ZEBRAFISH VON WILLEBRAND FACTOR

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In humans, von Willebrand factor (vWF) is a key component in hemostasis and acts as a 'cellular adhesive' by letting the circulating platelets bind to exposed subendothelium. It also acts as a carrier and stabilizer of factor VIII (FVIII). A dysfunction or reduction of vWF leads to von Willebrand disease (vWD), resulting in bleeding phenotype which affects 1% of the population. Currently there are a variety of animal models used for the study of vWF and vWD; however, they do not possess the advantages found in zebrafish. Therefore, we set out to establish zebrafish as a model for the investigation of vWF and vWD through the use of bioinformatics and various molecular techniques. Using bioinformatics we found that the vWF gene is located on chromosome 18, that the GPIbβ protein sequence is conserved. Confirmation of vWF production was shown by means of immunostaining and by RT-PCR, in thrombocytes as well as in veins and arteries. Evidence of vWF involvement in hemostasis and thrombosis was shown using MO and VMO technology to produce a vWD like phenotype, resulting in an increase in TTO and TTA, as well as a reduction in FVIII when blood was tested using the kPTT assay, coinciding with a decrease in vWF. Stimate treatment provided opposite results of MO and VMO, showing a decrease in TTO and TTA. Investigation of the role of microparticles in hemostasis and their interaction with vWF resulted in a conclusion that the GPIbα receptor should exist on MPs and that it may interact not only with zebrafish vWF but also with human UL-vWF. Agglutination of MPs in the presence of UL-vWF but in the absence of ristocetin and plasma, treatment with ADAMTS-13 abolishing the interaction between MPs and UL-

vWF provided evidence that vWF interacts with MPs probably with the GPIbα. We also found that TMPs agglutinate within the vessel wall *in vivo* when treated with Stimate. In conclusion, this research provided evidence for the presence of vWF in zebrafish and its conserved role in hemostasis. In addition to this we also showed that MPs also participation in hemostasis.

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The results of this research resulted in two publications: [1] M. Carrillo, S. Kim, S.K. Rajpurohit, V. Kulkarni, and P. Jagadeeswaran, Zebrafish von Willebrand factor. Blood Cells Mol Dis 45 326-33; and [2] S. Kim, M. Carrillo, U.P. Radhakrishnan, and P. Jagadeeswaran, Role of zebrafish thrombocyte and non-thrombocyte microparticles in hemostasis. Blood Cells Mol Dis 48 188-96.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Page

CHAPTER 1

GENERAL INTRODUCTION

Background

von Willebrand Factor History

In 1926 Erik von Willebrand, a Finnish physician, first described a bleeding disease within a family on the Åland archipelago in the Baltic Sea. Later in 1957 Inga Marie Nilsson at Malmö University Hospital discovered the protein responsible for the bleeding disease previously described by Erik von Willebrand and thus named the protein von Willebrand factor (VWF), in his honor. In the disease described by Dr. von Willebrand, there is a decrease in coagulation factor VIII (FVIII), described below. Due to the decrease in FVIII the disease was termed pseudo-hemophilia prior to the name von Willebrand disease (vWD) [1; 2]. Identification of the protein causing the bleeding disease by Inga Marie allowed for patients to be given differential treatment for vWD versus hemophilia.

VWF Protein

VWF is a large multimeric glycoprotein composed of 2813 amino acids and three subunits: a 22 residue signal peptide, a 741 residue propeptide, and a 2050 residue mature subunit (Figure 1.1*)* [3]. This protein is heavily glycosylated, providing protection from degradation [4]. VWF has a multimeric structure found in varying degrees of multimerization, with subunits beginning with 2 to greater than 50, and can range in mass from 250 kDa (monomeric VWF) to the 500 kDa (dimeric/protomer) and up to 10,000 kDa, or more [5; 6]. Large molecular weight VWF is termed, ultra large VWF

(UL-VWF), whereas a lower molecular weight VWF is merely termed VWF. Multimerization occurs in a series of steps, which includes the following: first protomer formation, the interaction of two VWF monomers coming together at the carboxy terminal end, within the endoplasmic reticulum via disulfide bond [6; 7]. This protomer then interacts with another VWF protomer at the amino terminal end, beginning multimerization; this occurs within the golgi apparatus [4; 6]. Following multimerization, the propeptide is cleaved from the mature portion of VWF; however, the propeptide and mature VWF remain non-covalently associated, necessary for proper multimer formation (Figure 1.2) [8]. Thus, the process of dimerization and multimerization are two independent events that involve different regions of VWF [9].

The VWF domains are organized in the following way, beginning with the amino terminal end: the D1 and D2 comprise the pro-peptide; followed by the mature portion of VWF composed of the D′, D3, A1-A3, D4, B1-B3, followed by C1 and C2 and CK (Figure 1.2) [5]. Each domain is important for proper function of VWF, playing an important role in thrombus formation. The A1 domain is involved in binding to the GPIbα receptor located on the surface of platelets, as well as a binding site for collagen [5; 10; 11]. The A3 domain is involved in collagen binding important for anchoring VWF in high shear rates [12]. The A2 domain is the site for cleavage by ADAMTS-13 as described below. Domains D1, D2 and D3 are all involved in multimer formation and storage; and D′ is involved in FVIII binding [9; 13; 14; 15; 16; 17]. The C domains are involved in binding with thrombospondin and collagen; C1, in particular, is the site for binding GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$). The CK domain is involved in dimerization at the carboxy terminal end as previously mentioned [18; 19]. The C terminal domain that

includes the D4 and B domains is thought to be a docking site for ADAMTS-13; however, it has a lower affinity than the A2 domain and is not cleaved by the protease [20].

ADAMTS-13 and VWF Multimer Cleavage

ADAMTS-13, a metalloprotease, is important for the cleavage of large and UL-VWF to lower molecular weight multimer forms. The site of VWF processing by ADAMTS-13 is located in the A2 domain, and it is important for maintaining lower molecular weight forms of VWF normally found in circulation [21]. This cleavage is important because UL-VWF is more reactive than VWF found in circulation, and can lead to spontaneous thrombus formation [22].

Synthesis and Secretion

VWF is synthesized in megakaryocytes and stored within the α-granules of platelets, it is also synthesized in endothelial cells and stored in Weibel-Palade bodies [16; 23]. Megakaryocyte and endothelial VWF secretion and storage differ in that endothelial cells will store VWF as well as constitutively release the protein, whereas megakaryocytes package VWF into platelets where they are stored in α-granules and released upon platelet activation [23; 24; 25]. The VWF constitutively secreted by endothelial cells is of a lower-molecular weight, compared to the UL-VWF which is stored. UL-VWF is also packaged into platelets when produced from megakaryocytes [24; 26; 27]. Due to the large amount of multimerization UL-VWF is more reactive in nature and is secreted upon injury rather than constitutively secreted. UL-VWF is

particularly important in vessels with high shear stress such as arteries and arterioles and the degree of multimerization is an important determinant of how reactive it will be with platelets. High shear blood flow causes UL-VWF bound to the subendothelium at the site of injury to extend and expose GPIbα binding sites, allowing for platelet binding and consequently platelet activation [27; 28; 29]. Based on this information we know that both UL-VWF and VWF found in circulation are important for maintaining balance in hemostasis.

von Willebrand Disease

If there is a defect in this balance whether it be a dysfunctional protein or a decrease in the amount of VWF present it is known as von Willebrand disease (vWD). In 1985 the first cDNA was synthesized by several laboratories; synthesis of the cDNA provided the sequence for determination of the various domains encoded by the VWF gene and the organization of the domains, as well as pinpointing the VWF gene location to chromosome 12 [30; 31; 32; 33]. The VWF gene contains 52 exons with exon 28 being the largest, encoding for the A1 and A2 domain. A pseudogene which includes exons 23-34 is located on chromosome 22 [5; 34; 35]. Cloning and characterization of the VWF gene has allowed for the observation of mutations in the protein that cause vWD disease. Many patients with vWD as well as individuals with a defect in ADAMTS-13 have been found to suffer from thrombotic thrombocytopenic purpura (TTP), which is characterized by the formation of microthrombi [36]. The possibility of 'overactive binding' by UL-VWF to platelets can result in platelet consumption and clumping, causing microthrombi which leads to thrombocytopenia that can then result in bleeding,

this has been identified in patients with TTP [37].

vWD manifests as a prolonged bleeding upon injury, the severity of the disease ranges, from very little mild bleeding to severe spontaneous bleeding and affects 1-2% of the population [2; 38; 39]. Bleeding usually occurs in mucocutaneous regions of the body, such as the nose and gums; a severe case can result in bleeding in the joints as well as in the gastrointestinal area. There are two classes of vWD, qualitative and quantitative. Qualitative vWD is due to a defect in the protein itself, whereas quantitative vWD is a decrease in the amount of VWF. The quantitative class of vWD is called Type 1 vWD and is characterized by a decrease in the amount of VWF, resulting in type 1 or type 3 vWD.

Type 1 vWD is the most prevalent of the vWD types affecting >70% of the population with the disease, it is characterized by a mild to moderate bleeding phenotype, and is inherited in an autosomal dominant fashion [40]. Individuals with Type 1 vWD respond well to the use of desmopressin (DDAVP), a treatment that causes the release of VWF from storage organelles [41]. The other quantitative type of vWD is type 3; unlike type 1 vWD, type 3 vWD is characterized by a significant reduction in VWF resulting in severe bleeding and is inherited in an autosomal recessive fashion and in some cases caused by deletions of portions of the entire gene [42; 43]. Along with the reduction in VWF there is also a reduction in FVIII due to an increase in degradation. This is important to note because as previously mentioned VWF binds FVIII to protect it from degradation while in circulation, until needed for thrombus formation and delivers it to the site of injury [17].

The qualitative class of vWD is characterized by a dysfunction in VWF, resulting

in Type 2 vWD. Type 2 vWD is further classified into the following subtypes: 2A, 2B, 2M, and 2N. These qualitative defects are characterized by different defects in the interaction of VWF with various factors involved in thrombus formation. Most of the mutations for type 2A and 2B occur in the A1 domain of VWF with the exception of type 2N which occur in the D′ and D3 domain. The A1 domain is of great significance because it encodes for platelet GPIbα binding site [44].

Type 2A vWD is the most common form of type 2 vWD, it is characterized by a decrease in adhesion to platelets due to a lower concentration of high-molecular-weight VWF [45]. It is typically inherited in an autosomal dominant fashion, but has also been inherited in a recessive manner [46; 47]. Individuals with this type of vWD do not respond well to DDAVP and tend to experience moderate bleeding. Type 2A differs from the next type of vWD, 2B in that rather than a decrease in platelet binding there is an increase in affinity for binding.

The characteristic increase in binding affinity to platelet GPIbα in type 2B is inherited in an autosomal dominant fashion [48]. The cause of this increased binding is due to a variety of different mutations as well as degree of increased interaction between VWF and GPIbα. The severity of bleeding can vary significantly among family members [49]. The next vWD subtype is type 2M, which is similar to 2A, also having a reduction in adhesion to platelets.

The reduction in platelet adhesion is different from 2A in that it is not due to loss of high-molecular-weight multimers; however, it is inherited in an autosomal dominant fashion [50]. In some cases there was an increase in high-molecular weight VWF known as vWD “Vincenza” [51]. This form of vWD can be differentiated from the other

types based on different aggregation assays based on binding of two antibiotics namely ristocetin and botrocetin [50]. The last subtype is 2N vWD which is different from the other subtypes in that the mutation is not in the A1 domain.

The final qualitative form of vWD is 2N which is characterized by a significant decrease in FVIII binding to VWF. This was once called vWD Normandy due to one of the first observations of this form of vWD in a woman from Normandy [52; 53]. This individual when tested had a prolonged aPTT, an assay that is used to test for proper function of the intrinsic pathway that involves FVIII [53]. The mutations in type 2N are not located in exon 28, but instead are in the FVIII binding site, which is located in the D′ and part of the D3 domain of VWF [54]. Multimerization is normal and inheritance seems to be in an autosomal recessive manner [54].

Role of VWF and FVIII in Hemostasis

The ability to stop bleeding after injury is called hemostasis; this includes thrombus/clot formation. The process of thrombus formation is complex in humans and involves many different factors and two different coagulation pathways, the intrinsic and extrinsic pathway [55]. The intrinsic pathway is also known as the contact activation pathway, whereas the extrinsic pathway is activated when TF is exposed upon injury. The two pathways converge with activation of factor X to amplify the production of thrombin, increasing thrombus formation [56]. One of the key components in the increase of thrombin is FVIIIa, the activated form of FVIII. It acts as a cofactor to factor IXa, a member of the intrinsic pathway, amplifying thrombin production [57; 58]. FVIII plays an important role in the coagulation cascade, but it can be degraded by activated

protein C (APC). Factor VIII is stabilized by VWF which is important for thrombus formation. VWF acts as an adhesive, binding platelets to the subendothelial matrix [34; 59]. Once VWF is bound at the site of injury it is extended due to blood flow exposing the platelet binding site, A1 domain [60]. Platelet binding to VWF causes platelet activation which then leads to platelet/platelet interaction which also involves VWF leading to stable thrombus formation and thus VWF's contribution to hemostasis [61].

Platelets versus Thrombocytes

Human Platelets

Platelets are anucleate cells approximately 2.0 μm in size derived from megakaryocytes, and are found circulating in the blood of humans. They are essential in thrombus formation within vessels upon injury, adhering to the vessel wall to form a platelet plug when in contact with VWF. As previously mentioned platelets store UL-VWF in α-granules for release upon activation. The interaction site for VWF on platelets is the GPIbα receptor which interacts with the VWF A1 domain [62]. The activation of one platelet leads to secretion of its contents and promotes activation of other neighboring platelets. The equivalent to human platelets in the zebrafish model are thrombocytes, which also posses some characteristics similar to megakaryocytes.

Zebrafish Thrombocytes

Zebrafish do not have platelets or megakaryocytes, but instead have nucleated thrombocytes, which play an important role in thrombus formation. Thrombocytes are about 5 μm, they have been characterized and show many similarities to platelets and

megakaryocytes such as: conserved receptors that include GPIIb/IIIa, GPIbα and $P2Y_1$ receptors as well as structural characteristics, like the open canalicular system, and are also present in two populations in circulation, young and mature thrombocytes [63; 64; 65; 66; 67]. Thrombocytes are transcriptionally active having transcription factors such as GATA-1 and Fli-1 which are present in megakaryocytes. In addition to these characteristics, thrombocytes also form aggregates similarly to platelets when exposed to collagen, arachadonic acid, and ristocetin [63]. Thrombocytes have also been shown to produce microparticles similarly to human platelets.

Microparticles

Microparticles are small microvesicles found in normal human blood circulation, which contain membrane proteins as well as cellular components of the cell from which they are derived [68]. There are a variety of microparticles found in circulation such as endothelial cell microparticles, and platelet microparticles (PMP) [69]. Platelet microparticles range in size from 0.1-1.0 μm and are derived from platelets, as well as megakaryocytes [25]. Although the role of microparticles in hemostasis has been studied recently, their role in initiation of thrombus formation has not been addressed. Microparticles are also found in zebrafish, ranging in size from 0.1 to 2 μm, currently the involvement if any of microparticles in zebrafish are not known in hemostasis and thrombosis. In the later section of this thesis, studies on the role of zebrafish microparticles derived from thrombocytes and other cells in hemostasis and thrombosis will be described.

