Published in final edited form as:

Blood Cells Mol Dis. 2010 March 15; 44(3): 169–174. doi:10.1016/j.bcmd.2009.12.004.

Vivo-Morpholino knockdown of α IIb: A novel approach to inhibit thrombocyte function in adult zebrafish

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SUMMARY

Knockdown of protein function by antisense oligonucleotides has been used to understand the protein function not only in development but also in human diseases. Recently, Vivo-Morpholinos, chemically modified morpholinos which penetrate the cells, have been used in adult experimental animal models to alter the splicing and thereby change the protein expression. Until now, there have been no such studies using Vivo-Morpholinos, to evaluate hemostatic function in adult animals. We injected α IIb Vivo-Morpholinos intravenously into adult zebrafish. Thrombocyte function was assayed by time to aggregation assay of the citrated blood, annexin V binding to thrombocytes, and gill bleeding. The thrombocyte functional inhibition occurred in 24 hrs after α IIb Vivo-Morpholinos injection and reached a maximum in 48 hrs. However, in 72 hrs, the inhibition was no longer observed. Reduction of annexin V binding to thrombocytes and increased gill bleeding were observed 48 hrs after α IIb Vivo-Morpholino injections. The action of the α IIb Vivo-Morpholino was demonstrated by the presence of an alternatively spliced α IIb mRNA and the reduction of α IIb in thrombocytes of fish treated with α IIb Vivo-Morpholino. These results provide the first proof of principle that thrombocyte function can be inhibited by thrombocyte-specific Vivo-Morpholinos in adult zebrafish and presents an approach to knockdown thrombocyte-specific genes to conduct biochemical studies in thrombocytes. This study also provides the first antisense antithrombotic approach to inhibit thrombocyte function in adult zebrafish.

INTRODUCTION

Antisense oligonucleotides have recently been used to knockdown protein levels by either translational blocking or splice blocking to control cancer and viral infections with the goal to treat human diseases[1;2]. This knockdown inhibition has been exploited widely in model organisms such as zebrafish predominantly through use of morpholino oligonucleotides (MOs) to study functions of proteins in both development and disease particularly as a gene discovery tool[3;4]. These MOs are introduced into the yolks of 1-8-cell-stage zebrafish embryos. Due to the cytoplasmic bridges, it has been thought that MOs rapidly diffuse into these cells allowing ubiquitous cytosolic delivery. However, direct cytosolic delivery of MOs into cells has been difficult to achieve with the exception of microinjections. Recently, photoactivatable MOs have been introduced to achieve tissue-specific knockdowns in embryos[5]. Another recent development is the conjugation of dendrimeric octaguanidine to MOs (Vivo-MOs) which has

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resulted in permeability of MOs into cells[6]. Because of this membrane diffusible nature and lack of toxicity, it has been suggested for the use in human therapy[7]. In fact, recently Vivo-MOs have been used to evaluate their use in treatment of Duchenne muscular dystrophy[8].

Platelets play a central role in primary hemostasis[9]. During injury, they adhere to subendothelial matrix, become activated, and aggregate to form the primary hemostatic plug. This plug formation involves $\alpha\text{IIb}\beta 3$ activation followed by fibrinogen binding[10;11]. We have shown that primary hemostasis in zebrafish also involves thrombocyte aggregation and that the fibrinogen receptors as well as thrombocyte functions are all intact in fish[12;13]. These results suggest the technology developed in zebrafish should be translatable to other animal models as well as to humans.

