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Role of zebrafish thrombocyte and non-thrombocyte microparticles in hemostasis

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Abstract

Hemostasis is a defense mechanism that protects an organism from bleeding in the event of injury. We have previously demonstrated the utility of the zebrafish as a model to study human hemostasis. However, there are no studies on the role of microparticles in hemostasis in early vertebrates. Studying microparticles in zebrafish may provide insight into the evolution of microparticle function in hemostasis and may lead to direct observation of these microparticles in zebrafish larvae due to transparency of the vessels. In this investigation we demonstrate the presence of cellular microparticles in fish blood by both immunostaining as well as by using zebrafish whose thrombocytes are labeled with green fluorescent protein. Further investigation showed that microparticles were also labeled by fluorescein isothiocyanate annexin V, suggesting that these particles are derived via apoptosis. A portion of the fluorescein isothiocyanate annexin V labeled microparticles was also labeled by DiI-C₁₈. Labeling by DiI-C₁₈ suggests that some microparticles are derived from young thrombocytes. Additionally, GpIIb antibody labels almost all thrombocyte-derived microparticles and a greater percentage of microparticles are labeled by GpIIb antibody than by DiI-C₁₈. This suggests that thrombocyte microparticles are derived from both young and mature thrombocytes. Furthermore, the increase of microparticles by adding excessive microparticles into blood *in vitro* and through intravenous injections led to an increased hemostatic response. In addition, treatment with tumor necrosis factor alpha resulted in an increased number of thrombocyte microparticles and enhanced hemostasis; in contrast, treatment with zVAD-FMK, a caspase inhibitor, resulted in a decrease in thrombocyte microparticles and decreased hemostasis. We also found that thrombocyte microparticles agglutinate, along with other cells and cellular microparticles, in the presence of an excess of either ristocetin or ultra-large von Willebrand factor. Also, stimulation of von Willebrand factor release *in vivo* resulted in clusters of thrombocyte microparticles in the veins. Moreover, thrombocyte microparticles were the first to appear at the site of arterial injury. We found that thrombocyte microparticles are functionally equivalent to platelet microparticles. The microparticles initiate arterial thrombus formation in a von Willebrand factor-dependent manner and further enhance thrombus formation by forming clusters of microparticles in venous thrombosis. This finding may have applications for understanding the role of platelet microparticles in humans and may have diagnostic applications.

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Keywords

Thrombocytes; Microparticles; vWF; Thrombosis; Platelets; Zebrafish

Introduction

Microparticles are membrane vesicles produced from cells by either cellular activation or apoptosis. Blood carries several types of microparticles derived from a variety of cells, e.g. platelets, leukocytes/monocytes, and endothelial cells. In humans, it has been shown that the majority of microparticles are of platelet origin [1]. Microparticles carry the corresponding membrane proteins from which they originate. For example, platelet microparticles carry membrane receptors similar to those present on platelets, such as P selectin, which is translocated to the cell surface after activation, as well as GpIIb, GpIIIa, and GpIb [2]. Platelet derived microparticles also carry phosphatidylserine (PS) on the membrane surface [3]. In fact, PS on the membrane surface has been suggested to indicate that cells are undergoing apoptosis. Studies on platelet microparticles have suggested that they may be involved in functions such as induction of coagulation by activated protein C resistant factor Va, which is bound to microparticles [4]. Binding studies on artificially generated platelet microparticles revealed that platelet microparticles may promote platelet interaction with subendothelial matrix in a GpIIb/IIIa-dependent mechanism [5]. However, little is known about microparticles in zebrafish or other early vertebrates. This information may provide insight into the evolution of microparticle function in hemostasis. Furthermore, such knowledge will also be helpful in understanding the role of microparticles *in vivo* in greater detail due to the transparency of zebrafish larvae and easy visualization of blood vessels.