Zebrafish as an Animal Model

Zebrafish (*Danio rerio)* is a small vertebrate fish that has been used as an animal for a variety of topics in research such as: cardiac development, cancer, and hemostasis and thrombosis [70]. The zebrafish as a model has many benefits which include: cost efficient, rapid progression through development, high fecundity, large embryo size, and the larvae and embryos are both transparent. A zebrafish facility can be easily assembled and the fish maintained at a much lower cost compared to many other models such as mice, or rats, and even chickens [71]. The transparency of the embryos facilitates observation of development from the moment of fertilization up to hatching and even after hatching, for a total of approximately 7 days post fertilization (dpf) [72]. Observations of arteries, veins and circulating blood cells in larvae and embryos can be accomplished using a microscope or lower power dissecting microscope [73]. Zebrafish can also be used for large scale screening using saturation mutagenesis, to create mutants and screen for desired phenotypes in an unbiased way [70]. Knockdown technology using antisense morpholino (MO) and Vivo-morpholino (VMO) can also be utilized in zebrafish to examine function of a particular protein, adding more power to this excellent genetic vertebrate model [74; 75]. Due to the transparency of the larvae direct observation of clot formation upon laser induced injury to a blood vessel is possible [76]. Images and recordings of the injury and clotting process can be recorded using a high powered microscope attached to a computer. The transparency and ease with which zebrafish can be genetically manipulated has also given rise to thrombocytes that are GFP labeled as well as vessels that are GFP labeled [77; 78]. These labeled vessels and thrombocytes provide easily observable

development of vessels as well as clot formation initiation, clotting, and circulation.

Based on all of the advantages zebrafish provides I have utilized zebrafish for the purpose of creating an animal model for vWD, such that the model could be used to detect modifier genes for vWD. Currently zebrafish is established as a model for the study of mammalian bleeding disorders such as hemophilia; however, it has not yet been used for the study of VWF and vWD [79]. Because zebrafish is an established model for the study of hemostasis and thrombosis, previous work has shown that the extrinsic pathway and intrinsic pathway are both conserved, and that thrombocytes resemble human platelets physically as well as megakaryocytes due to nucleation of the cells. Thrombocytes also have functions similar to platelets and almost all receptors are conserved from human platelets to thrombocytes [80; 81]. One of the physical characteristics conserved in thrombocytes is the open canalicular system (OCS), also found in platelets. The OCS is important for increasing surface area upon activation exposing receptors not expressed on the surface of inactive platelets.

Zebrafish have become a powerful tool not only to as a models for the study of hemostatic disorders but also for identifying novel genes involved in hematopoeisis [79; 82]. Such knowledge can be translated into the human setting providing great clinical value and further understanding of disease as well as treatment.

Current Models for the Study of von Willebrand Factor

Currently there are a variety of animal models used to study vWD such as: murine, porcine, and canine. One murine model in use is a VWF knockout in which the mice have a prolonged bleeding time as well as spontaneous bleeding, mimicking

patients with severe vWD type 3, a quantitative form of vWD [83]. The investigators of this particular model are able to visualize thrombus formation in these mice; however, the procedure is invasive and requiring a surgical procedure, in which mesentery must be “exteriorized” [83]. In addition to performing a surgical procedure the investigators also collected and labeled platelets, which were then injected back into the mouse to visualize clot formation [83]. While there are several models for the type 3 vWD there is a murine model for the quantitative vWD type 1 vWD using the RIIIS/J inbred mouse, which has been treated successfully using DDAVP [84]. This particular model does provide some similarities between human vWD, and while there is survival of neonates homozygous for VWF knockout there is a reduction in survival rate. The homozygous knockout of VWF provides a model that is absent of VWF, important for the investigation of treatment for type 3 vWD individuals. Thus, although this mouse model provides a setting for the examination of the defects in VWF, disadvantages such as low fecundity and lack of easy visualization of thrombus formation and manipulation still remain. In addition to the mouse model the canine and porcine model has also been used for the investigation of vWD. Both of these animal models like the murine model are used for the study of vWD type 3 [85]. The canine model has also been used for the study of α-granule formation in endothelial cells [86]. The porcine model has been used in bone marrow transplantation studies and the effects of VWF on atherosclerosis [87; 88].

As for the study of vWD type 2 there are few models for this qualitative form. One such model is the found in German shorthair pointers, while rare, they do have the characteristic reduction of the higher molecular weight multimers similar to type 2A [89].

A second model is the murine model used for examining type 2B; however, this is performed in an animal that is a homozygous knockout for VWF and is injected with an expression vector so that the dysfunctional form of the protein is synthesized *in vivo* and subsequently be examined and treated [90].

It is clear that despite the all of the above models currently being utilized for the study of vWD that zebrafish maintain the upper hand. This is exemplified in the fact that in order to observe thrombosis *in vivo*, vessels must be exteriorized, which is an extremely invasive procedure that can also increase incidence of infection. In zebrafish observation of thrombus formation does not require a surgical procedure due to the transparent nature of the larvae as well as the availability of transparent adult zebrafish developed by White *et. al*. [91]. Another advantage over these models is that zebrafish thrombocytes can be labeled by allowing the fish to sit in water containing mepacrine, a dye which labels thrombocytes, a noninvasive procedure. Furthermore, it has been difficult to identify modifier genes with the mouse model in contrast to zebrafish which has the advantage of large scale ENU mutagenesis. Through the use of ENU mutagenesis several models for type 2 vWD have the potential to be discovered. This screening can be performed quickly and on a large scale with a relatively high through to identify a large number of zebrafish with a mutation in the VWF gene causing not just a quantitative vWD but also a qualitative form. Despite the current models in use having provided a useful tool for the study of VWF and vWD they simply do not provide a cost effect and easily manipulated model like the zebrafish [83; 86; 92; 93]. Furthermore, the cell biology of VWF could be better studied in zebrafish due to fact the GFP fusion proteins can be made with the VWF domains and easily visualized [94]. In

addition, the developmental role of VWF can be studied using the zebrafish model easily compared to the other vertebrate models, which are viviparous. All of the models currently employed pose a problem in that they are larger, more expensive and difficult to manipulate, requiring invasive procedures for visualization of thrombus formation, or in require tail cutting, or ear clipping to observe bleeding time, whereas zebrafish can be tested for time to occlusion using a laser induced injury.

Current Hypotheses and Aims

Hypotheses

1. At the time of the initiation of this research, the zebrafish genome was mostly sequenced; however, the location and sequence of VWF in zebrafish had not been determined. We hypothesize that through the use of bioinformatics we can sift through the genome to identify the VWF gene and subsequently confirm the identity of the gene with the help of synteny. We also hypothesize that VWF does exist in zebrafish and will have some homology when compared to human VWF.

2. Currently there is no information regarding the cell type that synthesizes VWF in zebrafish. However, because of the similarities between thrombocytes and human platelets/megakaryocytes we hypothesize that zebrafish VWF is synthesized in endothelial cells as well as in thrombocytes.

3. At this time there is no vWD model in zebrafish but we hypothesize that a knockdown of VWF using MOs and VMOs will yield a vWD phenotype in zebrafish larvae and adults.

4. There are a variety of microparticles found in circulation in humans; however,

the involvement of these particles in hemostasis if any is not known. Specifically we are interested in thrombocyte microparticles involvement in hemostasis. We hypothesis that since thrombocyte microparticles are much smaller in size compared to other circulating blood cells they will probably be circulating closer to the vessel wall and when injury occurs these particles may adhere to the subendothelial matrix much before larger cells. Because one of the major components of the subendothelial matrix is VWF we hypothesize that the microparticles may adhere to VWF and agglutinate at the site of injury. In addition, because these microparticles have been shown to carry GPIIb/IIIa thrombocyte receptors we also hypothesized that the GPIbα receptor will also be present and involved in VWF interaction.

Aims

The specific aims of this study are:

1. To identify VWF in the zebrafish genome through the utilization of bioinformatics to locate the gene sequence of VWF and determine whether or not homology exists between human VWF and zebrafish VWF.

2. To determine where VWF is synthesized through immunostaining and RT-PCR analysis. We expect to find VWF synthesized in thrombocytes as well as in the endothelium of the blood vessels.

3. To determine whether morpholino targeting of zebrafish VWF will produce a bleeding phenotype, and to determine whether VWF is stored and released upon treatment with Stimate (desmopressin-acetate; DDAVP) as well as establish whether FVIII deficiency also occurs in zebrafish with low VWF.

4. To determine whether or not zebrafish TMPs participate in hemostasis and thrombosis in VWF dependent manner. We also wish to explore whether the microparticles agglutinate *in vitro*.

All of the hypotheses and aims are addressed in the following chapters.

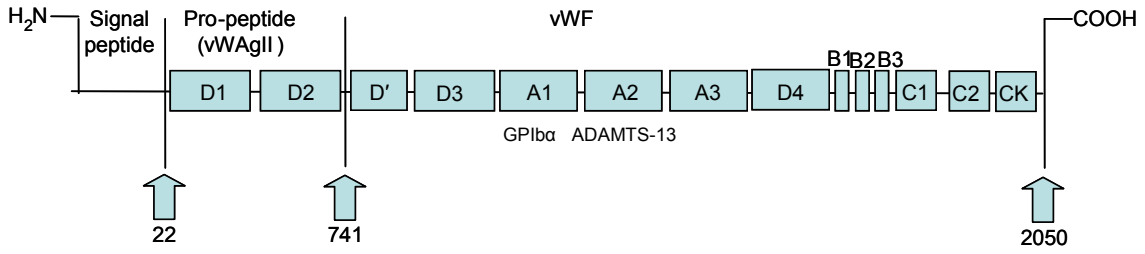

Figure 1.1: Schematic representation of VWF protein. Blue boxes show the various domains involved in hemostasis. Arrows indicate the parts of the protein beginning with the 22 aa signal peptide, followed by the 741 aa pro-peptide, and the 2050 aa mature VWF sequence. Domain A1 is the site for GPIbα binding and the A2 domain is targeted for cleavage by ADAMTS-13.

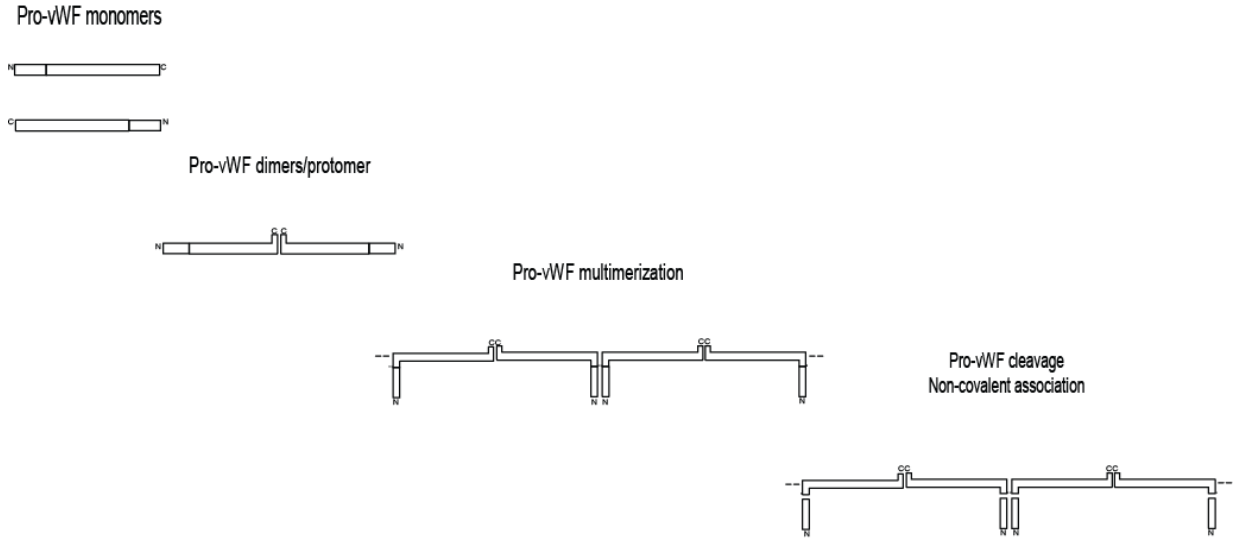

Figure 1.2: VWF multimerization. Multimerization begins with Pro-VWF monomers interacting at the carboxy (C) terminal end, creating dimers/protomers. These protomers then interact at the amino (N) terminal end. After this association at the amino terminal end the Pro-VWF strand is cleaved; however, the propeptide and mature VWF remain non-covalently associated.

CHAPTER 2

BIOINFORMATIC SEARCH FOR ZEBRAFISH VON WILLEBRAND FACTOR AND GPIB-BETA AND CHARACTERIZATION OF THE VWF GENE

Introduction

Bioinformatics Search for VWF in Zebrafish

Bioinformatic tools such as Ensembl and NCBI are utilized to locate and compare gene sequences as well as protein sequences in a variety of organisms including zebrafish. Ensembl and NCBI are both online library banks of genetic information, including gene, protein, and mRNA sequences [95; 96]. Information on orthologs and paralogs can also be found on these data bases. These online banks may or may not be complete; this means that the organism in the data base may have all of the genome information sequenced but not have annotations to all of the possible genes and gene locations, or proteins being expressed within the genome. These online banks are a collaborative effort and information is submitted from researchers from various institutes [95; 96]. In addition to the protein and gene information NCBI and Ensembl also provide tools that allow a researcher to compare nucleotide and protein sequences of several organisms at a time; or within the organism of interest to determine whether homologies exist. DNA sequences can also be used to identify possible open reading frames (ORF) using the ORF finder available on NCBI. The ORF finder provides a possible protein sequence coinciding with the nucleotide sequence. The information on Ensembl and NCBI are always being updated so that the information gathered is as relevant and reliable as possible. An examination of protein sequences between organisms can provide information on conserved domains in the proteins that persisted

through evolution and therefore may provide clues to as to how the protein functions. If many of the regions or domains in the proteins are conserved then it is likely that the structural conformations are similar indicating the proteins may function similarly and their potential interactions with other proteins if any can be predicted.

Bioinformatics also allows investigators to compare homology among many organisms, as well as view whether or not the gene of interest is flanked by the same genes found in another organism, termed synteny which means “on the same thread”. Examination of synteny between organisms provides information about evolutionary changes, gene duplication, chromosomal evolution and conservation of protein function [97]. Conserved synteny indicates that the organisms are likely to have a common ancestor and there may be some sort of selective pressure as to why the synteny is conserved [98]. This synteny also helps in determining whether a gene is truly homologous. For example in duplicated genes that are dispersed to different chromosomes it is difficult to determine whether or not the two genes are in fact different. In such situations and in the absence of functional assays synteny provides the identity of the gene. When I began the my research the VWF gene was not yet annotated. This chapter will begin with an examination of the zebrafish genome sequence.

Gene Characterization

The increasing information found in genomic databases allows for scientists to move more quickly than in the past because it allows for a direct comparison of known gene sequences to genomic sequences that may not yet be annotated to determine

whether or not a homologous gene sequence is present. Once the gene is identified using the bioinformatic approach primers can be made and the genome amplified by PCR and subsequently sequenced to confirm the genomic sequence. Since the bioinformatic approach predicts exon intron organization the biological evidence for the exon intron organization will be lacking. Therefore, the VWF mRNA should be sequenced so that by comparing the mRNA and genomic sequence the exon intron boundaries can be defined

Aims and Hypothesis Tested

When this research began the zebrafish genome was mostly sequenced; however, the location and sequence of VWF in zebrafish had not been determined. The hypothesis of this chapter is that by sifting through available bioinformatics and comparing synteny, I will be able to identify the VWF gene. We also hypothesize that VWF does exist in zebrafish and will have some homology when compared to human VWF. Therefore, the goal here is to utilize bioinformatics to locate the gene sequence of VWF and determine whether or not homology exists between human VWF and zebrafish VWF.

