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Separation of young and mature thrombocytes by a novel immuno-selection method

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Abstract

Our earlier studies on the structural and functional properties of zebrafish thrombocytes have shown that they have many similarities to mammalian platelets. We have also shown that zebrafish have both young and mature thrombocytes as do mammalian platelets. In addition, we have distinguished young thrombocytes from mature thrombocytes microscopically using lipophilic DiI-C18, and have shown that young thrombocytes have higher GPIIb receptor levels. However, at present, there is no immunoselection method to separate young thrombocytes from mature thrombocytes in order to study differences among them, such as mRNA expression levels of thrombocyte specific genes. We developed a novel technique employing specific biotinylated anti-Cy3 antibody against the chromophore of DiI-C18 and using streptavidin magnetic beads to separate young thrombocytes from mature thrombocytes. Our technique separates and differentiates young and mature thrombocytes from whole blood. This method is specific and is effective with small amounts of blood.

Keywords

Immuno-fluorescence; Mature thrombocyte; Young thrombocyte; Streptavidin magnetic beads; DiI-C18; Zebrafish

Introduction

Young mammalian platelets, also known as reticulated platelets, are the platelets newly released into circulation. Their levels have been used to study the rates of thrombopoiesis and platelet turnover[1,2]. An increased reticulated platelet population is observed in patients with idiopathic thrombocytopenic purpura, although their total platelet counts are decreased. [3,4]. Recently, we have shown that zebrafish thrombocytes could be used to simulate mammalian hemostasis by nature of their open canalicular system, filopodia formation, adhesion, aggregation, and secretion, as well as the presence of a number of receptors similar to those found on the mammalian platelet surface. Furthermore, we determined that young and mature zebrafish thrombocytes correspond to young and mature mammalian platelets and have shown that young thrombocytes have more adhesive and fibrinogen receptors on their membrane surface than those found on mature thrombocytes

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[5]. We have also shown that young thrombocytes are more hemostatically active than mature thrombocytes. In addition, we have shown that during arterial thrombosis, young thrombocyte form clusters first and are then followed by mature thrombocyte clusters [5]. Recently, we demonstrated that mRNA for GATA1 decreases with concomitant increases in Fli1 mRNA and also suggested that these young and mature thrombocytes could be used to study thrombocyte maturation [6]. However, no systematic studies have been conducted on the differences between mRNA expression among young and mature thrombocytes. This has primarily been due to a lack of technology to rapidly separate young and mature thrombocytes. In order to study such differences, we set out to isolate the young and mature thrombocyte populations from zebrafish whole blood. Currently available methods of separation include flow cell sorting and gradient centrifugation. The flow cell sorting methods are laborious and time consuming. Furthermore, this method would require several runs to obtain sufficient numbers of cells to perform expression studies [7]. Gradient centrifugation methods typically yield cell populations with low purity. In order to circumvent these problems, we developed a novel technique to separate young and mature thrombocytes from small amounts of zebrafish whole blood by using biotinylated anti-Cy3 and anti-GPIIb antibodies. This technique is specific, yields a pure population of thrombocytes, and, most importantly, provides rapid results. Using this method, we isolated young and mature thrombocytes from zebrafish whole blood. The RNA from the thrombocytes isolated by this procedure was found to be highly pure and of sufficient levels to perform RT-PCR studies.

Materials and methods

Materials

DiI-C18 (1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate), also called DiI, was purchased from Invitrogen, Carlsbad, CA. Monoclonal Anti-Cy3/5-Biotin purified mouse immunoglobulin antibody was purchased from Sigma-Aldrich, St. Louis, MO. Custom Antisera GPIIb, biotinylated Anti-GpIIb rabbit antibody, and Custom Polyclonal Pre-immune Serum were custom ordered from Alpha Diagnostic Intl. Inc., San Antonio, TX. Streptavidin-FITC was purchased from Sigma-Aldrich, St. Louis, MO. Streptavidin magnetic beads and 6 Tube Magnetic Separation Rack were purchased from New England Biolabs Inc., Ipswich, MA. Oligonucleotide primers were custom ordered from Biosynthesis, Lewisville, TX. Absolutely RNA miniprep kit was purchased from Stratagene, La Jolla, CA. Superscript One-step RT-PCR kit with Platinum Taq was purchased from Invitrogen, Carlsbad, CA. Agarose gel DNA extraction kit was purchased from Roche Diagnostics, Indianapolis, IN.