In this investigation, we found that zebrafish carry microparticles derived from both thrombocyte and non-thrombocyte cells. We then discovered that, in the absence of blood cells, these microparticles participate in agglutination response to ristocetin; whereas, in the presence of blood cells, the microparticles disperse among the cells of the agglutinate. The microparticle production was significantly reduced when fish were treated with an irreversible, pan-specific caspase inhibitor, zVAD-FMK, and was increased when treated with tumor necrosis factor alpha (TNF- α). These changes in microparticle production resulted in corresponding increases and decreases of hemostatic function both *in vitro* and *in vivo*. We also demonstrated that the microparticles appear as clusters inside the vein when fish were treated with Stimat. In these Stimat-treated fish we have also shown enhancement of hemostatic function. We then found that the thrombocyte microparticles are the first responders in arterial injury but not in venous injury. Taken together, these findings suggest that thrombocyte microparticles are involved in both initiation and propagation of thrombus formation.

Materials and methods

Zebrafish aquaculture

The following methods of zebrafish aquaculture were conducted similar to those previously described [6]. Briefly, adult zebrafish, larvae, and embryos were raised in a circulating water

system. The water used was deionized, supplemented with Instant Ocean, and kept at 28 °C. Embryos were collected.

Blood collection, intravenous injection and nasal spray

Blood was collected from adult zebrafish as described earlier [7]. Intravenous (IV) injections into adult zebrafish were performed using 5 µl of either 100 µg/ml TNF-α, 1 mg/ml zVAD-FMK, or 10 µM DiI-C₁₈ (DiI) [8]. IV injections into 4 day old larvae used 5 nl of plasma free microparticle suspension or each of the above reagents [9]. We also utilized 1.5 mg/ml Stimite (Desmopressin acetate) nasal spray (gift from Shelly Crary, UT Southwestern Medical School) [10]. The Stimite was first sprayed into an Eppendorf centrifuge tube and centrifuged at 10,000g for 1 min. With all the Stimite now in the bottom of the Eppendorf tube, the solution was measured using a pipetteman and diluted five-fold with distilled water. After the dilution, larvae were placed in this solution for 30 seconds and then returned to water to wash away excess Stimite.

Labeling, detection, and quantification of thrombocyte microparticles

Labeling of microparticles using GpIIb antibody with fluorescein isothiocyanate (FITC) conjugated secondary antibody, FITC annexin V, and DiI was performed according to the procedures previously established for thrombocyte [7,8,11]. For direct FITC labeling of microparticles, 1 µl FITC (1 mg/ml in PBS) was mixed with 2 µl blood and incubated for 2 min. For *in vitro* microparticle detection, 2 µl of blood from both DiI injected and CD41-GFP transgenic zebrafish [12] was placed on a microscopic slide, smeared, and examined for fluorescence using either a Nikon Optiphot microscope or Nikon 80i eclipse microscope equipped with NIS Elements AR 2.30 software. The DiI labeled microparticles were detected by excitation at 510–560 nm; GFP labeled microparticles excited at 450–490 nm. In addition, FITC labeled microparticles were also detected at excitation frequencies similar to green fluorescent protein (GFP) particles. For *in vivo* detection, adults were anesthetized with 125 µM tricaine (Sigma-Aldrich, Saint Louis, MO), placed on a slide, and observed under the microscope [11]. DiI and GFP labeled microparticles inside the vessel were detected using the same frequencies as described above. For observation of these particles in larvae, larvae were prepared according to previously published procedures [9]. The thrombocyte microparticles were quantified in a Becton-Dickinson FACSCalibur flow cytometer. Acquisition was gated to include only those particles of a certain size of those labeled with either GpIIb antibody (custom ordered from Alpha Diagnostic, Inc., San Antonio, TX) followed by FITC conjugated secondary antibody, DiI, FITC annexin V (BD Biosciences, San Jose, CA), or GFP. All signals were detected using FITC channel except DiI, which was detected in phycoerythrin channel. Ten-thousand positive particles from each sample were analyzed for forward and right angle light scatter and for fluorescence intensities.