Materials and Methods

Bioinformatic Search for VWF in Zebrafish

In order to establish the location of zebrafish VWF first the human VWF sequence was located on Ensembl and subsequently BLASTed against the zebrafish whole genome. The presence of synteny between the genes surrounding the VWF

gene of human and zebrafish was determined by examining the contigs present in the Ensembl database. Sometimes the sequences were downloaded from the Ensembl database and then BLASTed using NCBI and Multialin, another online bioinformatic tool and used to identify the exons of VWF gene. Possible exon sequences were put into the ORF finder and the predicted protein sequences from the exons were combined to derive the complete protein sequence and then compared to human VWF.

Comparison of Sections of Vertebrate VWF in Vertebrate Fish to Human VWF

Comparison of the protein sequences corresponding to an exon was performed using Biology Workbench 3.2 available through San Diego Super Computer Center. Using BLAST and Multialin the VWF sequences in the genomes of *Gasterosteus aculeatus* (stickleback), *Orysias latipes* (medaka), *Takifugu rubripes* (fugu), *Tetraodon nigroviridis* (tetradon), and human were searched for and then used for comparison. Ensembl and NCBI databases were also used for the comparison.

Comparison of Zebrafish and Human GPIbβ

Human GPIbβ was used to BLAST Ensembl and NCBI databases as described above.

Genomic PCR and RT-PCR

Genomic PCR was performed using zebrafish genomic DNA collected from whole larvae, with primers designed across the exon homologous for human exon 28. Genomic DNA was prepared using the Wizard Genomic DNA Purification Kit (Promega;

Madison, WI) and subsequently used for PCR amplification. RNA was collected from whole larvae using the Absolutely RNA miniprep kit (Stratagene, Inc.; Santa Clara, CA). The PCR and RT-PCR sequences were then sent for sequencing (Lone Star Labs, Lewisville, TX). For RT-PCR amplification of VWF mRNA with the following primers: Forward primers: 5'-TGAGTGGAGATATAACACCTGTGC-3' (F1), 5'-CAGTAACTGGTTTAACCTCCACACT-3' (F2), 5'-CTGTTGACGGCAAGTGCTAA-3' (F3), 5'-GAAGCTTTGAGCATTACTGACTACC-3' (F4), and 5'-CACAGAGTCCTCCAACTGACG-3' (F5). Reverse primers: 5'-TCATCCATGAATGCGACATC-3' (R1), 5'-GAGGTCAGAAGGGTCATCCA-3' (R2), 5'-ATGTTTTCAAGTCCTCAAACTG-3' (R3), and 5'-GTTTTCACAAATGTTTTCAAGTCCT-3' (R4) (Biosynthesis; Lewisville, TX). F1 is located in the exon corresponding to human exon 26. F2, F3, F4, F5, R1 and R2 are located in the exon corresponding to human exon 28. R3 and R4 are located in the exon corresponding to human exon 29. The following primers were used for mRNA amplification of EF1-α: forward primer 5'-CGGTGACAACATGCTGGAGG-3' and reverse primer 5'-ACCAGTCTCCACACGACCCA-3' were used. Genomic DNA from adult zebrafish was prepared using the Wizard Genomic DNA Purification Kit (Promega; Madison, WI) and was amplified by PCR using two independent primer sets F5R3 and F1R1. These sequences were then compared to each other to determine whether or not the intron is present in the VWF Ensembl sequence is truly present.

Zebrafish Aquaculture

The following methods of zebrafish aquaculture were conducted similarly to

those previously described [99]. Briefly, adult zebrafish, larvae, and embryos were kept at 28^{o}C in deionized water, supplemented with Instant Ocean, in a circulating water system. Embryos were collected as previously described [99].

Results

Bioinformatic Search for VWF in Zebrafish

We first used full length human VWF cDNA to BLAST NCBI and Ensembl databases. Unfortunately the BLAST search resulted in an output of no significant homology from the databases. Since the gene is huge containing 52 exons and most of the exons are short we felt that we may be missing the gene due to the short sequences. Because these programs were not designed to BLAST short sequences at the time we searched the database after splitting the cDNA into multiple short stretches of sequences and then BLASTed the above databases. This time we found exons 13, 15 and 28 were aligned; however, finding the remaining sequences was not trivial. Therefore, we downloaded the large zebrafish contig sequences that contained the exons, 13, 15 and 28 and then used these sequences to BLAST them using programs designed to align two sequences. At times a visual examination of the sequence was required to align short exon sequence between human and VWF genes. Such searches resulted in the building of the entire zebrafish VWF gene except for exons 1 and 2. By combining all of the exon sequences from the zebrafish VWF gene we were able to deduce the VWF protein sequence. We then BLASTed using this compiled cDNA to the zebrafish genome which resulted in a number of locations that appeared to be homologous to certain domains of the human VWF sequence. Upon further

examination of the sequences using BLAST we found that the gene residing on chromosome 18 was identified to have the most homology with human VWF. In addition to this we found that the CD9 gene and the VWF gene are syntenic between human and zebrafish chromosomal loci (Figure 2.1). A comparison of the whole protein encoded by zebrafish and human VWF showed 46% identity between human VWF with 63% positives.

Genomic PCR and RT-PCR

In order to confirm the sequence of the most importance, exon 28, a PCR reaction was performed using zebrafish genomic DNA. It was confirmed that exon 28 is consistent with the bioinformatic results from the sequences that were down loaded but not yet not annotated. Shortly after I identified the zebrafish VWF gene delineating the exons encoding the protein as well as confirming the exon 28 sequence by PCR, the fully amended sequence was posted on Ensembl at the same genomic location. At this time I then compared my sequence with the annotated Ensembl sequence. The comparison indicated that there is a discrepancy in the sequence posted on Ensembl versus the sequence identified regarding exon 28. In the newly amended Ensembl database the exon 28 I found is listed as exons 26 and 27 with an intron. To further examine whether or not the intron was present as reported by Ensembl RT-PCR was performed and then compared the RT-PCR product sequences with the human and zebrafish VWF sequence present in Ensembl database. The comparison showed that my observations were correct; there is either an error in Ensembl database or the sequences might have been obtained from a different zebrafish strain (Figure 2.2).

These results led to the question of whether or not other vertebrate fish might have separate exons that are homologous to the important GPIbα binding region of VWF. The location of the proposed intron is in the GPIbα binding domain in human VWF encoded by exon 28.

Bioinformatic Comparison of Vertebrate VWF in Vertebrate Fish to Human VWF

To investigate whether or not there are other vertebrate fish with an intron in this particularly important region we used an alignment program to compare the protein sequences among stickleback, medaka, fugu, tetraodon, zebrafish and human VWF (Figure 2.3). All of the fish with the exception of zebrafish, the exon 28 is split into more than one exon thus splitting the GP1bα binding region. In addition to splitting the GP1bα binding region I also found that the GPIbα binding region is composed of three exons in stickleback and medaka. The region where Ensembl indicates the proposed intron is found in zebrafish is close to the region of the second intron found in stickleback and medaka (Figure 2.3).

Comparison of Zebrafish and Human GPIbβ

Even though previously shown, by immunostaining, that GP1bα exists on the thrombocyte surface, it was not yet shown using molecular methods that there is the presence of genes for GPIbβ. Therefore, we searched the ENSEMBL database and found the GPIbβ proteins, which is part of the complex of GPIbα. Furthermore, we also observed that the Cysteine122 of GP1bβ, which forms a disulphide bridge with GP1bα is conserved (Figure 2.4).

Discussion

The bioinformatics performed provided information regarding the conservation of the VWF protein in zebrafish as well as insight into whether or not function is also conserved based on homology of the two protein sequences. The bioinformatics also showed that indeed synteny does exist between VWF and CD9 in humans and zebrafish, indicating that evolutionarily there has not been much of a change between VWF and CD9. The significance of the syntenic conservation of these two genes is not known. Conservation of the protein in zebrafish also indicates that VWF is important and that there has also been selective pressure to preserve the gene most likely for the function of hemostatic defense.

Finding that the GP1bβ gene exists and has important conserved regions lends further support to earlier findings that the receptors for VWF are conserved in zebrafish and are likely to be the point of interaction between zebrafish VWF and thrombocytes. The protein comparison between human and zebrafish GPIbβ shows that there is homology between the protein as a whole as well as the conservation of Cysteine122 which is important for forming a disulphide bridge with GP1bα [100].

When a comparison of human VWF against other vertebrate fish including zebrafish was examined we again observed that the GPIbα binding site is conserved. As previously mentioned the GPIbα is found in the A1 domain encoded by exon 28, which also encodes the A2 domain. In all the fish examined there was an intron in the A1 region homologous to exon 28 of humans with the exception of zebrafish. In addition to this intron there is a second intron found in stickleback and medaka, making the GPIbα binding region encoded by three exons rather than one or two. We also

found that the exon 28 homologous region in all these fish encodes an A2 domain similar to the human A2 domain, the site for ADAMTS-13 cleavage in human VWF. This slightly departs from Gilbert and Go's hypothesis that individual exons code for individual domains in the proteins [101]. Despite the presence of the introns in this homologous region the binding site is conserved indicating that the interaction between VWF and GPIbα is important across species and is conserved for the hemostatic function.

The 'intron' in exon 28 is indeed part of the exon sequence; but if the exon were to be an unspliced product, then the 'correctly' spliced product should have been obtained in other reactions. Therefore, the intron suggested by the database does not exist and the VWF protein carries the additional peptide sequence. Interestingly, the binding site for GPIbα is located in the amino-terminal region of the protein coded by exon 28 and this region also contains two of the three A domains of VWF. Thus, the additional peptide sequence appears to separate these two domains. It is noteworthy that in human vWD many of the mutations are clustered in exon 28 resulting in a qualitative form of vWD. Because many of the mutations in human vWD are found in this region it will be interesting to see whether or not loss of this particular exon in zebrafish will result in a bleeding phenotype.

Results with genomic PCR combined with RT-PCR provided evidence that the region homologous to human exon 28 of zebrafish is composed of one exon rather than two as suggested by the Ensembl sequence database. These results also suggest that it is important to exercise caution in using the sequences present in the current

databases because there can be minor errors in the databases and databases must be viewed as starting point for research.

Conclusions

Through the use of bioinformatics I was able to locate the VWF gene in zebrafish as well as derive the protein sequence. At the time this search for VWF began the VWF gene location had not yet been listed in the database. However, this listing enabled a confirmation of the experimental findings. I was also able to again confirm that the correct gene sequence for VWF had been found thus emphasizing the power of bioinformatics.

The experiments showed that the GPIbα binding site is conserved in zebrafish VWF indicating that zebrafish VWF may behave similarly to human VWF. The bioinformatics also showed that exon 28 is split in the Ensembl database in zebrafish and is not split in our sequencing results. In subsequent chapters I will examine the effect of morpholino targeting on the GPIbα binding region to determine whether or not the zebrafish VWF plays a similar role in thrombosis as human VWF. The finding that GPIbβ exists lends support for VWF and its receptor interaction.

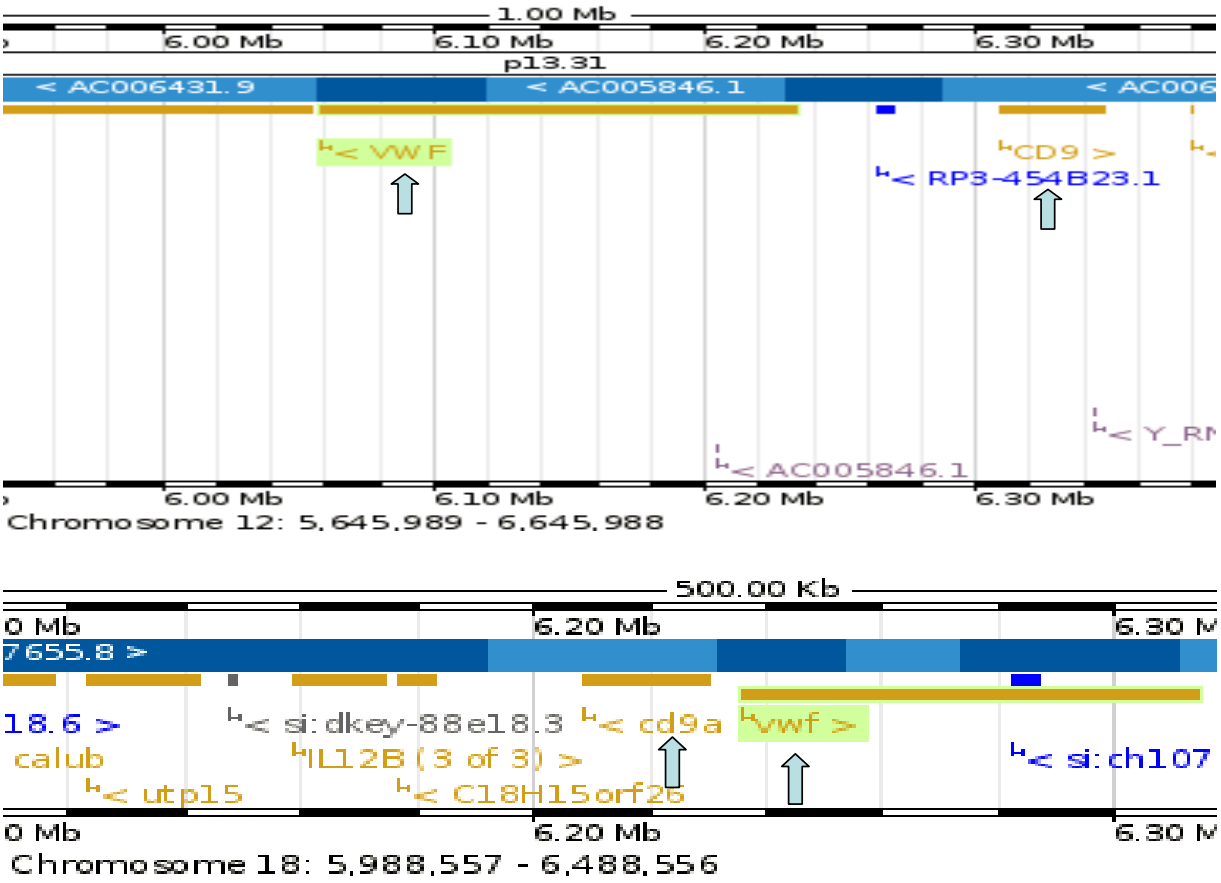

Figure 2.1: Human and zebrafish VWF and CD9 synteny. Image from Ensembl showing synteny between the human chromosome 12 (top panel) and zebrafish chromosome 18 (bottom panel) VWF and CD9. Blue arrows in both panels are pointing to the VWF and CD9 gene location. This image is from the Ensembl online database.