Methods

In vivo labeling of zebrafish thrombocytes using DiI-C18—Zebrafish were injected with 10 μ l of 20 μ M DiI solution in phosphate buffered saline (PBS) [8]. After a 30–40 minute incubation period, blood was drawn from each fish with a micropipette tip by making a cut on the lateral side, just above the pelvic fin of the fish [9]. 2–4 μ l blood was collected in a 1.5 ml centrifuge tube containing 1 μ l of 3.8% sodium citrate.

Immunofluorescence on blood thrombocytes—Young thrombocytes were labeled using the anti-Cy3 antibody. For this, 1 µl of zebrafish blood was collected in a tube containing 0.5 µl 3.8% sodium citrate and 1 µl of 20 µM DiI was added. 20 µl of 1:20 dilution of 1 mg/ml biotinylated anti-Cy3 antibody was added and the tube was incubated on ice for 10 min. Unbound antibody was removed by centrifuging at 200 g for 5 min. The pellet was washed with 100 µl of PBS twice and supernatant was removed. 20 µl of 1:100 dilution of 1 mg/ml streptavidin-FITC was added. The cells were centrifuged for 5 min at 200 g, the pellet was washed twice with 100 µl of PBS and resuspended in 10 µl of PBS. 5 µl of this sample was put on a glass slide under a coverslip and was then observed under both bright field and fluorescent light. The DiI labeled cells were observed under 510–560 nm and FITC labeled cells were observed under 450–490 nm.

Mature thrombocytes were labeled using the anti-GPIIb antibody. For this, 1 µl of blood was collected from the zebrafish that were not injected with DiI and smeared on a glass slide. The blood cells were fixed with methanol for 5 s and rinsed with PBS. The cells were incubated under a coverslip with 20 µl of 1:100 dilution of the 1.7 mg/ml biotinylated anti-GPIIb antibody for 30 min at 4 °C [9]. After removing the coverslip, the blood smear was washed 3 times with PBS, each wash for 15 s. Then the smear was incubated with 20 µl of 1:100 dilution of 1 mg/ml streptavidin-FITC for 30 min at 4 °C. The smear was washed with PBS, dried, and observed under bright field and fluorescence light. IgG was used at similar concentrations as a control for the GPIIb antibody.

Fluorescence images were taken using either a Nikon Optiphot microscope or Nikon 80i eclipse microscope equipped with NIS Elements AR 2.30 software.

Isolation of young and mature thrombocytes—To obtain a sufficient number of thrombocytes, 2 or 3 DiI labeled blood samples from different fish were pooled together to reach 10 µl of total blood volume. 3 µl of 1 mg/ml biotinylated anti-Cy3/ Cy5 antibody was added to 10 µl of DiI labeled blood and the blood sample was incubated for 30 min on ice, with intermittent vortexing, approximately every 10 min. 100 µl of streptavidin coated magnetic beads was added to this sample, gently mixed, and again incubated for 30 min on ice. The tube containing this mixture was kept on a magnetic stand for 30 s. Since streptavidin and biotin form a strong bond, the streptavidin magnetic beads, biotinylated antibody, and attached DiI labeled young thrombocytes were all pulled towards the magnet while the tube was on the stand. The beads with young thrombocytes were washed 8 times with 400 µl of PBS and, after the final wash, were resuspended in 100 µl of PBS to prepare for RNA extraction. The remaining blood was aspirated in another tube for mature thrombocyte separation. 10 µl of 1.7 mg/ml biotinylated anti-GPIIb antibody was added to the tube containing remainder of the blood sample and incubated for 30 min on ice. This mixture was subjected to magnetic bead separation and washing to obtain mature thrombocytes using the protocols described above.

RNA preparation from thrombocytes and zebrafish whole blood—Stratagene RNA miniprep kit was used to prepare RNA from the isolated young and mature thrombocytes. RNA prep procedure: 200 µl Lysis buffer with 1.4 µl of β-mercaptoethanol was added to the bead suspension. After 5 min incubation, beads were separated out using

magnetic stand and the remaining cell suspension was filtered through pre-filter spin cup. 70% ice cold ethanol was mixed with the filtrates and this mixture was then centrifuged using the RNA binding spin cup. The RNA spin cup was washed with low salt wash buffer and DNase solution was added; the contents were then incubated. The spin cup was then washed with high and low salt buffers. After washing, 40 µl of elution buffer was added to elute the RNA.