Therefore, it is our hypothesis that Vivo-MOs can be used to inhibit hemostatic functions in adult zebrafish thrombocytes. αIIb is an unparalleled target candidate for this inhibition in the primary hemostatic pathway for the following reasons: First, its inhibition by Vivo-MOs will not cause any non-specific effects of inhibition in other cell types because thrombocytes are the only cells which have αIIb on their membrane surface. Second, its down regulation could be controlled by the dose of MOs so proper balance of hemostasis could be achieved. Third, since newly generated young thrombocytes will undergo the effect of MOs and the young thrombocytes have previously been shown to initiate thrombus formation at the site of injury, reduction of αIIb in these young thrombocytes could have an immediate effect. Fourth, the αIIb is the ultimate molecule which receives signals from many pathways and therefore controlled inhibition of this molecule will block the effect of all the pathways. Fifth, if the proof of principle is established for inhibiting thrombocyte function in adult zebrafish, any other candidate factors such as thrombocyte receptors could be targeted for evaluating the function of novel genes involved in hemostatic pathways by studying the biochemical events in thrombocytes because it is possible to collect adult thrombocytes.

In this paper, we provide the first demonstration that the Vivo-MOs can be used to inhibit thrombocyte function in adult zebrafish. We also show that in adult zebrafish only a single intravenous injection is necessary to achieve effective targeting of αIIb in thrombocytes and down-regulation of thrombocyte aggregation concomitant with reduced hemostatic activity.

MATERIALS AND METHODS

Zebrafish antisense injections to generate knockdowns

We designed an antisense αIIb Vivo-MO, 5'- GGAAGTGAATAACCCTCACCTCAT-3' against the donor splice site of exon 20 of the zebrafish αIIb gene and submitted it to Gene-Tools LLC, Philomath, OR for synthesis. A control Vivo-MO 5'- CCTCTTACCTCAGTTACAATTTATA-3' was also purchased from Gene-Tools. Zebrafish were anesthetized and approximately 5 μL Vivo-MOs (either the original solution supplied by the vendor, 0.5 mM or 0.05 mM diluted with phosphate buffered saline, pH 7.4 (PBS)) were taken in the 27G1_{1/4} needle such that the only Vivo-MO solution remained in the needle. For injection, the needle was placed into the region that is located between the second and third body stripes closer to the anal pore and at right angles to the location of the inferior vena cava. It was then gently pushed to insert into the vessel; the syringe piston was immediately pushed gently to inject the contents.

Blood sample preparation and thrombocyte functional assays

Blood collection was performed by gently poking the lateral surface of the fish body where the caudal artery and the caudal vein anastomose with the 27G1_{1/4} needle. A micropipette set was used for collecting 1 μL blood welling out from the vessel. This 1 μL blood was immediately

dispensed into a 0.5 ml Eppendorf tube containing 1 μ l 3.8% sodium citrate in PBS. For qualitative study, the thrombocyte aggregation assay was performed by adding 1 μ l of citrate-buffered blood to 8 μ l 0.63% sodium citrate in PBS and 1 μ l ADP reagent (200 μ M, Sigma-Aldrich, St. Louis, MO) in a conical well of the microtiter plate[12]. The plate was tilted manually every 5 min for 1 to 1.5 hrs at 25°C to determine the time taken to stop the flow of blood down the walls of the well i.e. time taken for aggregation of thrombocytes (TTA). For quantitative study, the thrombocyte aggregation assay was performed without ADP reagent.

For detecting annexin V binding, cells from heparinized blood (2 μ l blood collected into 2 μ l 20 mg heparin in 1 ml PBS pH 7.4) were used. To this blood, 1 μ l of ADP reagent or 1 μ l of PBS as the control was added, and the cells were incubated for 3 min at 25°C. The cells were fixed immediately with 10 μ l 4% paraformaldehyde and to this mixture 2 μ l of 10X annexin binding buffer and 3.5 μ l of annexin V-FITC (BD Biosciences, San Jose, CA) were added and incubated in the dark at 25°C for 1 minute. After annexin V probing, the cells were smeared on a slide and kept under a cover slip, and the fluorescent images of thirty thrombocytes were taken using Nikon Eclipse 80i microscope with excitation at 450-490 nm with constant exposure times. The average intensity of thrombocytes was plotted as levels of annexin V binding[14].