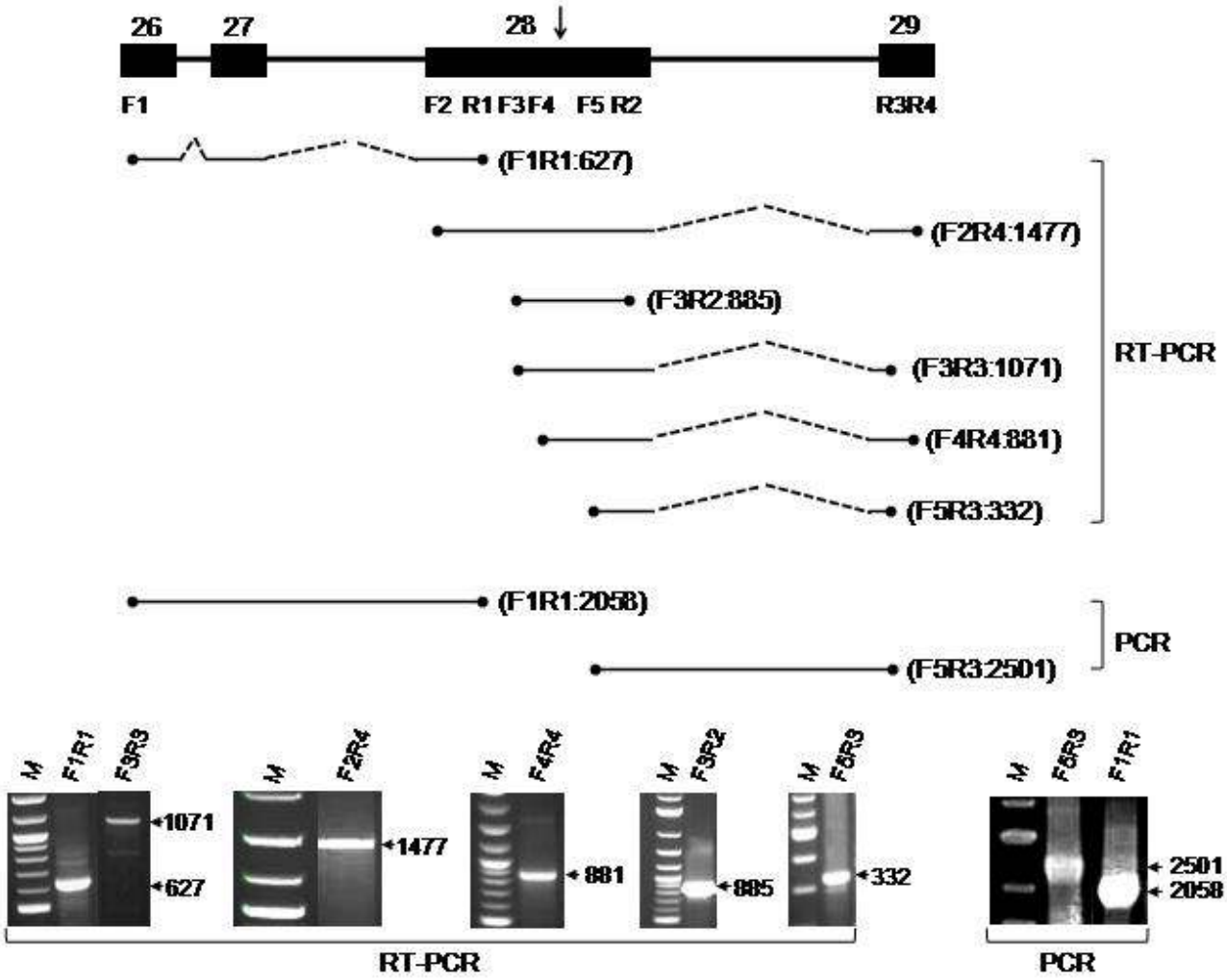

Figure 2.2: Schematic diagram of regions amplified in PCR and RT-PCR. PCR and RT-PCR performed using genomic DNA and RNA, respectively. Forward primers are denoted as F1 to F5 and reverse primers are denoted by R1 to R4. Primers F3:R2 spanned the region where Ensembl showed an intron separating the homologous GPIbα binding region. Primers F5:R3 were used for the Genomic PCR and also span the region where the intron is shown in the Ensembl VWF sequence. Exons denoted as black boxes with the numbering on top corresponding to the human exon numbering. The arrow points to the location of the 'intron' according to the ENSEMBL database. RT-PCR and PCR product sizes are shown by lines flanked by solid circles (not drawn to scale). Forward and reverse primer combinations are shown in parenthesis followed by the size of the product in base pairs. The agarose gel photographs show the amplified products corresponding to those shown in the schematic diagram. Lanes of the amplified products are marked with the combination of primers; M shows the DNA size markers. The cDNA products generated by RT-PCR and the genomic products generated by PCR are marked separately. Dashed lines indicate the introns removed during splicing. Agarose gel photos are of the amplified products corresponding to those shown in the diagram.