Purity of the isolated RNA was checked by RT-PCR. Young thrombocyte RNA was amplified using primers for cell specific markers. Zebrafish Pu.1 (forward and reverse) was used as a neutrophil marker, β -globin was used as red cell marker, and GPIIb was used as thrombocyte specific marker. Elongation factor 1- α (EF1- α) was used as an internal control for RT-PCR [10]. Zebrafish whole blood RNA was amplified using the same cell markers and used as a positive control. The following primers were used for RT-PCRs. For EF1- α , (5' CGGTGACAACATGCTGGAGG 3', 5'-ACCAGTCTCCACACGACCCA-3') [13], Pu. 1 (5'-TCAAATGAAAAGCAGCGTCATATTC-3', 5'-CCATAGCACATCATGAAAGTTCAC-3') [11], β -globin (5' GGCCTGTGGGGAAAGCTC 3', 5' GTTGTCGGGATCCACATGCAG 3') [12], and GPIIb (5' CAGCTGGACAGAATGAAGCA 3', 5' GGGAGTCAGCCAAGCTGTAG 3') were used. RT-PCR was performed for 40 cycles, at a melting temperature specific for each of the primer pairs. The RT-PCR products were electrophoresed on 2% agarose gel. The product sizes were 220 base pair (bp) for EF1- α , 200 bp for Pu.1, 269 bp for β -globin, and 199 bp for GPIIb primers. The GPIIb primers were designed using Primer 3 software to amplify zebrafish GPIIb gene. The EF1- α , Pu.1, and β -globin primer sequences were designed from previously published reports.

Results

Principle of separation of young and mature thrombocytes

At low concentrations, DiI-C18 labels only young thrombocytes and both young and mature thrombocytes can be labeled with antibody raised against GpIIb. Thus, we reasoned that if DiI-C18 binds to the cell membrane lipid bilayer, and if an antibody is present against the DiI chromophore, we should be able to separate young thrombocytes from blood cells and that we should be able to separate the remaining mature thrombocytes from the blood cells by using the antibody against GpIIb. We hypothesized that the anti-Cy3 antibody should bind to DiI-C18 since it has a chromophore similar to Cy3 (Fig. 1). Furthermore, since antibodies can be biotinylated, separating these cells from whole blood should be susceptible to magnetic separation using streptavidin conjugated beads. Such cells should be pure and utilizable for biochemical and molecular studies (Fig. 2).

Recognition of DiI-C18 by anti-Cy3 antibody

Zebrafish thrombocytes were labeled by intravenous DiI injections. Once the fish were injected and incubated, blood was collected and a small aliquot was checked for DiI labeled thrombocytes. When the remaining blood was probed with biotinylated anti-Cy3 antibody and observed under fluorescence, labeled thrombocytes exhibited a green fluorescence. Green cells were not present on the control slide, not labeled with DiI (data not shown).

Thrombocytes can be differentiated from other blood cells in the bright field image seen in Fig. 3A.

Labeling of thrombocytes with biotinylated anti-GPIIb antibody

To separate mature thrombocytes from the remaining blood cells, we developed a biotinylated anti-GPIIb antibody. To test the efficacy of this antibody, we incubated zebrafish blood smear with biotinylated anti-GPIIb antibody and then with streptavidin FITC. We found that all thrombocytes were labeled by this antibody and demonstrated green fluorescence. The control slide, in which pre-immune rabbit antisera was substituted for the anti-GPIIb antibody, did not show any green fluorescent thrombocytes (data not shown). Labeling of young thrombocytes alone by anti-cy3 and also all thrombocytes by biotinylated anti-GPIIb antibody provided the basis for separation of young and mature thrombocytes by this dual labeling method.

Isolation of young and mature thrombocytes

To test whether streptavidin coated magnetic beads will pull DiI labeled young thrombocytes labeled with biotinylated anti-Cy3 antibody, we spread the magnetic beads along with these cells on slides and examined whether DiI labeled cells are found in those magnetic beads. As predicted we found the DiI labeled cells attached to beads after the separation using anti-Cy3 antibodies (Fig. 4). When a large number of beads were spread on slides, we noticed the cells under these beads (Fig. 4, top panels). In contrast, when a small number of beads were spread, we could see individual cells attached to beads, although the cell morphology changed relative to blood smears (Fig. 4, bottom panels). Subsequently, the mature thrombocytes were pulled using the biotinylated anti-GpIIb antibody and streptavidin magnetic beads. For this, after separation of the young thrombocytes from the whole blood, the remaining blood cells were used to separate the mature thrombocytes using the anti-GPIIb antibody and the magnetic beads. The beads were washed several times to get rid of the contaminating red cells and the white cells.