Microparticle preparation

Plasma free microparticles were collected from 2 µl citrated blood of wild type fish by centrifugation at 500g for 10 min. The supernatant plasma was centrifuged at 20,000g for 15 min and the pellet was washed with 1 ml, pH 7.4 phosphate buffer saline (PBS) and centrifuged again at 20,000g for 15 min. The washed pellet was then suspended in 2 µl PBS

for further use. FITC microparticles were also prepared as described above. Microparticle rich plasma was prepared from 2 μ l citrated blood by centrifugation at 500g for 10 min and the supernatant plasma was used. We also generated thrombocyte microparticles *in vitro*. For this we centrifuged 2 μ l citrated blood of CD41-GFP fish at 150g for 15 min. The supernatant devoid of red cells was centrifuged at 500g for 10 min, and the white cell pellet including thrombocytes was suspended in PBS. The washed white cells containing thrombocytes were stimulated with 0.5 μ g/ml thrombin, 10 μ g/ml collagen, or a combination of these two agonists to generate thrombocyte microparticles and were then collected as described above.

Functional evaluation assays

1 μ l plasma free microparticle suspension was added to 2 μ l blood and 1 μ l of this blood was used in whole blood aggregation reaction using a plate tilt assay as described [7,11]. For blood-free microparticle agglutination/aggregation experiments, 1 μ l of plasma rich microparticles or plasma free microparticle suspension was used directly in the plate-tilt assay, in the place of whole blood. For these assays, the concentration of agonists (ADP, collagen, and ristocetin) was the same as described earlier [7,10,11]. For agglutinations involving ultra-large von Willebrand factor (ULVWF), we used human ULVWF and ADAMTS-13 metalloproteinase (a gift from Jing-Fei Dong, Baylor College of Medicine) [13,14]. For this assay, 1 μ l of plasma-free microparticles was mixed with 0.1 μ l of 100 μ g/ml ULVWF and incubated for 5 minutes. To demonstrate that ADAMTS-13 inhibits agglutinate formation induced by ULVWF, we added 0.1 μ l of 50 μ g/ml ADAMTS-13 to the above reaction mixture. After aggregation/agglutination, an aliquot of either the whole blood agglutinate/aggregate or microparticle agglutinate/aggregate was observed under a cover slip. The images were photographed using a Nikon E995 CoolPix digital camera. To quantify *in vitro* agglutination/aggregation, time taken to complete formation of agglutinate/aggregate (TTA) was measured according to the published method [11]. For *in vivo* quantification of the effect of microparticles on hemostatic function, laser induced time to occlusion (TTO) assay was performed on larvae according to the previously published procedures [15] using the above described injections. TTO was recorded using a Nikon Optiphot microscope as described previously [15]. For laser injury of DiI injected adult zebrafish, fish were anesthetized as described above and placed on a microscopic slide. Under a microscope, a clearly visible tail artery was targeted for laser injury. Using the above excitation frequencies, time-lapse images were taken each second after injury. A Nikon Optiphot microscope and one second exposure times were used. In both *in vitro* and *in vivo* experiments, PBS was used for controls.

Results

Identification of non-thrombocyte and thrombocyte microparticles in zebrafish

To determine whether thrombocyte microparticles are present in zebrafish blood, we used zebrafish blood smears from DiI injected zebrafish because we have shown in our earlier work that DiI specifically labels young thrombocytes [16]. We also used blood smears from CD41-GFP transgenic zebrafish because, in these fish, only thrombocytes are labeled by GFP. The blood smears from the above fish showed microparticles (Fig. 1). In addition to the

above microscopic analysis, we also performed fluorescence-activated cell sorting (FACS) analysis of the GpIIb immunolabeled (because GpIIb antibody is specific for thrombocytes) and the GFP labeled thrombocyte particles. GpIIb labeled particles made up approximately 45% of the total microparticles, while GFP labeled particles were about 15% of the total (Fig. 2a and b). By FACS analysis, we then quantified the DiI positive microparticles in the zebrafish blood and found that they make up about 14% of the total microparticles. Considering human platelet microparticles also show FITC annexin V binding *via* PS, we investigated whether there are FITC annexin V positive microparticles in zebrafish and found that they are indeed present in zebrafish blood and represented approximately 20% of total microparticles. We then performed double labeling, with both FITC annexin V and DiI, and found that almost all DiI positive microparticles were labeled with FITC annexin V (Fig. 2c). However, not all FITC annexin V labeled microparticles were DiI positive.