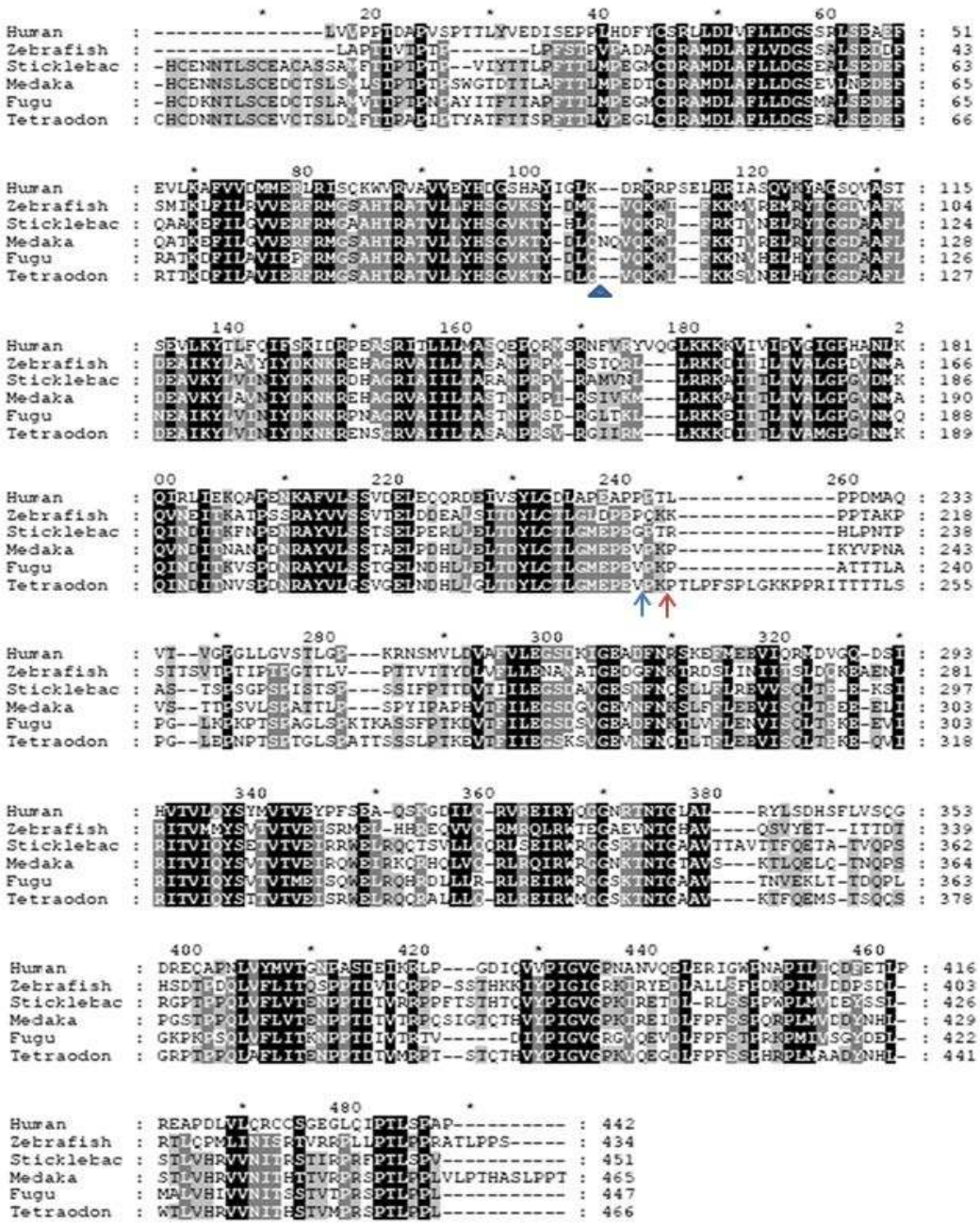

Figure 2.3: Alignment of human, zebrafish, stickleback, medaka, fugu and tetraodon VWF. The arrow head indicates the region where human exon 28 is split in all fishes with the exception of zebrafish. The blue arrow indicates the region where human exon 28 is split in stickleback and medaka. The red arrow indicates the region of human exon 28 that is split in the zebrafish VWF according to the Ensembl database.

```
Query  8   ALSLLLLLLAPPSRPAAGCPAPCSCAGTLVDCGRRGLTWASLPTAFPVDTTELVLTGNNL  67
           ++ L+  L A  +     CP  CSC+  +VDC  R LT A+LP++FP  TTEL+L  N+L
Sbjct  3   SVVLVFFLSAMAAVVQGSCPHVCSCSAGVVDCSNRALTTATLPSSFPASTTELLLNENHL  62

Query  68  TALPPGLLDALPALRTAHLGANPWRCDCRLVPLRAWLAGRPERAPYRDLRCVAPPALRGR  127
           TALP GLLDALPALR   L  N W CDC ++ LR W+  R      R++ C +P  LRGR
Sbjct  63  TALPTGLLDALPALRRVALHGNSWACDCAILYLRGWMLKRGSDPSMRNVSCSSPAHLRGR  122

Query  128 LLPYLAEDELRAACAPGPLCWGALAAQLALLGLGLLHALLL---VLLLCRLRRLRARARA  184
           L+ YL E EL  +C    LC  ALA+Q++LL    + ALLL   +  L R  RL   A+
Sbjct  123 LIVYLPEQELLDSCRYW-LCNLALASQISLLVFIGVQALLLASVIFFLRRFERLTEEAQR  181

Query  185 RAAARLS  191
            AA   +
Sbjct  182 TAAESFT  188
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Figure 2.4 Amino acid alignment of human and zebrafish GPIbβ

CHAPTER 3

EXPRESSION OF VWF IN ZEBRAFISH

Introduction

Biosynthesis of Human VWF

Human VWF is synthesized in endothelial cells of blood vessels where it is either stored in Weibel-Palade bodies or constitutively secreted [102]. Weibel-Palade bodies are small rod shaped storage organelles that contain VWF and P-selectin [102]. VWF is necessary for Weibel-Palade body formation and has been shown to interact with P-selectin to promote its storage [86; 103]. A second site of VWF synthesis is the megakaryocyte. The VWF synthesized in these cells are stored in α-granules (another type of storage organelle, also containing P-selectin) then packaged into newly forming platelets [24; 104]. The VWF within these organelles is released upon injury or activation along with P-selectin and any other proteins found in storage [105] Expression of P-selectin is used as a marker for detecting platelet activation because once it is released from storage it is found on the surface of the cell [106].

Zebrafish endothelial cells and thrombocytes have both been shown to contain dense bodies that appear to be similar to Weibel-Palade bodies and α-granules in humans [107; 108]. Unlike humans, zebrafish do not have platelets but instead have nucleated thrombocytes that are transcriptionally active. Because these cells are transcriptionally active, they are able to produce proteins important for hemostasis. Up to now there has been no detection of VWF storage in the endothelium or in thrombocytes; however, Jagadeeswaran et al. have shown using immunostaining that P-selectin is detected on the surface of zebrafish, thrombocytes upon activation,

whereas before activation it was not detected [65]. This indicates that P-selectin is being released from storage and may be stored with VWF similarly to humans. To date there has been no immunostaining to address where VWF is expressed in zebrafish.

Immunostaining

Immunostaining is a common technique used to determine where a protein of interest is either: expressed, or stored. This is a technique that is well established and used in a variety of organisms and laboratories. The process of immunostaining is accomplished by the use of an antibody specific for the protein of interest. This antibody is either conjugated to a chromophore, or it is later treated with a secondary antibody conjugated with a chromophore. The primary antibodies can be organism specific or it can be an antibody from one organism that reacts with a protein in another organism, provided that the region for which the antibody is designed is similar. If an antibody is not available because it has not been developed or for some reason is not reacting across species then and antibody can be produced. To produce a polyclonal antibody the protein or a portion of the protein for targeting is isolated and subsequently injected into an animal along with an adjuvant, used to boost the immune system for antibody production; after which time the injected animal will produce antibodies that can be later collected [109]. Monoclonal antibodies can be produced for targeting a specific epitope through the use of hybridomas; this is a hybrid of a myeloma cell and mouse spleen cell. The spleen cells are collected from a mouse previously immunized with an antigen; subsequently the cells are fused with the myeloma cells creating a population of cells that will divide indefinitely producing a mixture of monoclonal

antibodies. These cells are then grown as clones and screened for the antibody of choice and subsequently cultured, and later the antibody is isolated [110; 111]. The technique of immunostaining can be used in a variety of staining procedures: staining of the entire organism for tissue localization of protein, western blot analysis to determine whether a protein is present, for instance in plasma or cell lysates, as well as in whole blood smears [112]. Zebrafish larvae as well as blood cells can be stained by this technique.

Aims and Hypothesis Tested

The hypothesis in this investigation is that zebrafish VWF is synthesized in endothelial cells as well as in thrombocytes. The goals of the experiments are to determine where VWF is synthesized through immunostaining and RT-PCR analysis. I expect to find that VWF is synthesized in thrombocytes as well as in the endothelium of the blood vessels.

Materials and Methods

Immunostaining of Thrombocytes

To determine whether VWF is present in thrombocytes immunostaining was performed using whole blood smears. A blood smear was made using whole blood from adult zebrafish and allowed to dry for 10 minutes. The slide was immersed in 70% cold ethanol for 10 minutes. The slides were then rinsed three times in phosphate buffered saline (PBS) and incubated in VWF-Ab (Sigma; St Louis, MI) diluted 20 fold in PBS in a total volume of 60 µl, which was used to cover the blood smear under a

coverslip and incubated for 2 hours. After incubation, the slides were rinsed as described above and then incubated with FITC conjugated anti-rabbit IgG (Jackson Immuno Research; West Grove, PA) and diluted 20 times in 1xPBS for 1 hour. Once the second incubation was complete the slides were rinsed with 1xPBS three times; then, the slides were subjected to one final rinse in double distilled water. These slides were then examined using the Nikon 80i eclipse microscope and the NIS Elements AR 2.30 software.

Immunostaining of Whole Larvae

To determine whether VWF is synthesized in blood vessels, we used whole larvae for immunostaining. Whole larvae were fixed in 4% paraformaldehyde for 6 hours at 4^{o}C, then washed with 0.1 M phosphate buffer (pH of 7.3) for 5 minutes The larvae were then washed in distilled water for 5 minutes, incubated at -20^{o}C for 7 minutes in acetone, and washed in distilled water for 5 minutes followed by a 5 minute wash in 0.1 M phosphate buffer (pH of 7.3). Subsequently, these larvae were blocked in 2% goat serum in PBS with 3% BSA and 1% DMSO for 1 hour. After blocking, larvae were incubated overnight at 4^{o}C in a solution of 1% DMSO containing either anti-human VWF antibody (VWF-Ab) 8 mg/ml at a 1:200 dilution (Sigma; St Louis, MI) or control purified rabbit IgG (primary antibody) from non-Immune Sera 10 mg/ml at a 1:200 dilution (Affinity Biologicals; Ancaster, ON, Canada). After incubation, larvae were rinsed with a solution containing PBS with 3% BSA and 1% DMSO for 2 hours with a change to fresh solution every 30 minutes. Larvae were incubated for 4 hours at 20^{o}C in PBS with 3% BSA and 1% DMSO with FITC conjugated anti-rabbit IgG (secondary

antibody) 2 mg/ml at a dilution of 1:200 (Jackson Immuno Research; West Grove, PA). The larvae were observed as described above.

RT-PCR using Zebrafish Thrombocytes

RT-PCR was performed using zebrafish thrombocytes alone to show that there is indeed mRNA synthesis of VWF. Thrombocytes were collected from whole blood using a method developed in the Jagadeeswaran laboratory for separating young and mature thrombocyte populations [113]. These thrombocytes were subsequently used for RT-PCR targeting the exon homologous to human exon 28. The following primers were used forward: 5′-CACAGAGTCCTCCAACTGACG-3′, and reverse: 5′-AATGTTTTCAGTCCTCAAACTG-3′.

Results

Expression of VWF in Thrombocytes

Immunostaining of whole blood showed that thrombocytes were positive for the presence of VWF; this was performed on 10 independent smears using control and VWF-Ab. The VWF-Ab stained slides showed 100% of the thrombocytes positive for staining, compared to the control, which showed no immunostaining in any of the thrombocytes. These results indicate that VWF is indeed present within zebrafish thrombocytes (Figure 3.1). Since thrombocytes in earlier work have shown that granules exist in these cells and since thrombocytes synthesize VWF it suggests that the synthesized VWF may be stored in these granules.

To confirm that VWF is being synthesized in thrombocytes we performed RT-

PCR using only thrombocytes, this resulted in positive amplification of VWF mRNA (Figure 3.2). This positive amplification shows that VWF is indeed being synthesized in thrombocytes. No other cells in the blood smear were positive for VWF immunostaining. RT-PCR using both young and mature thrombocytes showed that VWF mRNA is being synthesized.

Expression of VWF in Endothelium

Next immunostaining was performed, using whole larvae to determine whether VWF is present in the vasculature. I found that the blood vessels were stained with VWF antibody and no staining was observed in the controls (Figure 3.3). These results provide the first evidence that VWF is synthesized in zebrafish endothelium.

Discussion

Previously in chapter 2 RT-PCR was performed using whole larvae to show that VWF mRNA is indeed being synthesized; however, these results did not provide information about the location of VWF synthesis. In this chapter experiments showed for the first time staining for VWF in zebrafish thrombocytes and blood vessels. The fact that there was antibody staining in the arteries and veins, as well as intersegmental vessels, serves as evidence that VWF is present in all endothelial cells irrespective of the vessel type. This is consistent with the finding in mammals, where it has also been shown that endothelial cells express VWF in both veins and arteries [114]. At present, it is not know whether VWF is stored in the baso-lateral area of the endothelial cells, although the endothelial cells of the fish have been shown to have electron dense

organelles that may represent Weibel-Palade bodies [115]. These Weibel-Palade body like organelles have also been detected in the blood vessels in another teleost fish *Pimelodus maculates* [108; 115]. The possible presence and storage of VWF is important because it provides further evidence that VWF synthesis, storage and secretion is likely to be similar to that of humans. The storage of VWF in blood vessels would allow for rapid thrombus formation at the site of injury by the release of VWF. The VWF stored in the endothelium may likely be UL-VWF, and it may be highly reactive with thrombocytes. However, this remains to be further studied.