Purity of isolated RNA

The young thrombocytes obtained by the above streptavidin selection were used in preparation of RNA. Purity of these thrombocytes was checked by RT-PCR using cell specific markers such as thrombocyte marker GPIIb, neutrophil marker Pu.1, and red cell marker β -globin. As an internal control, EF1- α was used. Whole blood RNA was used as a positive control. The whole blood RNA control showed positive amplification for all three cell markers (Pu.1, β -globin, and GPIIb) and the internal control EF1- α , whereas the young thrombocyte RNA showed only GPIIb amplification and the internal control EF1- α (Fig. 5). We then verified these results by determining if the mature thrombocytes purified by this method were positive for GPIIb. We found that they were in fact intensely positive for the GPIIb amplification product (Fig. 6).

Discussion

The principle established in this paper separates young and mature thrombocytes from whole blood based upon the detection of the DiI chromophore by the anti-Cy3 antibody .

This method is novel and has application for any cell type that can specifically be labeled by DiI, including some brain cells. Panning techniques, such as this one, are superior to the FACS method in quickly obtaining a cell sample, since FACS sorting is time consuming. The methods established here have an innate capability to sort thrombocytes in a very rapid fashion. Furthermore, when sorting live cells, the time delay involved in FACS, makes this quick separation method advantageous.

The novelty of this paper is the observation that the chromophores of Cy3 and DiI are similar. Based upon this observation, we checked for the recognition of DiI-C18 by anti-Cy3 antibody. The results indicated that the anti-Cy3 antibody reacted with DiI and was bound to the DiI chromophore on the surface of thrombocytes and did not bind to the DiI negative thrombocytes in the control smear. These results provided proof for the specificity of the Cy3 antibody for DiI. We also generated a biotinylated anti-GPIIb antibody and tested the specificity of this antibody. Our results demonstrated that this antibody is specific for both young and mature thrombocytes. Since the DiI labeled thrombocytes are eliminated in the first round of streptavidin magnetic bead selection, mature thrombocytes can easily be selected from the remaining blood cells with this biotinylated anti-GPIIb antibody. The results of the biotinylated GPIIb antibody experiments provided the basis for second round of selection.

Using the above principles, we separated young and mature thrombocytes and isolated the RNA from them. We checked for purity using RT-PCR and the results demonstrated that in the young thrombocytes prepared by first round selection, only GPIIb RNA was detected while the neutrophil-specific Pu.1 and red cell-specific β -globin, RNAs were not detected. These results provided solid evidence that the young thrombocytes obtained by this method were not contaminated with any neutrophils or red blood cells, suggesting that this method can separate young thrombocytes from blood cells with high purity. In the second round of selection, we observed that the enriched mature thrombocytes also showed intense GPIIb RNA amplification, suggesting that the second round of selection also provided pure mature thrombocyte populations.

In conclusion, in this paper we have described a method to separate young and mature thrombocytes from whole blood by modulating the concentrations of DiI-C18 and by using DiI specific anti-Cy3 and GPIIb specific anti-GPIIb antibodies and have demonstrated its application for the first time. The novel technique we developed here separates these thrombocytes to the highest purity from miniature amounts of blood, as little as 1 μ l. In this paper we have shown that the anti-Cy3 antibody detects DiI-C18 and have also developed a GPIIb zebrafish specific antibody. These reagents should be useful for the analysis of thrombocyte function. This methodology opens a new avenue to study differences in signaling pathways between these different populations of thrombocytes. For example, the use of specific inhibitors or knockdown approaches allows for the comparison of specific pathway differences between young and mature thrombocytes. Since this method of dye labeling is not only feasible in fish but also in other animals which have thrombocytes, this method can be generalized and applied towards the separation of thrombocytes from any thrombocyte bearing animal. As we have shown in our previous work, human reticulated platelets could be specifically labeled in vitro by appropriately mixing human blood and DiI

[5]. Thus, the method developed here can be applied for the separation of reticulated and mature platelets in humans. Because of the small volumes of blood necessary for this technique, it should especially have application to analyze platelets from neonates, where blood samples understandably yield limited amounts. Furthermore, the methods presented in this paper can be applied to separate other cell types that respond to DiI-C18 labeling, including certain neuron populations.