Platelet microparticles can be produced by stimulation with collagen and thrombin, thus, we explored the affects of collagen and thrombin on thrombocyte microparticles. For this we generated microparticles by stimulating the CD41-GFP fish white cells, with collagen and thrombin. Microparticle production was measured by FACS. We found either thrombin or collagen stimulation of thrombocytes produced microparticles 1- or 2-fold, respectively, above the control level whereas the simultaneous stimulation by collagen and thrombin resulted in a greater than 70-fold induction of microparticle production (Fig. 3a).

Role of microparticles in hemostasis

To test whether the microparticles enhance thrombocyte aggregation/agglutination, we used the microplate tilt assay, developed earlier in our laboratory [7,11]. We added microparticles to the zebrafish blood in the above assay and measured TTA. The results showed a shorter TTA, and thus greater aggregation/agglutination, for the sample to which microparticles were added, compared to control blood (Fig. 3b). To test whether microparticles enhance hemostasis *in vivo*, we injected these microparticles into 4–5 days old zebrafish larvae and found, upon laser injury, the treated larvae demonstrated significantly shorter TTOs as compared to control larvae (Fig. 3c and d). To test whether thrombocyte microparticles play a direct role in hemostasis, adult fish were given intravenous injections of either zVAD-FMK, which should decrease microparticles generated from apoptosis, or TNF- α , an agent that has been shown to increase platelet microparticles [17]. Twenty-four hours after treatment we estimated the thrombocyte microparticles. The results show the thrombocyte microparticles were significantly reduced in the fish blood treated with zVAD-FMK whereas there was a significant increase in these particles under TNF- α treatment (Fig. 4a). When we performed whole blood aggregation on blood taken from the above treated fish, there were similar reductions and increases in TTA (Fig. 4b). Additionally, larvae were given intravenous injections with zVAD-FMK and TNF- α . Again, after 24 h, laser induced TTO was measured. The larvae had significant reduction and increases in TTO, respectively, when compared to controls (Fig. 4c and d).

von Willebrand factor (vWF) mediated agglutination of microparticles

Our results above indicated thrombocyte microparticles carry surface receptors (labeled by GpIIb antibody) and cytoplasm (labeled by GFP). Since both receptors and cytoplasm are

necessary for signaling of thrombocyte aggregation, we tested whether thrombocyte microparticles themselves could form agglutinates/aggregates in response to agonists. Using blood from CD41-GFP fish and the whole blood aggregation assay, we tested whether microparticle aggregates/agglutinates formed in response to agonists. We found that, though ADP and collagen formed thrombocyte aggregates, the aggregates did not contain any thrombocyte microparticles or any other cell type (data not shown). However, the ristocetin induced agglutination yielded agglutinates, which contained not only thrombocytes but also red cells, thrombocyte microparticles, and non-thrombocyte microparticles (Fig. 5a). The GFP labeled microparticles were interspersed throughout the agglutinate and seemed to close gaps between blood cells. These observations demonstrated that microparticles respond to ristocetin. To facilitate better visualization of the microparticles, we used FITC to label whole blood from wild-type fish and found that FITC labels all cells including microparticles. Microparticles were isolated by centrifugation and the microparticle pellet was found to be mostly devoid of cells (Fig. 5b). Then, we tested whether the microparticles themselves would agglutinate in presence of ristocetin and found that they form agglutinates (Fig. 5c). We then investigated the effect of plasma on agglutinate formation using plasma free microparticles and microparticle rich plasma isolated from FITC labeled blood. We found that these microparticles did not form any ristocetin induced agglutinates in the absence of plasma; however, they formed agglutinates in presence of plasma (Fig. 5d). To test whether the zebrafish microparticles agglutinate in the presence of human ULVWF, we incubated plasma-free microparticles with ULVWF and found large agglutinates of microparticles similar to ristocetin-mediated agglutination of microparticles in the presence of plasma as shown in Fig. 5d, left panel. Subsequently, we tested whether the presence of human ADAMTS-13 metalloproteinase reduces the agglutinates induced by ULVWF. In the presence of both ULVWF and ADAMTS-13, plasma-free microparticles did not form agglutinates similar to Fig. 5d, right panel. We also found similar agglutinates using microparticles derived from DiI labeled blood; in addition to small microparticles, these agglutinates also had large clusters of DiI particles (Fig. 5e). This ristocetin-mediated agglutination suggested that vWF may play a role in this agglutination. To further demonstrate whether an increase of vWF *in vivo* also resulted in such microparticle clusters, we coinjected Stimate and DiI into zebrafish larvae and found DiI labeled clusters of 3–4 microns in the vein; however, we did not observe such clusters in the artery (Fig. 6). Since individual thrombocytes are >5 microns, the cluster size of 3–4 microns confirms that these clusters are made of microparticles and eliminates the possibility that these clusters are made of thrombocytes.