Gill bleeding was induced by placing the fish in 50 μ M NaOH. The fish were anesthetized with 2 mM tricaine (Sigma-Aldrich, St. Louis, MO) for 3 minutes prior to placing them in NaOH. The fish were photographed with a Nikon E995 Coolpix camera and the red pixels were counted by Adobe Photoshop software 7.0 as a measure of bleeding.

RT-PCR

Zebrafish thrombocytes in whole blood were labeled with mepacrine as described earlier[14]. The blood was diluted and placed on the microscopic slide so that thrombocytes were well separated from other cells. Five hundred thrombocytes were pipetted using a Nanoject II micropipette (Drummond Scientific Company, Broomal, PA) under Nikon Eclipse 80 microscope (with excitation at 450-490 nm) and were used in cell to cDNA kit (Agilent Technologies, LaJolla, CA) to amplify the α IIb mRNA. We designed forward 5'-AGTGCTGCATGGACAAAGTG-3' and reverse 5'-GGTTCTCCACCTGTTCCAGA-3' primers for exons 18 and 22, respectively; these were synthesized by Biosynthesis, Lewisville, TX. They were used to amplify the 396 bp product. In the case of exon skipping, the predicted product is 149 base pair. These RT-PCR products were resolved on 1.5% agarose gels and their DNA sequences were determined using sequencing service by Lone Star Labs, Houston, TX. The density of RT-PCR products was measured by Quantity One software from Bio-Rad Laboratories, Inc. Hercules, CA. Since the 149 bp band is 2.5 times smaller than the 396 bp band the intensity of 149 bp band was multiplied by 2.5 so the bands have molar equivalent intensities. Relative percentages were calculated by using the sum of the intensity of the 396 bp and the corrected intensity of 149 bp bands as 100%.

Immunostaining of thrombocytes

Immunofluorescence was performed on freshly prepared blood smears from control and α IIb Vivo-MO injected zebrafish. The slides were fixed with 70% cold ethanol for 15 minutes, and rinsed with PBS three times. 20 μ l of 1 mg/ml rabbit polyclonal antiserum against the zebrafish α IIb peptide RGGTDIDDNGYPDLIG (Custom made by Alpha Diagnostic, Inc., San Antonio, TX) were incubated with blood cells under a cover slip for 1.5 hrs at 25°C. To minimize evaporation the slides were kept in a sealed plastic bag. After removal of the cover slip, the slides were rinsed with PBS three times and then incubated with FITC-conjugated rabbit anti-sheep IgG (Sigma-Aldrich, St. Louis, MO) for 1.5 hr. The slides were rinsed again with PBS followed by a brief rinse with water, then photographed for immunofluorescence using a Nikon Eclipse 80i

microscope. The intensities of the immunofluorescent thrombocytes were quantitated by NIS-Elements AR 2.30 software from Nikon.

Statistical analysis

Statistical analysis was performed using Sigma Plot 10 with Sigma Stat integration software. Statistical significance was assessed by ANOVA and a P value <0.05 was considered significant.

RESULTS

To inhibit the synthesis of α IIb by knockdown method, we chose to splice out exon 20 of α IIb pre-mRNA because the fibrinogen binding site that is critical for the function of α IIb is within this exon. We hypothesized that by direct injection of α IIb Vivo-MOs into the bloodstream these MOs should penetrate thrombocytes and the exclusion of exon 20 in the newly synthesized α IIb should result in reduction in functional α IIb molecules thereby reducing the thrombocyte aggregation potential. To test this hypothesis, we injected 5 μ l of 0.5 mM α IIb Vivo-MO intravenously into adult zebrafish and after 24 hrs isolated thrombocytes by pipetting them individually under the microscope using the Nanoject II. These thrombocytes were analyzed for the alternative splicing by using primers designed from exon 18 and 22 on RNA prepared from these thrombocytes. If the normal splicing occurs this should yield a 396 bp product. If the exon skipping occurs, this should yield 149 bp of DNA. As expected, we obtained a 149 bp band in the thrombocytes of zebrafish where α IIb Vivo-MOs were injected compared to control Vivo-MOs. This result was also confirmed by sequencing the DNA from these bands (Figure 1).