Acknowledgments

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References

- [1]. Ault KA. Flow cytometric measurement of platelet function and reticulated platelets. *Ann. N. Y. Acad. Sci.* 1993; 677:293–308. [PubMed: 8494216]
- [2]. Harrison P, Goodall AH. “Message in the platelet” — more than just vestigial mRNA! *Platelets.* 2008; 19:395–404. [PubMed: 18925506]
- [3]. Saxon BR, Mody M, Blanchette VS, Freedman J. Reticulated platelet counts in the assessment of thrombocytopenic disorders. *Acta Paediatr. Suppl.* 1998; 424:65–70. [PubMed: 9736223]
- [4]. Abe Y, Wada H, Sakakura M, Nishioka J, Tomatsu H, Hamaguchi Y, Oguni S, Shiku H, Nobori T. Usefulness of fully automated measurement of reticulated platelets using whole blood. *Clin. Appl. Thromb. Hemost.* 2005; 11:263–270. [PubMed: 16015411]
- [5]. Thattaliyath B, Cykowski M, Jagadeeswaran P. Young thrombocytes initiate the formation of arterial thrombi in zebrafish. *Blood.* 2005; 106:118–124. [PubMed: 15769888]
- [6]. Jagadeeswaran P, Lin S, Weinstein B, Hutson A, Kim S. Loss of GATA1 and gain of FLI1 expression during thrombocyte maturation. *Blood Cells Mol. Dis.* 2010; 44:175–180. [PubMed: 20110178]
- [7]. Niessen J, Jedlitschky G, Grube M, Bien S, Strobel U, Ritter CA, Greinacher A, Kroemer HK. Subfractionation and purification of intracellular granule-structures of human platelets: an improved method based on magnetic sorting. *J. Immunol. Methods.* 2007; 328:89–96. [PubMed: 17884082]
- [8]. Gregory M, Jagadeeswaran P. Selective labeling of zebrafish thrombocytes: quantitation of thrombocyte function and detection during development. *Blood Cells Mol. Dis.* 2002; 28:418–427. [PubMed: 12367586]
- [9]. Jagadeeswaran P, Sheehan JP, Craig FE, Troyer D. Identification and characterization of zebrafish thrombocytes. *Br. J. Haematol.* 1999; 107:731–738. [PubMed: 10606877]
- [10]. Lin HF, Traver D, Zhu H, Dooley K, Paw BH, Zon LI, Handin RI. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. *Blood.* 2005; 106:3803–3810. [PubMed: 16099879]
- [11]. Lieschke GJ, Oates AC, Paw BH, Thompson MA, Hall NE, Ward AC, Ho RK, Zon LI, Layton JE. Zebrafish SPI-1 (PU.1) marks a site of myeloid development independent of primitive erythropoiesis: implications for axial patterning. *Dev. Biol.* 2002; 246:274–295. [PubMed: 12051816]
- [12]. Chan FY, Robinson J, Brownlie A, Shivdasani RA, Donovan A, Brugnara C, Kim J, Lau BC, Witkowska HE, Zon LI. Characterization of adult alpha- and beta-globin genes in the zebrafish. *Blood.* 1997; 89:688–700. [PubMed: 9002973]

Fig. 1.
Comparison of the structures of DiI-C18 (top) and Cy3 (bottom) dyes. Note the similarity between the heterocyclic chromophores of these two dyes.

Fig. 2.

Principle of thrombocyte isolation using magnetic beads. Y, young thrombocyte; M, mature thrombocyte; R, red cell; N, neutrophil; B, biotin labeled antibody; S, streptavidin beads attached to magnet. IgG/Cy3 and IgG/GPIIb are represented as Y shaped structures. Thrombocyte and neutrophil are shown in shaded circles. The triangular and square structures on the shaded circles represent Cy3 and GPIIb respectively. Red cells are shown in shaded ovals.

Fig. 3.

Immunofluorescence images of blood cells. A, images showing blood cells incubated with anti-Cy3 antibody kept under a cover slip. Left panel, bright field image; middle panel, fluorescence image of the DiI labeled thrombocyte; right panel, fluorescence image of the streptavidin FITC labeled thrombocyte. B, images showing blood smears probed with biotinylated anti-GPIIb antibody followed by streptavidin FITC. Left panel, bright field image; right panel, fluorescence image of the streptavidin FITC labeled thrombocyte. Arrows indicate the thrombocytes.

Fig. 4.
Slide showing streptavidin magnetic beads after pulling the DiI labeled young thrombocytes.
A, bright field images and B, fluorescence images.

Fig. 5. Purity of the isolated young thrombocytes. (A) Whole blood RNA control showing positive amplification for EF1- α (labeled as EF- α), Pu.1, GPIIb, and β -globin (labeled as β glo) genes. (B) Young thrombocyte RNA showing positive amplification for EF1- α and GPIIb genes.

Fig. 6.

Presence of GPIIb transcripts in mature thrombocytes. Left and right panels show the positive amplification for EF1- α and GPIIb in both young and mature thrombocytes, respectively.