In addition to the positive staining of the blood vessels, positive staining in thrombocytes provided more evidence for even more similarities between human platelets and zebrafish thrombocytes. For example the appearance of P-selectin on the surface of thrombocytes after activation indicates that this protein is stored and released when needed similarly too human platelets. The release of P-selectin upon activation supports the idea of storage granules in zebrafish thrombocytes which leads us to wonder whether VWF is also stored in thrombocytes. Because at this time granules have been identified but not stained for any particular protein, immunostaining has provided evidence that VWF is synthesized and may be stored in thrombocytes. This immunostaining combined with the RT-PCR evidence of mRNA production suggests VWF mRNA is being translated into protein. Young and mature thrombocytes used for RT-PCR analysis showed that VWF mRNA is being synthesized in both populations. However, the slight increase in the mature population suggests that more localized production of VWF during propagation of thrombus may be required in thrombus

growth. VWF production may also be a marker for thrombocyte maturation, this has been used in megakaryocytes a marker for maturation [116].

Conclusions

In this chapter the experiments showed that VWF is present in thrombocytes as well as in endothelium of vessels. This is the first time thrombocytes have been shown to have VWF transcripts. Experiments also confirmed the presence of VWF protein in thrombocytes which also provided evidence for the synthesis of the protein within thrombocytes. VWF synthesis in thrombocytes may be similar to the synthesis of VWF in megakaryocytes since thrombocytes have transcriptional machinery. In this context it is similar to the presence of transcription factors in thrombocytes that were also found in megakaryocytes. This also adds credence to the earlier observations of similarities between thrombocytes and megakaryocytes [117].

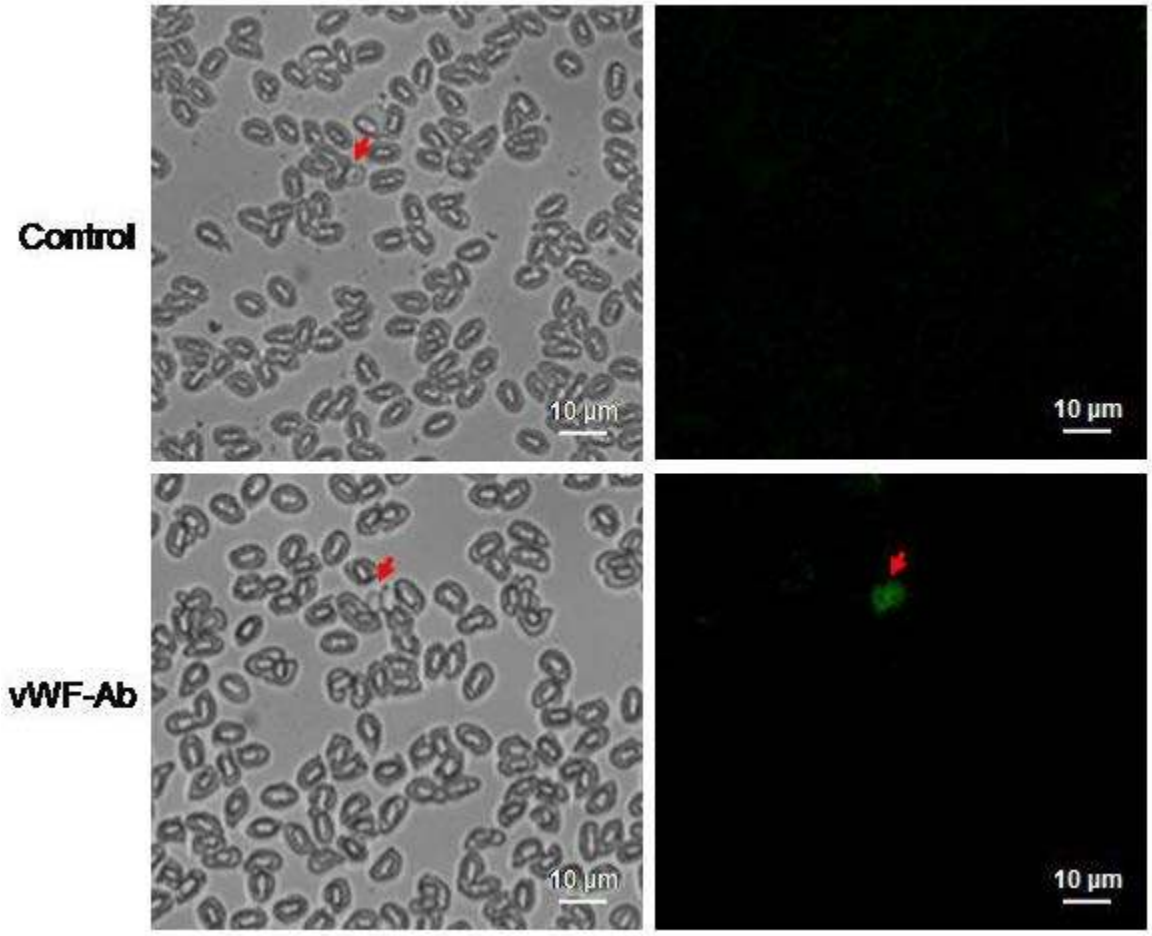

Figure 3.1: Immunostaining of thrombocytes. Human von Willebrand factor antibody (VWF-Ab) and rabbit IgG (Control) were used as primary antibodies followed by FITC conjugated secondary antibody for visualization. Left and right panels show the brightfield images and fluorescent images, respectively. Arrows point to thrombocytes, which are surrounded by other cells, including red cells.

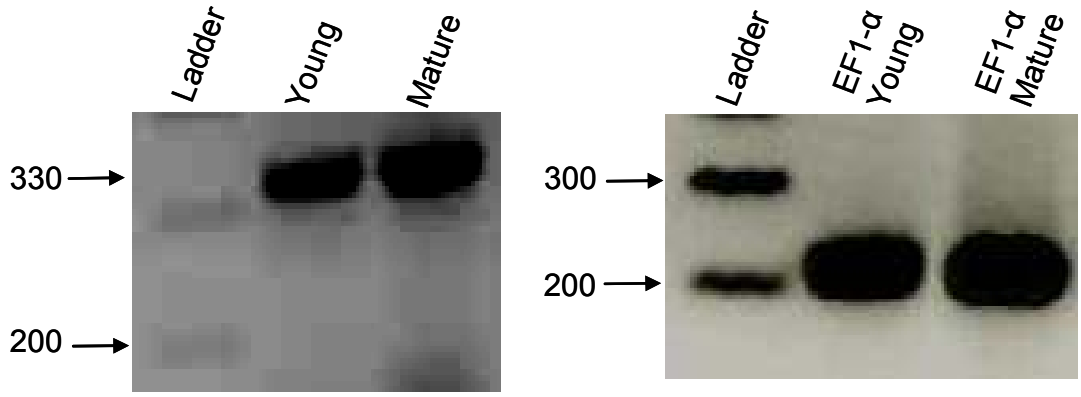

Figure 3.2: RT-PCR using zebrafish thrombocytes. Thrombocytes collected and used for RT-PCR amplification of the exon homologous to human exon 28 of VWF (left panel). Amplification of EF1-α in young and mature thrombocytes (right panel). The image shows that both populations of thrombocytes are transcribing VWF mRNA.

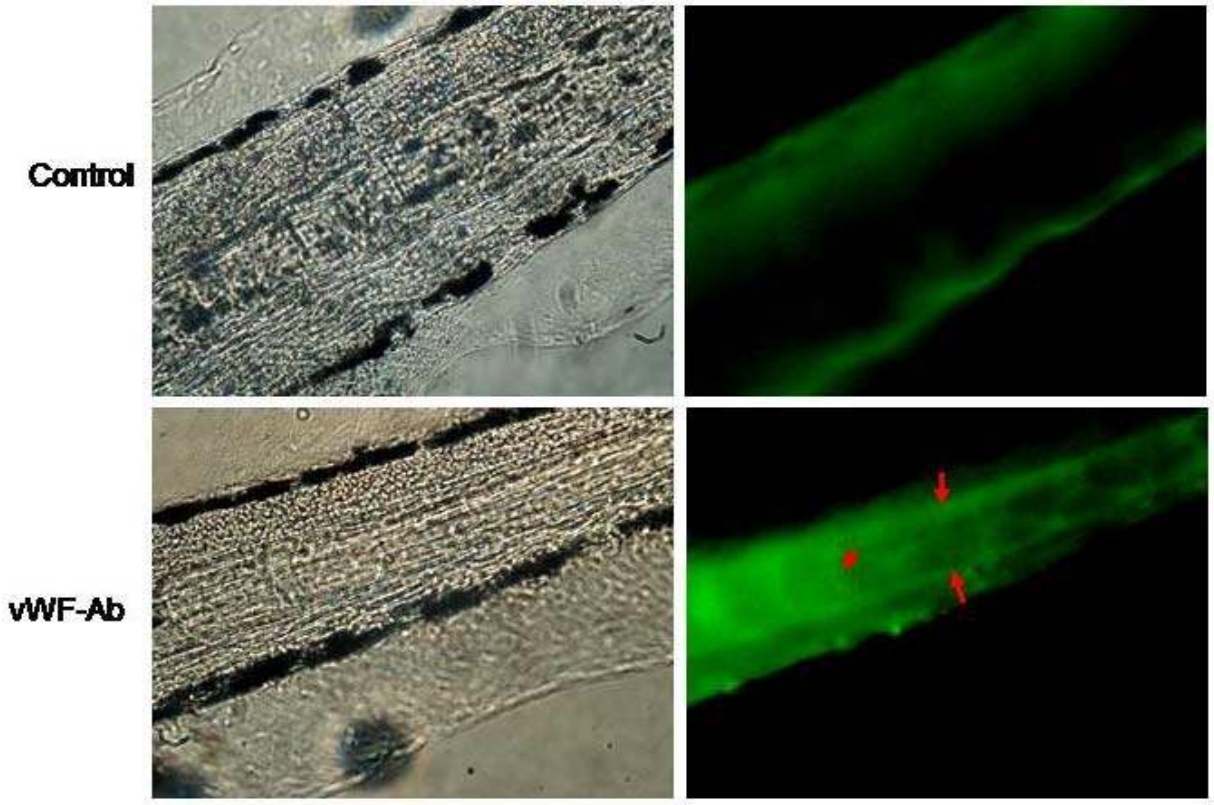

Figure 3.3: Immunostaining of whole larvae. Human von Willebrand factor antibody (VWF-Ab) and rabbit IgG (Control) were used as primary antibodies followed by FITC conjugated secondary antibody for visualization. Left and right panels show the brightfield and fluorescence images, respectively. Larger arrows show caudal artery (upper region) and caudal vein (lower region) whereas the smaller arrow shows the intersegmental vessels.

CHAPTER 4

MOPHOLINO TARGETING OF ZEBRAFISH VWF

Introduction

Antisense Morpholino

Morpholinos are powerful tools that can be easily administered by injection to prevent expression of protein by blocking translation or create a dysfunctional protein by removal of an exon during posttranscriptional processing. Two types of morpholinos in use today are the antisense morpholino (MO) and Vivo-morpholino (VMO) oligonucleotides. Both of these were developed by Gene Tools, LLC and have been employed to knockdown proteins.

MO is a modified oligonucleotide synthesized using modified ribonucleoside subunits called morpholine subunits. In this modification of the ribonucleoside nitrogen is inserted in between the 2′ and 3′ hydroxyls of the five ringed ribose sugar thus creating a six membered ring which is called a morpholino ring. In the final assembly of the MO these six membered rings are linked by a phosphoroamidate instead of phosphodiester bridge (Figure 4.1) [118; 119]. This MO analog is resistant to nucleases making it more stable while also maintaining high efficiency for binding to complimentary RNAs [118; 120]. Currently MOs are used in a variety of model organisms such as: zebrafish, *Xenopus*, seaurchin and even chick embryos. The studied included several topics such as studies on β-catenin signaling in *Xenopus* and seaurchin, angiogenesis in zebrafish, and transcription factor function in chick embryos [121; 122; 123; 124]. MOs are not taken up by cells and therefore, must be administered by injection or electroporation. For example they have been injected into

zebrafish at the 1 to 4 stages of development, when yolk is in contact directly with the newly developing cells. Thus all cells in subsequent divisions will acquire MO [125]. Injection of MO after the 1 to 4 cell stages may result in incomplete distribution of morpholino to all cells. However, once the MO is injected into 1-4 cell stage embryos MO will continue to persist in cells up to 8 day post fertilization (dpf). Therefore MO based knockdowns could not only be applied to silence genes that are important for development but also genes that are important in larvae even after hatching.

VMOs are essentially the MOs with a transporter moiety that allows the morpholino to move through the plasma membrane and into the cell [126; 127]. The transporter portion of the VMO is composed of 8 guanidinium heads, most effective for moving through the membrane, attached at the 3′ end of MO (Figure 4.2) [127; 128]. VMOs were first introduced to the zebrafish model system for the study of hemostasis, although prior to this VMOs have been used in mice and dogs to treat muscular dystrophy [74; 129]. While these MOs and VMOs have the similar mechanisms in the knockdown function VMOs differ from MOs in that they are better suited for use in older organisms although they can also be injected into embryos. The ability of VMOs to freely enter the cells makes them a powerful too to study adult or juvenile phenotypes. This is an advantage when investigating function of a protein that is developmentally lethal when a knockdown or knockout is performed and essentially similar to conditional knockouts. Thus, the use of the VMO after the critical stage of development allows for the examination of the effects of knocking down the protein that is lethal in early development at a later time to determine whether absence of the protein would still have a detrimental effect.

Once the MO or VMO is administered well developed phenotypic assays can be performed to determine whether or not there is a consequence to knockdown or a dysfunctional protein. If the protein of interest is important for proper functioning of a particular pathway or process, the effects should be easily assayed or observed. Also, by using knockdown approaches not only can pathways be delineated, but human diseases can be produced and their effects on the organism studied.

Time to Agglutination/Aggregation assay

The time to agglutination/aggregation assay (TTA) used in zebrafish was developed in the Jagadeeswaran laboratory to suit the smaller volume of blood available from zebrafish [63]. The assay is used to measure the time it takes for blood to form an agglutinate/aggregate [63]. This assay could be used to monitor the Ristocetin cofactor activity that assays VWF in humans. The TTA in presence of ristocetin should give a measure of VWF activity in the blood sample. Ristocetin is an antibiotic isolated from *Nocardia lurida*, which interacts with the A1 domain of VWF [130]. Agglutination/aggregation will only occur in the presence of VWF because ristocetin interacts with VWF inducing the VWF interaction with the GPIbα receptor on platelets. The information collected from this assay is used to determine whether an individual has vWD and provides insight as to the type of vWD it is. A sample that is lacking VWF or having a lower concentration of VWF will not aggregate/agglutinate or will have a prolonged TTA, thus indicating whether VWF is present or dysfunctional [131]. This particular assay occurs *in vitro* and does provide valuable information;

however, to test for VWF function *in vivo*, the time to occlusion assay (TTO) can be performed.

Time to Occlusion

Laser induced time to occlusion (TTO) assay is an *in vivo* hemostatic assay developed in Dr Jagadeeswaran's laboratory to observe thrombus formation *in vivo* under a microscope and can be used to target either the artery or vein [72]. Thrombus formation is induced by laser injury to a blood vessel in larvae immobilized in agarose and subsequently the time it takes to form an occlusive thrombus is measured in seconds. The formation of the thrombus can also be recorded using a video camera or computer attached to a microscope.

This method was first utilized to observe whether or not thrombocytes are involved in thrombus formation at the site if injury within blood vessels [76]. The TTO in arteries is about 60 sec. and in veins it is about 30 sec, these TTO's were established previously in Dr. Jagadeeswaran's laboratory [72]. Laser induction of an injury and subsequent observation of thrombus formation in zebrafish larvae have several advantages: the injury can be induced in a specific area and vessel, without having to exteriorize any of the vessels or organs such as in mouse to observe the occlusion. Despite the small size of the blood vessels they are easily targeted because injury is performed under the microscope. Zebrafish larvae are small and can easily be mounted in agarose on a microscope slide and then images can be captured and live footage recorded using a computer attached to the microscope. Larvae can even be

rescued from the agarose in which they are mounted to be saved for breeding when screening for mutants.

Stimate

Stimate (desmopressin acetate, DDAVP) is a synthetic analogue of hormone 8-arginine vasopressin (ADH), and is used to treat individuals with hemophilia A, and vWD, type 1 [132]. Treatment of individuals with Stimate causes the release of VWF from Weibel-Palade bodies found in the endothelial cells resulting in an increase in plasma VWF [133; 134]. The VWF release from Weibel-Palade bodies is of a higher molecular weight than the VWF found in circulation, and is more reactive in binding to platelets this type of VWF is termed UL-VWF [22]. Treatment using Stimate allows for individuals with a low VWF in circulation to be relieved of symptoms due to an increase of VWF in circulation after treatment as well as a stabilization effect to FVIII [132]. This type of treatment is good for individuals with a quantitative issue with VWF rather than individuals with a qualitative issue.

Activated Partial Thromboplastin Time

The activated partial thromboplastin time (aPTT) assay is a commonly used assay for determining whether the factors of the intrinsic and common pathway of coagulation are functioning properly [135]. This assay provides information about FVIII content in plasma, and some indication as to whether or not there is a deficiency in FVIII [136]. FVIII is part of the intrinsic pathway acting as a cofactor for Xa which results in fibrin production, which is important for stable clot formation, and is what is being