Real-time demonstration of microparticle role in hemostasis

To demonstrate the role of microparticles in the flowing blood, we first needed to establish microparticle presence in flowing blood. We injected DiI into adult fish and observed flowing DiI labeled microparticles under the microscope. Then, using these DiI injected fish, we tested whether thrombocyte microparticles play a role at the site of injury. We used the laser injury model and found that microparticles form string-like structures at the site of arterial injury. However, we did not find microparticle strings at the site of injury in venous thrombosis (Fig. 7). We also performed similar experiments with GFP labeled

microparticles; however, although we were able to observe microparticles in circulation, we did not find strings at the site of either arterial or venous injury.

Discussion

Platelet microparticles come in various sizes of approximately 0.1 μm to 1 μm diameter; they have been shown to have both cell surface receptors and cytoplasmic contents [2]. This report documents the first demonstration of the presence of similar microparticles in zebrafish. Microparticles were found using fish in which thrombocytes (platelet equivalents) were labeled with either DiI or GFP. We also found that approximately one third of the total microparticles were labeled by GpIIb antibody and that the DiI and GFP labeled particles were comparatively less in number. Since GpIIb antibody is specific to thrombocyte receptors, and since GpIIb labeled microparticles are the greatest in number compared to DiI or GFP particles, the GpIIb microparticles probably represent a fair assessment of the microparticles derived from thrombocytes. The smaller percentage of DiI particles is expected because DiI selectively labels only young thrombocytes, which constitute a small percentage of the total thrombocytes. Since GFP labels only cytoplasm and since microparticle size limits the amount of cytoplasmic contents, microparticles containing a small amount of GFP were undetected due to the sensitivity limits of GFP, thus yielding the smaller percentage of GFP microparticles. The presence of microparticles derived from young thrombocytes also suggests that microparticles could be derived from mature thrombocytes as well. Our findings that thrombocyte microparticle production is increased by collagen and thrombin stimulation are consistent with the earlier findings in humans [18] and provide additional evidence that these microparticles are derived from thrombocytes. The non-thrombocyte microparticles form the largest fraction in zebrafish. This finding is in contrast to those in humans where platelet microparticles dominate [19].

We have also noted that FITC annexin V labels microparticles. This corresponds with the observation that some platelet microparticles positively bind to FITC annexin V [20]. This finding suggests that some microparticles are derived as a result of apoptosis of blood cells, including thrombocytes. Interestingly, almost all DiI microparticles were labeled by FITC annexin V. This suggests that the microparticles derived from young thrombocytes are products of apoptosis. The loss of such microparticles may, in itself, result in maturation of these thrombocytes. One would then expect mature thrombocytes to be smaller than young thrombocytes; however, our earlier observations found that the mature thrombocytes are in fact larger in size than young thrombocytes [21]. These observations, coupled with earlier findings of loss of GATA1 and gain of Fli1 [21], suggest that the loss of microparticles from young thrombocytes may trigger the loss of certain proteins, such as transcription factors, and the synthesis of additional proteins, which, together, may ultimately result in the larger size of mature thrombocytes. Furthermore, since not all FITC annexin V microparticles were DiI positive, some microparticles may be derived from apoptosis of mature thrombocytes and other cell types. We did not perform any further experiments to differentiate whether the remainder of FITC annexin V labeled particles is derived from mature thrombocytes or from other cell types.