To test the aggregation potential of the thrombocytes treated with α IIb Vivo-MO, we collected blood twenty four hours after treatment with α IIb Vivo-MOs which was then used in whole blood thrombocyte aggregation assay in presence of ADP. We found that the blood collected from control Vivo-MO injected fish did not aggregate when the plate was tilted after 40 min, while it aggregated completely in the presence of ADP (Figure 2A). However, the α IIb Vivo-MO treated fish blood did not aggregate in either the absence or in the presence of ADP suggesting that the α IIb Vivo-MO is inhibiting thrombocyte aggregation (Figure 2B). To further quantitate the aggregation potential of the thrombocytes treated with α IIb Vivo-MO, we collected blood every twenty four hours after treatment with α IIb Vivo-MOs which was then used in whole blood thrombocyte aggregation assay in absence of ADP reagent. The blood from the treated fish took longer time to aggregate compared to control fish in samples obtained after 24 and 48 hours. In 48 hrs α IIb Vivo-MO was the most effective. However, at 72 hrs after injection the effect of α IIb Vivo-MOs was reduced and at 96 hrs it was not pronounced reaching almost control Vivo-MO values. Also, different doses of α IIb Vivo-MOs were used to show that the aggregation was proportionately decreased to the dose (Figure 3a). At 0.5 mM dose 72 hrs-sample, although demonstrated decreased aggregation compared to 48 hrs-sample there was still respectable inhibition of aggregation compared to the corresponding 72 hrs-sample that received 0.05 mM dose. We then tested whether a second dose of α IIb Vivo-MOs at 48 hrs would maintain the effect of MOs by injecting 0.5 mM α IIb Vivo-MOs 48 hrs after the first injection and found that the inhibition of thrombocyte aggregation was maintained at 96 hrs and was similar to that found at 48 hrs whereas the one that did not receive the second dose declined as observed in Figure 3a (Figure 3b).

The RT-PCR analysis of blood cells also showed an increase in 149 bp alternative splice product corresponding to the loss of thrombocyte function (Figure 4a). The intensities of the bands corresponding to unspliced and alternatively spliced mRNA were measured by image analysis. The results showed the alternatively spliced mRNA is about 50% in the second day

as shown in Figure 4b. The relative percentages of 396 bp band and the 149 bp band are shown in Figure 4b.

To test whether the above reduction in mRNA producing α IIb will also result in reduction in α IIb in thrombocytes, we probed the thrombocytes after treating the fish with 0.5 mM α IIb Vivo-MOs for 48 hrs with primary antibody raised against zebrafish α IIb peptide followed by secondary antibody that is labeled with FITC. They were then compared to the thrombocytes derived from fish injected with control Vivo-MOs probed similarly. The fluorescence intensities of thrombocytes representing the amount of α IIb were measured. The results demonstrated the intensity of thrombocytes treated with α IIb Vivo-MOs was about 65% of the control Vivo-MO treated fish (Figure 5). To provide further evidence on the inhibition of function of these thrombocytes treated with α IIb Vivo-MOs, we tested thrombocyte function by annexin V binding assay and found that the thrombocytes treated with α IIb Vivo-MOs for 48 hrs showed less annexin V binding compared to thrombocytes treated with control Vivo-MO (Figure 6). Similarly, in gill bleeding assay, the fish treated with α IIb Vivo-MOs exhibited more bleeding compared to the fish treated with control MOs (Figure 7).