measured in the assay. To examine fibrin formation the assay is activated by adding $CaCl_2$ in the presence of Dade ACTIN and plasma [135]. If there is a reduction in FVIII more time is being taken to form fibrin. This assay also provides information regarding FVIII content in relation to VWF. If there is a decrease in VWF or binding activity of VWF to FVIII then more time should be taken to form fibrin. To examine VWF interaction with FVIII in zebrafish modified version of aPTT assay developed in Jagadeeswaran's laboratory suitable for small amounts of blood. In this modified aPTT assay volumes as small as 1 µl of plasma can be used and is termed the kinetic partial thromboplastin time (kPTT) because it actually measures the time course of thrombin generation as a function of fibrin formation from exogenously added fibrinogen [137]. With the availability of this assay it should be possible to test whether or not a lack of VWF will result in a prolonged kPTT due to a decrease in FVIII. In this kinetic assay prolonged kPTT will result in shift of the fibrin formation kinetic curve to the right compared to controls. As previously mentioned this is because FVIII is closely associated with VWF. In some vWD patients there is a prolonged aPTT due to a decrease in FVIII due to [138]. The kPTT assay should help in establishing zebrafish as a relevant model for studying human VWF and vWD.

Aims and Hypotheses Tested

The major hypothesis in this chapter is that the knockdown using MOs and VMOs should yield vWD phenotype in zebrafish larvae and adults. The goals of these experiments proposed below are to determine whether morpholino targeting of zebrafish VWF will produce a bleeding phenotype, to determine whether VWF is stored

and released upon treatment with Stimate and to establish whether kPTT is altered in zebrafish with low VWF.

Materials and Methods

Morpholino and Vivo-Morpholino Injections

Embryos were injected with 3 nl of 1 mM antisense MO for VWF, 5'-ACTGTAGTGTTGATTCTGACCTGAA-3' (Gene Tools; Philomath, OR) for the exon-intron boundary of the exon homologous to exon 28 in humans (which encodes for the binding site, in VWF, to GpIbα), at the 1-4 cell stages using the picospritzer III (Parker Precision Fluidics; Hollis, NH) [75]. A standard control MO 5'-CCTCTTACCTCAGTTACAATTTATA-3' was also injected into embryos at the 1-4 cell stages.

Adult zebrafish were injected intravenously with either 5 µl of 0.5 mM VWF-VMO, or control VMO. The VWF-VMO was designed using the same sequence as the VWF-MO that was injected into embryos at the 1-4 cell stages; the control VMO is also the same sequence as the MO injected into embryos at the 1-4 cell stages (Gene Tools; Philomath, OR) as previously described [74]. The VMO was injected intravenously in the caudal region of the adults and the blood was collected after 48 hrs as described above. The blood was used in the ristocetin mediated thrombocyte aggregation/agglutination assay. The assay was performed as previously described [63].

RT-PCR and Zebrafish

Total RNA from whole larvae collected using Absolutely RNA miniprep kit (Stratagene, Inc.; Santa Clara, CA) was used for RT-PCR for amplification of VWF mRNA. The following primers were used: Forward primer 5'-CACAGAGTCCTCCAACTGACG-3' (F5) and reverse primer 5'-ATGTTTTCAAGTCCTCAAACTG-3' (R3) (Biosynthesis; Lewisville, TX) were used: A schematic of the primer design can be seen in Figure 2.2. F5 is located in the exon corresponding to human exon 28 and R3 is located in the exon corresponding to human exon 29. The following primers were used for mRNA amplification of EF1-α: forward primer 5'-CGGTGACAACATGCTGGAGG-3' and reverse primer 5'-ACCAGTCTCCACACGACCCA-3' were used.

Imaging

Images of bleeding larvae, thrombocytes and blood vessels were recorded using either a Nikon Optiphot microscope or Nikon 80i eclipse microscope equipped with NIS Elements AR 2.30 software. TTO was also recorded using the Nikon Optiphot microscope as previously described [72].

Effect of Morpholino on Time to Occlusion (TTO)

Larvae that were previously injected at the one to four cell stages with MO and which were at 5-6 dpf were uses in laser injury experiments. The TTO was then measured in sec. Laser injury was induced in the artery, using a nitrogen laser light pumped through coumarin 440 dye using a MicroPoint Laser System (Photonic

Instruments Inc.; St. Charles, IL) connected to a Nikon Optiphot microscope, after immobilization in 0.8% agarose by methods previously established [72].

Stimate Effect on TTO

For treatment with Stimate we used larvae at 5-6 dpf. The Stimate (desmopressin acetate), used for treatment of the zebrafish comes in the form of a nasal spray (1.5 mg/ml, gift from Shelly Crary, UT Southwestern Medical School). The larvae were treated with Stimate by placing them in a solution diluted eight fold in distilled water for 15 min. After treatment TTO was measured by placing larvae in agarose as described above.

Stimate Effect on TTA

Blood was collected from adult zebrafish treated with Stimate. The Stimate concentrations used were similar to those used for the TTO assay. Stimate was administered by first spraying it into an eppendorf centrifuge tube and then diluting it five fold with distilled water. After the dilution, 5 µl of the diluted solution was placed on the gills of the adult zebrafish. The fish was then returned to water for 30 minutes, after which time blood was collected and the above TTA assay was performed.

VWF-VMO effect on kPTT

Adult zebrafish were injected with either control VMO or VWF-VMO as described above. Blood was collected from these fish in 3.8% sodium citrate, and then centrifuged at 500 rcf for 2 min. After centrifugation the plasma was carefully collected

and placed on ice. This plasma was then used for the kPTT assay which was previously established [81; 137]. Readings were taken using the Synergy H1 hybrid reader (BioTek, Winooski, Vermont).

Results

Morpholino Injection

To establish whether the MO injections would lead to vWD phenotype in zebrafish, embryos injected with VWF-MO at the 1-4 cell stages were observed observed. The number of embryos injected was 50-100 per batch with control MO and another 50-100 embryos per batch with VWF-MO. For each control and VWF-MO I injected 10 seprate batches of embryos. Approximately 50% of these embryos in both control and VWF MO treatment survived. Approximately 30% showed bleeding, whereas 100% of the controls did not develop any bleeding. Larvae manifested a bleeding phenotype in the head and yolk region at 5-6 dpf. Those Larvae injected with control MO at the same embryonic stage did not manifest a bleeding phenotype at any stage in development (Figure 4.3). The observation of bleeding in larvae indicated that the VWF-MO was working and producing a vWD like phenotype. However, to determine whether or not the VWF-MO targeting VWF is producing the expected alternate splicing of mRNA we performed RT-PCR analysis using whole larvae.

RT-PCR

To confirm whether the phenotype was the result of improper splicing due to MO injections, larvae generated from embryos that received MO were collected and then

used for isolating RNA. Using the combination of F1 and R3, RT-PCRs were performed to check whether the alternatively spliced 327 bp along with an unspliced 1754 bp product can be amplified. The 1754 bp product along with several non specific bands were amplified; however, not the 327 bp product (data not shown). A schematic for all primers used are found in Figure 2.2. Other primers designed from the exon 27 also yielded similar results. Furthermore, primers F2 and R4, which should have yielded both 1477 and 811 bp bands, also did not yield an 811 bp band, whereas a 1477 bp band was present. In addition, compared to the control RNA, the 1477 bp band was lighter in those treated with VWF VMO. Therefore, I suspected the mRNA may be unstable due to the loss of exon 28 explaining the loss or reduction in amplification of bands using F5 and R3 primers, in which case amplification in larvae which developed after VWF-MO injections should not yield a band or instead yield a band with reduced intensity; however, EF1-α primers should yield a band. As predicted, the results showed that in some larvae produced after VWF MO injections, the amplification product disappeared whereas in some cases the product was lighter compared to controls (Figure 4.4). However, all larvae produced after control MO injections resulted in amplification of VWF. In both experiments, EF-1α control bands amplified and served as an internal control. We then quantified the ratios of densities for the VWF band and EF1-α band in both larvae produced after VWF MO and control MO injections. I found larvae resulting from control MO injections had significantly greater levels of VWF mRNA compared to the larvae produced after VWF MO injections.

Morpholino Effect on TTO

Since the VWF-MO injected embryos, produced a bleeding phenotype I then checked whether there will be an increase in the TTO of those larvae injected with VWF-MO. The results were positive; the embryos injected with VWF-MO did indeed have a prolonged TTO whereas the larvae injected with control MO experienced a normal TTO (Figure 4.5), a total of 14 larvae for control MO and VWF-MO. TTO was measured in the caudal artery, because VWF has been shown to be more important in arterial thrombosis due to the higher shear rates of the artery. In the artery a prolonged bleeding time is considered to be longer than 60 sec, while normal TTO is about 60 sec. We found that VWF-MO injected larvae had a TTO of approximately 130 sec compared to the normal 60 sec.

Stimate Effect on TTO

Next examined was whether or not Stimate might have an influence on the TTO in larvae. Larvae treated with Stimate had a significant decrease in the TTO forming a clot at approximately 10 sec compared to the normal 60 sec time (Figure 4.6), a total of 7 larvae were treated.

Injection of Vivo-Morpholino on Time to Agglutination (TTA)

To determine the VWF mediated agglutination of thrombocytes VWF-VMO was injected into adult zebrafish and blood collected. This blood was used to assay for ristocetin mediated agglutination by TTA assay. The TTA was affected by the VMO injections in to adult zebrafish in 60% of the samples, whereas the controls had a

normal TTA, a total of 10 fish were injected with control and VWF-VMO. Out of the 10 injected there were two deaths, one in control and one in VWF-VMO injected fish. Blood from zebrafish injected with the VWF-VMO had a prolonged TTA of >20 min whereas the blood from the control VMO injected fish agglutinated at approximately 15 min. This suggested that the VMO was sufficient for reducing the amount of VWF being synthesized by targeting mRNA for degradation (Figure 4.7). I next wanted to see whether treatment of adult zebrafish with Stimate would have the opposite affect of VWF-VMO.

Stimate Effect on TTA

Upon examination of Stimate on TTA there was a significant decrease in TTA of all adult zebrafish treated with Stimate compared to the control (Figure 4.7). This was expected based on the previous experiments using Stimate. The control zebrafish blood agglutinated at approximately 15 min whereas the blood from the fish injected with Stimate agglutinated in approximately 5 min.

Activated Partial Thromboplastin Time

While all the above results indicated that there is a decrease in VWF I also wanted to determine whether or not the decrease in VWF is going to affect FVIII levels. To do test this we performed the kPTT assay and found that there was a slight shift to the right of the control curve showing that it did take more time for thrombin generation compared to the control VMO treated blood (Figure 4.8).

Discussion

The above experiments were able to use VMO to examine whether or not creating a dysfunctional protein or a knockdown of VWF will have an affect on thrombus formation upon injury. This is the second hemostatic gene knockdown by VMO in zebrafish which adds credence to the VMO technology and its application to zebrafish hemostasis. The use of two different types of morpholino provides different modes of investigation while both providing the same efficacy and ability to target the same protein at any stage of development and also providing confirmation of the mutual results.

