockdown of critical genes in platelet function. Prior studies have utilized detergents to permeabilized platelets to introduce proteins,[12] and these platelets are known to retain the ability to undergo agonist-induced platelet aggregation and secretion, but it is not known whether the conditions described in this report will likewise retain platelet reactivity. If so, this approach would have the potential for enhancing platelet function in stored platelets in the Blood Bank or increasing platelet lifespan, both of which could ultimately reduce platelet transfusion need and alloantigen exposure.

Acknowledgments

This work was supported by NIH/NHLBI HL088458.

REFERENCE LIST

1. Thon JN, Schubert P, Devine DV. Platelet storage lesion: a new understanding from a proteomic perspective. Transfus Med Rev. Oct; 2008 22(4):268–279. [PubMed: 18848154]
2. Weyrich AS, Lindemann S, Tolley ND, et al. Change in protein phenotype without a nucleus: translational control in platelets. Semin Thromb Hemost. Aug; 2004 30(4):491–498. [PubMed: 15354270]
3. Booyse FM, Rafelson ME Jr. Studies on human platelets. I. synthesis of platelet protein in a cell-free system. Biochim Biophys Acta. Oct 29; 1968 166(3):689–697. [PubMed: 5722699]
4. Kieffer N, Guichard J, Farcet JP, Vainchenker W, Breton-Gorius J. Biosynthesis of major platelet proteins in human blood platelets. Eur J Biochem. Apr 1; 1987 164(1):189–195. [PubMed: 3830180]
5. Feng X, Clark RA, Galanakis D, Tonnesen MG. Fibrin and collagen differentially regulate human dermal microvascular endothelial cell integrins: stabilization of alphav/beta3 mRNA by fibrin1. J Invest Dermatol. Dec; 1999 113(6):913–919. [PubMed: 10594730]
6. Retta SF, Cassara G, D'Amato M, et al. Cross talk between beta(1) and alpha(V) integrins: beta(1) affects beta(3) mRNA stability. Mol Biol Cell. Oct; 2001 12(10):3126–3138. [PubMed: 11598197]
7. Thon JN, Devine DV. Translation of glycoprotein IIIa in stored blood platelets. Transfusion. Dec; 2007 47(12):2260–2270. [PubMed: 17714413]
8. Bruchova H, Yoon D, Agarwal AM, Mendell J, Prchal JT. Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis. Exp Hematol. Nov; 2007 35(11):1657–1667. [PubMed: 17976518]
9. Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. Nat Struct Mol Biol. Sep; 2009 16(9):961–966. [PubMed: 19668211]
10. Kondkar AA, Bray MS, Leal SM, et al. VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA. J Thromb Haemost. Feb; 2010 8(2):369–378. [PubMed: 19943878]
11. Felgner PL, Gadek TR, Holm M, et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A. Nov; 1987 84(21):7413–7417. [PubMed: 2823261]
12. Lapetina EG, Silio J, Ruggiero M. Thrombin induces serotonin secretion and aggregation independently of inositol phospholipids hydrolysis and protein phosphorylation in human platelets permeabilized with saponin. J Biol Chem. Jun 10; 1985 260(11):7078–7083. [PubMed: 2987242]

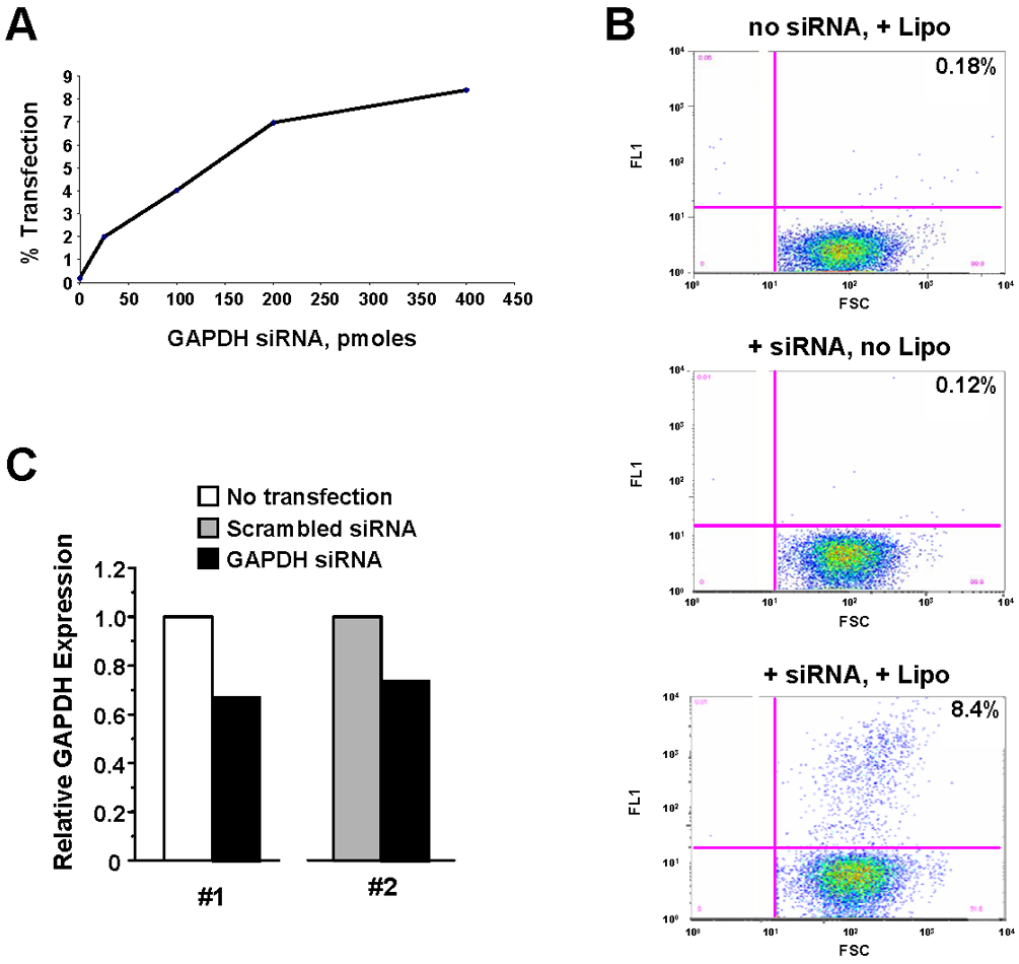

Figure 1. Transfection of siRNA into normal human platelets

Panel A, 2×10^8 washed platelets suspended in 1 mL Tyrode's buffer were transfected with 6 μL lipofectamine and 0, 25, 100, 200 or 400 pmole FAM-labeled GAPDH siRNA, and analyzed at 24 hours by flow cytometry. *Panel B*, Flow cytometric histogram analysis of platelets incubated with 6 μL lipofectamine but no siRNA (upper graph), with FAM-labeled GAPDH siRNA but no lipofectamine (middle graph), and with both FAM-labeled GAPDH siRNA and 6 μL lipofectamine (lower graph). X-axis of histograms is fluorescence intensity and Y-axis is the number of platelets. Percentages in each graph indicate the percent of transfected platelets (number in upper right quadrant divided by total number of platelets times 100%). *Panel C*, siRNA-induced knock down of GAPDH mRNA compared to non-transfected platelets (experiment #1) and compared to scrambled siRNA (experiment #2). GAPDH mRNA expression was first normalized to 18S RNA expression and fold-changes between GAPDH-siRNA and controls were made using the $2^{-\Delta\Delta Ct}$ method.[10]