DISCUSSION

In this paper, we demonstrate that direct injection of α IIb Vivo-MOs intravenously into zebrafish will inhibit α IIb and thus reduce thrombocyte aggregation. The RT-PCR results show that alternatively spliced α IIb mRNA is generated resulting in a 149 bp product (confirmed by sequence analysis) which provides evidence that Vivo-MO is effectively penetrating thrombocytes. Interestingly the effect of α IIb Vivo-MO on thrombocyte aggregation was observed in 24 hrs. This result suggests that the α IIb produced from the alternatively spliced product is replaced within this short period of time. One interpretation is that since thrombocytes have a shorter half life of 4.5 days (unpublished data), they are being rapidly replaced. Another explanation is that young thrombocytes which are newly synthesized contain reduced levels of functional α IIb. Since young thrombocytes initiate aggregation and may have functionally reduced α IIb levels, the overall aggregation is reduced. However, since α IIb Vivo-MO treated thrombocytes had overall reduction of annexin V binding to thrombocytes, the former explanation is more likely. In fact, our results of overall reduction of antibody binding to all thrombocytes in contrast to a reduction in a few thrombocytes support this explanation. Thrombocytes are produced in kidney marrow in fishes in contrast to bone marrow in mammals. Indeed, it has been shown that the transcription factor controlling megakaryocyte production is present in thrombocytes and blocking this factor reduced the synthesis of thrombocytes. Thus, the principle of reducing the thrombocytes' function by direct injection of Vivo-MOs into the blood should be translatable for mammalian megakaryocytes and thereby the platelet function could be modulated.

The results also showed that the thrombocyte-aggregation effect of α IIb Vivo-MOs was reduced significantly by the third day after injection. It is possible that Vivo-MOs are being cleared rapidly by either kidneys or by detoxification mechanisms and therefore the thrombocyte-aggregation effect of α IIb Vivo-MOs was not observed. In fact, the increased inhibition of aggregation in 72 hrs-sample that received higher dose of α IIb Vivo-MO compared to the 72 hrs-sample that received the low dose argues in favor of clearance because the higher dose probably takes longer time to clear.

Various α IIb antagonists such as abciximab, tirofiban, eptifibatide have been used as inhibitors of α IIb function[15]. Also in diabetic individuals who have thrombotic tendencies, these inhibitors have been suggested[16]. The current demonstration that Vivo-MOs are non-toxic and inhibit α IIb synthesis provides another opportunity to investigate morpholino based approaches to treat thrombosis. This is particularly suitable because the thrombocyte or

megakaryocyte/platelet is the only cell type making detectable levels of α IIB. It should be possible to use this approach of inhibition of α IIB in mammalian models because targeting megakaryocytes before the production of platelets may occur with α IIB Vivo-MO.

The inhibition of α IIB in adult zebrafish not only provides therapeutic possibilities but also provides the ability to inhibit the function of any thrombocyte specific gene and perform biochemical studies because thrombocytes are readily accessible. For example, thrombocyte surface receptors such as ADP receptors, thromboxane receptors, other GPCRs and several signaling molecules such as kinases could be inhibited. Thus, this method has the advantage of inhibiting molecules for which small molecule inhibitors are not currently available in resolving biochemical pathways. Furthermore, because of the ease in injections and thrombocyte assays, large scale high throughput knockdowns can be designed to identify novel genes participating in thrombocyte development and function. In addition, the same principles could be used to understand other hematological disorders as well as disorders that are amenable for studies by simple injections of MOs.

In summary, we have demonstrated that it is possible to inhibit thrombocyte specific function by exon skipping using α IIB Vivo-MOs in adult zebrafish. This proof of principle of inhibition of α IIB in adult zebrafish by Vivo-MOs should have tremendous applicability not only to identify functions of novel genes in thrombocytes but also in other accessible blood cells. Since it is possible to deliver Vivo-MOs to any hematopoietic cells it should be possible to use these reagents not only as an antithrombotic agent but also in correcting other hematological disorders.