Addition of microparticles to zebrafish blood resulted in a shortening of TTA which indicates that microparticles do enhance thrombocyte aggregation/agglutination and, thus, hemostasis. These *in vitro* findings have also been corroborated by *in vivo* injection of microparticles into the zebrafish larvae. Furthermore, the reduction and enhancement of TTO by zVAD-FMK and TNF- α injections further lends support that the microparticles are playing a role in hemostasis. In addition, since zVAD-FMK is an inhibitor of apoptosis and TNF- α induces apoptosis, our results suggest that the particles generated via apoptotic mechanism are probably important for hemostasis.

Our observation that all microparticles, i.e., those derived from both thrombocyte and non-thrombocyte cells, are dependent on plasma to agglutinate in presence of ristocetin and not in presence of other agonists suggests that the microparticles are agglutinating in a vWF dependent manner. Likewise, the agglutination of the microparticles induced by human ULVWF and the inhibition of this agglutination by human ADAMTS-13 also supports that the microparticles agglutinate in a vWF dependent manner. In the CD41-GFP fish agglutinates, particles were randomly dispersed and were not as large as those of DiI agglutinates. This may be either because the DiI particles are more efficient in agglutination or because of the small number of GFP particles. The finding that DiI microparticles form clusters during Stimat treatment in the larval vein, but not the artery, suggests that, in venous thrombosis, the microparticles may be interspersed between the cells. This is the first demonstration that the microparticles themselves form agglutinates.

Furthermore, our finding that the DiI microparticles form string-like structures at the site of arterial injury is novel as well. This finding also suggests that young thrombocyte microparticles play a role in initiation of arterial thrombus formation. Although we could observe GFP microparticles in circulation, we could not find such strings with GFP microparticles because the longer exposure times may have quenched these microparticles more than with DiI microparticles. It has been well established that exposure of subendothelium results in adherence of platelets via vWF [22]. Therefore, it is likely that the vWF may form the initial glue for the flowing microparticles when arterial injury occurs; this is then followed by the formation of young thrombocyte clusters [16]. The lack of such string microparticles in venous injury may be because red cells play a significant role in initiation of venous thrombosis and, as observed in our *in vitro* ristocetin mediated agglutination, the microparticles may later intersperse between the cells. However, in the later stages of venous thrombosis it is difficult to capture images of these particles *in vivo* because thrombocytes are large and their fluorescence may interfere with microparticle image capture. This is also true with regard to the image capture of microparticles in the later stages of arterial thrombosis. Taken together, our results indicate that zebrafish microparticles play a role in hemostasis by both initiating thrombus formation in arterial injury as well as forming clusters with thrombocytes and red cells in venous thrombosis. Furthermore, our finding that microparticles agglutinate in the presence of ristocetin may provide a relatively inexpensive diagnostic assay for detecting increased numbers of platelet microparticles.

In conclusion, we discovered thrombocyte microparticles in zebrafish and found that they are functionally equivalent to platelet microparticles. We also found that thrombocyte

microparticles agglutinate in a vWF dependent manner. We have demonstrated that thrombocyte microparticles are the first to appear at the site of arterial injury before young thrombocyte clusters appear.

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Fig. 1. Detection of thrombocyte microparticles. Images of blood smears from a) zebrafish injected with DiI and b) CD41-GFP zebrafish. Left and right panels are brightfield and fluorescence images, respectively. Black and white arrows show thrombocytes and their microparticles, respectively.

Fig. 2.