Acknowledgments

We thank Dr. Denise Simmons for critical reading of the manuscript. This research was supported by a grant from the National Institutes of Health, HL077910 (to P.J.).

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Figure 1.
 α IIb Vivo-MO induced alternative splicing resulting in deletion of exon 20 encoding fibrinogen binding site. Agarose gel showing the RT-PCR products from thrombocytes isolated from adult zebrafish injected with control Vivo-MO (Control) and α IIb Vivo-MO (α IIb). Arrows show bands corresponding to the normal splice product (396 bp) and alternatively spliced product (149 bp). 2-log DNA Ladder (New England Biolabs, Ipswich, MA) used as DNA size markers (Marker) are in left lane.

Figure 2.

Thrombocyte aggregation assay using adult fish citrate-buffered blood collected after treatment with α IIb Vivo-MOs. Panels A and B show comparison of blood samples obtained from fish injected with control Vivo-MOs and α IIb Vivo-MOs without ADP agonist (Control and α IIb) and with ADP agonist (Control/ADP and α IIb/ADP) respectively.

Figure 3.

Time and dose-dependent inhibition of thrombocyte function by α IIb Vivo-MOs. **a.** Time after injection of Vivo-MOs and time taken to complete aggregation (TTA) are shown on X and Y-axes respectively. Control represents the control Vivo-MO injections and α IIb represents α IIb Vivo-MO injections. P values show the significance between respective controls and samples collected after 24 hrs, 48 hrs, 72 hrs and 96 hrs and they are 0.009, 0.003, 0.234, and 0.502 (for 0.05 mM Vivo-MO treatment) and 0.009, <.001, 0.006 and 0.242 (for 0.5 mM Vivo-MO treatment) respectively. (b) N=6 for all the samples. **b.** TTA at 96 hrs after Vivo-MO injections. Control represents the control Vivo-MO injections and α IIb represents α IIb Vivo-MO injections.

Figure 4.

Time and dose-dependent inhibition of normal splicing of α IIb transcripts by α IIb Vivo-MOs.

a. Agarose gel showing the time and dose-dependent inhibition of normal splicing represented by the presence of 149 bp band corresponding to alternatively spliced product. Electrophoretic samples from the two different concentrations (0.05 mM and 0.5 mM) of α IIb Vivo-MOs used in intravenous injections. Arrows show bands corresponding to the normal splice product (396 bp) and alternatively spliced product (149 bp). 2-log DNA Ladder (New England Biolabs, Ipswich, MA) used as DNA size markers (Marker) are in left lane. **b.** Table showing the quantitation of 396 bp and 149 bp bands. The numbers in bold are the actual intensities and the numbers in parentheses are the relative percentages.

Figure 5.

Immunofluorescence of thrombocytes **a.** Representative images of immunofluorescence of thrombocytes probed with rabbit antisera zebrafish α IIb peptide polyclonal antibodies. A, Images of thrombocytes treated with control Vivo-MOs and B, Images of thrombocytes treated with α IIb Vivo-MOs. Left and right panels in A and B are bright field and fluorescence images respectively. Arrows show thrombocytes which are translucent with mostly nucleus and scanty cytoplasm; the other cells are erythrocytes. **b.** Intensity of fluorescence of thrombocytes (N=100) in control Vivo-MO treated (control) and α IIb Vivo-MO (α IIb) measured using image analysis programs.

Figure 6. Annexin binding assay. Intensity of fluorescence of thrombocytes (N=30) in control Vivo-MO treated (control) and α IIb Vivo-MO (α IIb) measured using image analysis programs. P value is < 0.001 between the control and α IIb.

Figure 7. Gill bleeding assay. Fish were photographed and the number of red pixels representing the bleeding were measured in control Vivo-MO treated (control) and α IIb Vivo-MO treated (α IIb) zebrafish (N=6). P value is <0.001 between the control and α IIb.