FACS analysis of thrombocyte microparticles. a) Representative dot plots depicting fluorescence intensity versus forward scatter intensity of GpIIb antibody stained microparticles. From left to right panels show gating (R1) used in counting, unstained control, FITC-conjugated secondary antibody alone, IgG control, and GpIIb antibody followed by FITC-conjugated secondary antibody. b) Representative dot plots depicting fluorescence intensity versus forward scatter intensity of GFP labeled microparticles. From left to right panels show gating (R1) used in counting, unstained control, and GFP labeled

microparticles. c) Representative dot plots depicting fluorescence intensity versus forward scatter intensity of DiI and FITC annexin V labeled microparticles. From left to right panels show gating (R1) used in counting, unstained control, DiI labeling, FITC annexin V labeling, and double labeling with both DiI and FITC annexin V. FS, forward scatter; SS, side scatter; UNST, unstained.

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Fig. 3. Enhancement of hemostasis by microparticles. a) Stimulation of thrombocytes with thrombin, collagen, and a combination of collagen and thrombin. The values are the percentage of thrombocyte microparticles generated from total white cells of CD41-GFP fish and represent an average of three independent experiments. b) TTA of whole blood in absence (C, control) and in presence of total blood cell microparticles (M) ($n = 6$, $p = 0.005$). c) Venous TTO ($n = 6$, $p < 0.001$). d) Arterial TTO ($n = 6$, $p < 0.001$). Both c and d

represent data for 4 day old zebrafish larvae where C and M stand for larvae injected with PBS (control) and with microparticles, respectively.

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Fig. 4.

Effect of zVAD-FMK and TNF-α on microparticle level and hemostasis. a) Relative percentage of microparticles from FACS analysis after zVAD-FMK (ZVAD) or TNF-α injections. Control percentages are taken as 100% for plotting the graph ($n = 6$, Control vs. ZVAD, $p = 0.009$, Control vs. TNF-α, $p = 0.001$). b) TTA of whole blood from fish injected with PBS (Control), zVAD-FMK (ZVAD), and TNF-α injected fish ($n = 6$, Control vs. ZVAD, $p = 0.008$, Control vs. TNF-α, $p = 0.005$). c) Venous TTO ($n = 6$, Control vs. ZVAD $p = 0.004$, Control vs. TNF-α, $p < 0.001$). d) Arterial TTO ($n = 6$, Control vs. ZVAD $p <$

0.001, Control vs. TNF- α , $p < 0.001$). Both c and d represent data for 5 day old zebrafish larvae in PBS (Control), zVAD-FMK (ZVAD), and TNF- α .

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Fig. 5. Ristocetin-mediated agglutination of microparticles. a) Representative agglutinates under a cover slip when citrated whole blood from CD41-GFP fish was treated with ristocetin. Left and right panels are brightfield and fluorescence images, respectively. b) Fluorescence images of FITC labeled wild type zebrafish whole blood (left panel) and enriched microparticles (right panel) in plasma under a cover slip. c) Representative agglutinates of the enriched microparticles in plasma shown in b in presence of ristocetin. Left and right panels are brightfield and fluorescence images, respectively. d) Fluorescence images of

representative agglutinates of the enriched microparticles in presence (left panel) and absence (right panel) of plasma. e) Representative agglutinates of enriched microparticles from DiI injected fish in presence of ristocetin. Left and right panels are brightfield and fluorescence images, respectively.

Fig. 6. Effect of Stimat treatment on thrombocyte microparticles: formation of microparticle agglutinates/aggregates in larvae. a) Injected with DiI. b) Injected with DiI plus Stimat. Left and right panels are brightfield and fluorescence images, respectively. Closed and open arrowheads point to vein and artery, respectively.

Fig. 7. Initial accumulation of thrombocyte microparticles in laser induced arterial injury. Time lapse images of accumulation of microparticles at the site of laser injury (shown by arrow). Top panel is the brightfield image of an adult zebrafish tail artery. The black portion of the panel shows melanin pigment. Bottom panels from left to right are the sequential fluorescence images taken every second. Note the large thrombocyte (approximately five microns) at the bottom flowing in a different vessel for size comparison of microparticle accumulation.