Here the employment of antisense morpholino was used to examine the effects of alternate splicing on VWF function; however, upon examination of the RT-PCR results it appears that an unstable VWF mRNA was created by the VWF-MO based alternative splicing. A knockdown of VWF is similar to the human vWD type 1, where individuals have very little VWF [139]. Because the resulting effect was a knockdown, this provided a convenient way to observe whether or not VWF is functioning similarly to human VWF, using a variety of bleeding assays. The assays include determining whether or not VWF is essential for a firm clot formation as well as determining whether or not VWF might be stored, and whether FVIII is being affected by a loss of VWF. Creating a model for type 1 vWD on zebrafish is significant despite the fact other type 1 models exist. This is significant because zebrafish provide advantages that no other animal models posses. A high fecundity allows for a researcher to investigate possible drugs that might initiate transcription of new VWF on a large scale with a variety of treatments all at the same time. The ease with which zebrafish adults and larvae can

be tested using a variety of assays also makes them a desirable model for the study of vWD.

To test whether in fact the bleeding manifested by VWF-MO was the cause of bleeding tested thrombus formation was tested by performing the TTO assay targeting the artery. In this assay indeed the larvae injected as embryos with VWF-MO did have a prolonged TTO. A prolonged TTO indicates that zebrafish VWF is important for thrombus formation similar to that found in humans. The measurement of arterial thrombus is of relevance because there is a much faster rate of flow in these vessels with higher shear stress compared to veins. This is an important difference between the two vessels because VWF acts as a docking site for platelets flowing past the site of injury. VWF first anchors itself to the exposed subendothelium and then unravels to expose platelet binding sites. VWF-MO effects were efficient in increasing the TTO by removing VWF from circulation, while Stimate treatment had the opposite effect.

In humans, Stimate initiates the release of UL-VWF stored in endothelial cells. This UL-VWF is more reactive than the VWF found in normal circulation and can spontaneously interact with platelets. The decrease in TTO in zebrafish indicates that UL-VWF might in fact be stored in zebrafish endothelial cells as well as thrombocytes. This reaction to Stimate is positive for zebrafish as a model for vWD because it provides further evidence that zebrafish VWF is not only functioning similarly to human VWF but storage and multimerization also seem to be occurring similarly to human VWF storage and multimerization. It would be interesting to try and create a vWD type 1 in zebrafish and treat the fish with Stimate, which is currently being used for the treatment of individuals with Type 1 vWD.

To further confirm the involvement of VWF in thrombus formation, blood from adults was injected with VWF-VMO. We found that there was an increase in TTA. These results further solidify the involvement of VWF in thrombus formation because aggregation/agglutination will not occur in the presence of ristocetin if VWF is not present. If the VWF is present in a lower concentration, only then a prolonged TTA is still expected. Thus, TTA results are consistent with the role of VWF in thrombus formation. In the corollary experiment of Stimate treatment a decrease in TTA was observed. This is probably due to the release of VWF in storage, as well as the fact that the VWF stored is of a higher molecular weight, UL-VWF and more reactive.

In humans it is well known that VWF interacts with FVIII to stabilize the protein and protect it from degradation while in circulation. Results showed in the kPTT assay a slight shift in the curve to the right in the blood samples prepared from VMO knockdown fish which indicated a longer time was needed for thrombin generation. These results will only occur if VWF is interacting with FVIII to stabilize the protein and protect it from degradation while in circulation. Thus, performing kPTT assay indicated that FVIII concentration is decreased as a result of VWF-VMO and provided with further evidence that zebrafish VWF does in fact behave similarly to human VWF by also interacting with FVIII and stabilizing it from degradation. In fact the kPTT assay detecting low factor levels was established in Jagadeeswaran's laboratory and was previously on human plasma that is deficient in FVIII [137]. This particular assay was performed using a very small amount of human plasma similar to the zebrafish kPTT. The shift in the kPTT to the right was also observed in their assays which are more pronounced. In our results the shift is modest nevertheless indicates low FVIII levels.

Conclusions

For the first time in this chapter experiments were able to show that by using VWF-MO a type 1 vWD could be induced in zebrafish. Experiments also showed that treatment of larvae using Stimate could decrease TTO and TTA suggesting that a form of UL-VWF may be present and is being released from storage. In addition to this these experiments also showed for the first time in zebrafish that a decrease in VWF appears to have an affect on the FVIII. Thus, I propose that the zebrafish model will prove to be an asset in the study of VWF function *in vivo* as well as for the study of vWD.

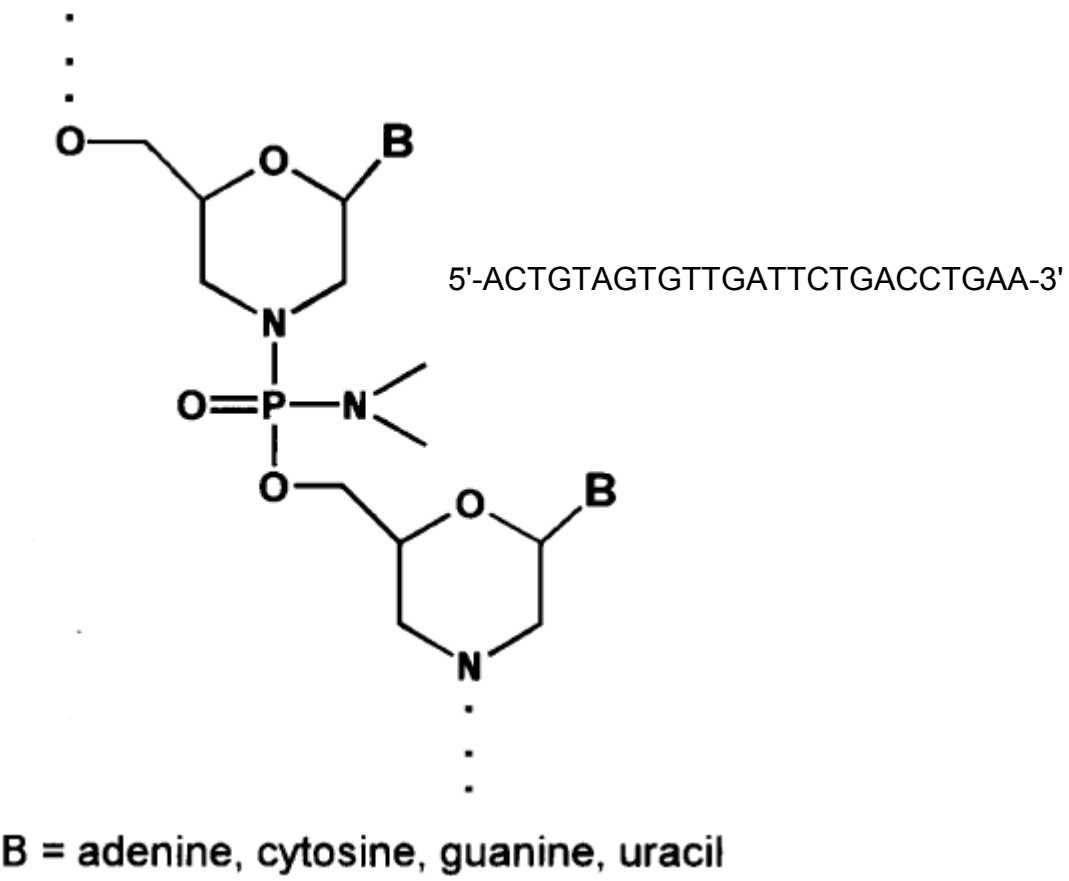

Figure 4.1: Morpholino structure. The image shows that nitrogen is inserted between the 2′ and 3′ position of an RNA molecule. Two separate nucleic acid analogs are joined by a phosphoroamidate. Sequence to the right of the MO image is the VWF-MO sequence used. Reprinted from Antisense & Nucleic Acid Drug Development, Volume 7, 1997, with permission from by Mary Ann Liebert, Inc.

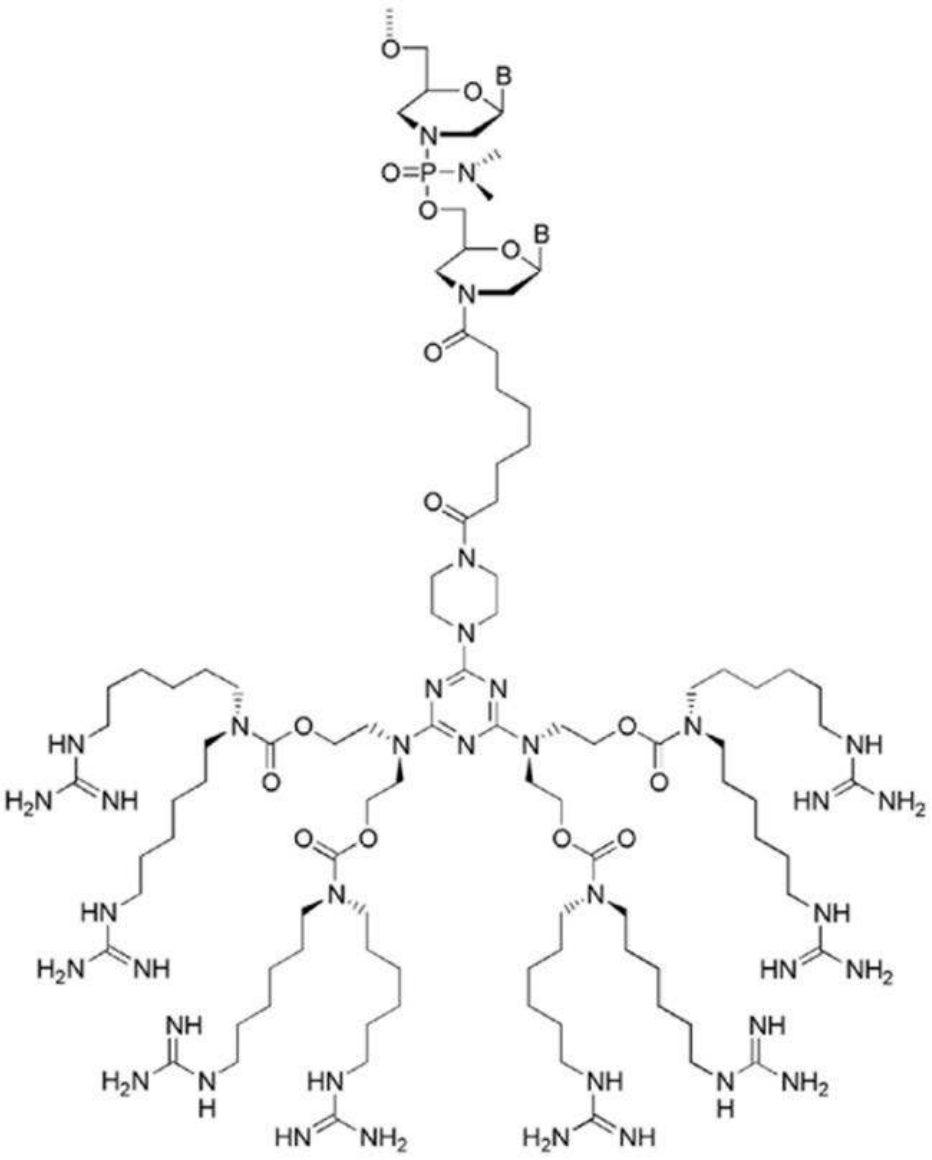

Figure 4.2: Vivo Morpholino structure. Morpholino oligonucleotide attached to triazine ring, with eight guanadinium head groups. These groups are what provide the penetrance of the cells. Reprinted with permission from, Y.F. Li, and P.A. Morcos, Design and synthesis of dendritic molecular transporter that achieves efficient in vivo delivery of morpholino antisense oligo. Bioconjug Chem 19 (2008) 1464-70, American Chemical society.

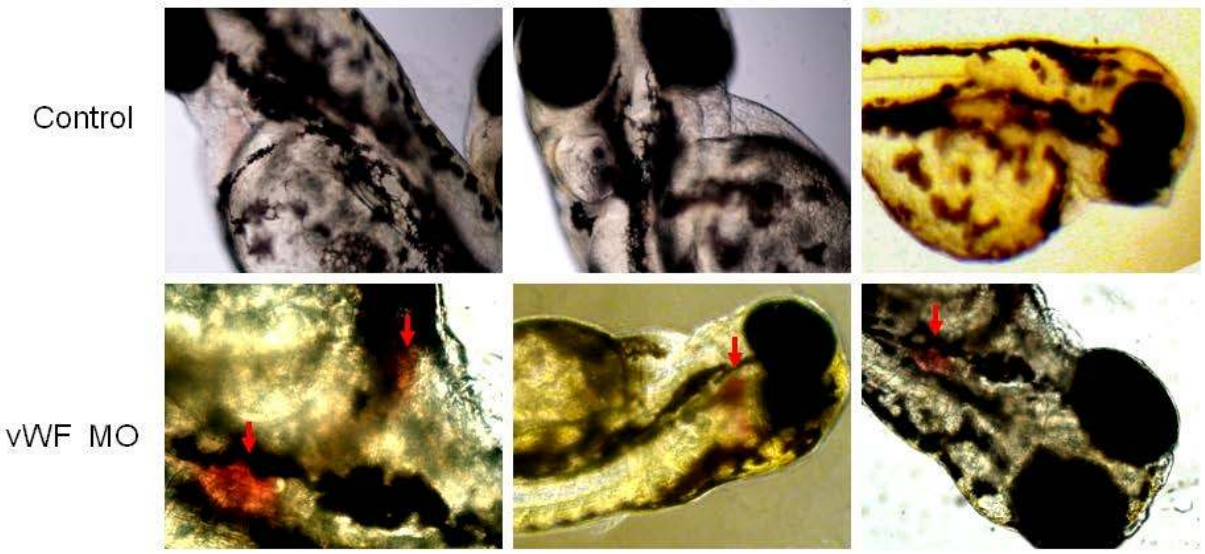

Figure 4.3: Larvae generated after injection of VWF MO (bottom panels) and control MO (top panels) into the 1-4 cell-stages of embryos. Arrows indicate the location of bleeding in the head and yolk region.

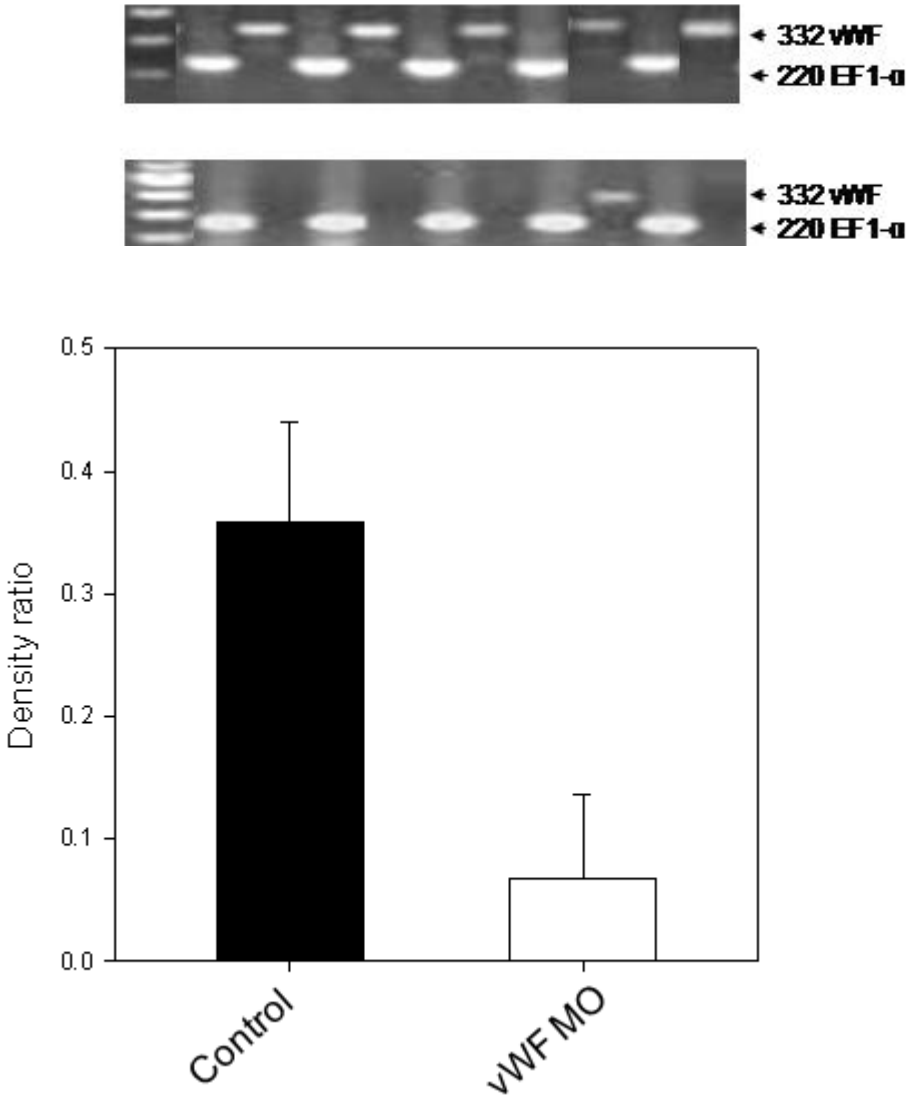

Figure 4.4: Analysis of splicing in VWF-VMO mRNA. Analysis of splicing after treating the embryos with VWF MO and control MO. Top panel shows representative RT-PCR products from RNA collected from embryos injected with control MO and VWF MO at 1-4 cell stages of embryos respectively. Arrows show the size of the RT-PCR products in base pairs (332 for VWF and 220 for EF1-α) on agarose gel photographs. Left most lanes show the DNA size markers. The bar graph shows the 220bp band 332 bp band densities n=30, p=<0.001.

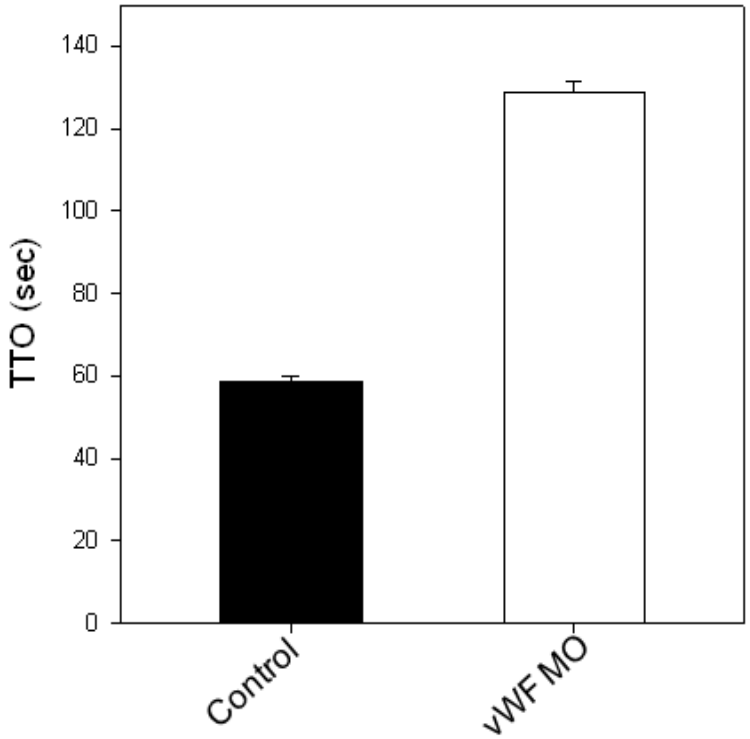

Figure 4.5: TTO in zebrafish larvae injected with VWF MO. TTO using larvae generated after injecting VWF MO and control Mo and control MO into 1-4 cell stages of embryos, n=14, p=<0.001 (left panel).

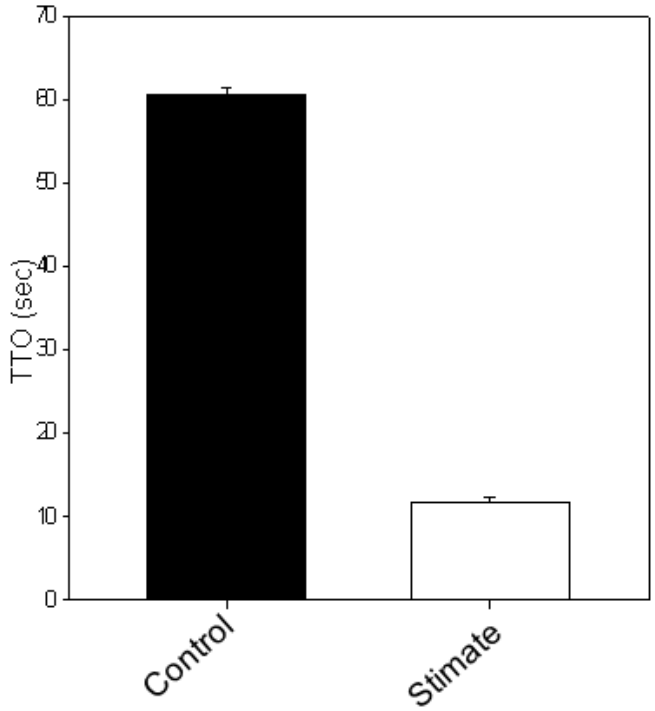

Figure 4.6: TTO in zebrafish larvae treated with Stimate. TTO using larvae 6 dpf treated with Stimate and untreated controls, n=7, p=<0.001 (right panel).

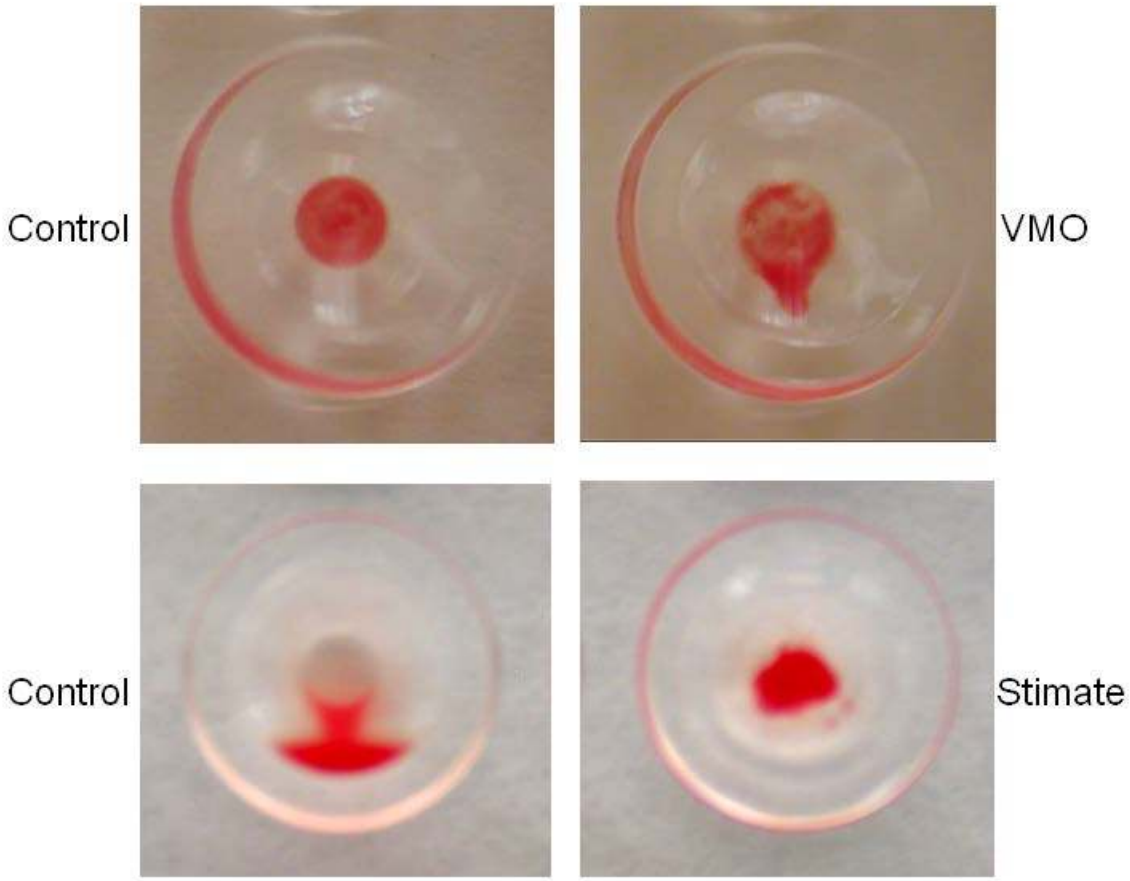

Figure 4.7: Ristocetin-mediated thrombocyte agglutination assay. Top panels show thrombocyte aggregation using whole blood from control MO and VWF-VMO injected adults. VWF-VMO treated adults took >20 to agglutinate whereas control were agglutinated at approximately 15, n=9. Bottom panels show thrombocyte aggregation using whole blood from Stimate treated and untreated control adult zebrafish. Stimate treated fish blood agglutinated at approximately 5 min and control blood at approximately 15, n=6.

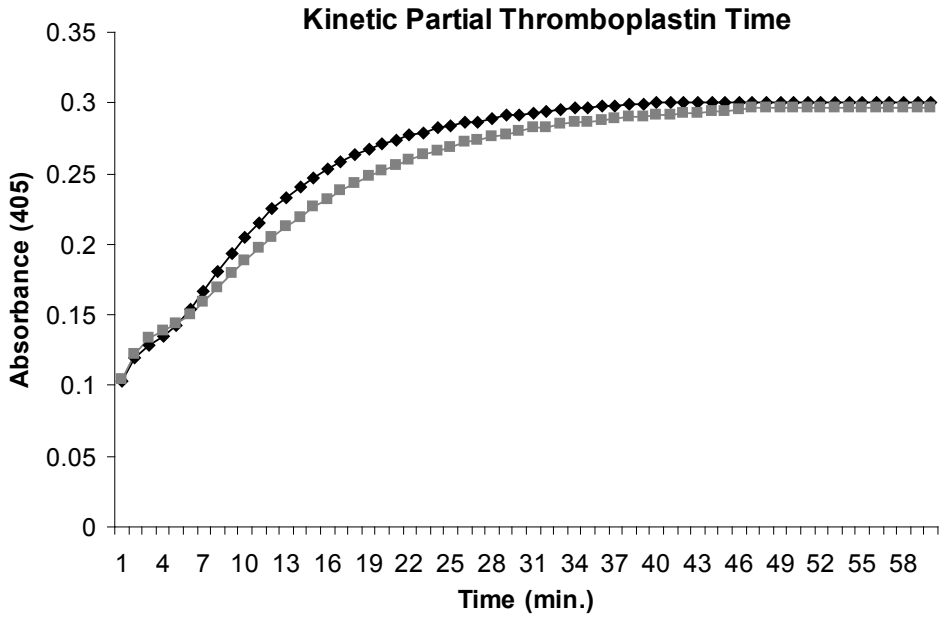

Figure 4.8: Zebrafish kPTT. Curves show a kPTT using plasma from zebrafish injected with VWF-VMO (grey curve) or control VMO (black curve). VWF-MO injected fish has slight shift to the right, compared to the control MO injected fish.

CHAPTER 5

MICROPARTICLES INTERACTION WITH VWF

Introduction

Microparticles

Microparticles are naturally occurring microvesicles found in blood circulation ranging in size from 0.1 – 1 μm in diameter and contain membrane proteins as well as cellular components of the cell from which they are derived [68]. Due to the fact that microparticles maintain many of the glycoprotein's and cellular components from the cells which they are derived from, it is reasonable to assume that they will also function similarly to the parent cell to some degree. While there are a variety of microparticles in circulation derived from a number of different cell types; our focus will be on thrombocyte microparticles (TMPs) found in zebrafish, which are similar to platelet microparticles (PMPs) in humans. Microparticles can be derived in a number of ways, either through activation, high shear stress or simply being shed from a parent cells such as megakaryocytes or platelets [25; 140; 141; 142]. *In vitro* experiments also indicate that the binding of platelets to VWF anchored to the site of injury results in microparticles expressing procoagulant receptors, which is also likely to help form a firm clot [143]. The method in which the PMPs are formed will determine to an extent the amount of reactivity of the microparticle, which is dependent on the proteins present on the surface [144]. Some PMPs have been shown to be more reactive than platelets due to presence of PS on the surface, as well as TF bearing which promotes procoagulant complexes to form on the surface of the microparticle [145; 146].

Platelet microparticles do indeed retain many of the receptors found on platelets

which includes the following glycoprotein's Ib, IIb, IIIa, IV, V and IX, to name a few [68]. Similar to platelet microparticles zebrafish thrombocyte microparticles have been detected in Jagadeeswaran's laboratory using CD41-GFP transgenic zebrafish as well as by DiI and FITC labeling. CD41 is the alpha subunit of the αIIb/β3 (CD41/CD61) integrin protein complex, which is also a platelet specific protein, detected in the earliest hematopoetic stem cells [147; 148]. These fish express green fluorescent protein (GFP) in thrombocytes, therefore microparticles derived from these thrombocytes should also be GFP labeled. This is one method by which Dr. Jagadeeswaran's laboratory has detected TMPs and in addition to this type of detection he has also previously shown that young thrombocytes can also be labeled using DiI, thus providing another method of microparticle identification [64; 65]. They also found that these thrombocyte microparticles are found in circulation as well as non-thrombocyte microparticles derived from other blood cells including red cells. Even though TMPs have been found in zebrafish their role in hemostasis has not been explored.

Aims and Hypothesis Tested

The major hypothesis explored in this chapter is that since thrombocyte microparticles are much smaller in size as compared to other circulating blood cells, they will probably be circulating closer to the vessel wall, when injury occurs these particles may adhere to the subendothelial matrix much before the larger cells. Since one of the major components of the subendothelial matrix is VWF, we hypothesize that the microparticles may adhere to VWF and agglutinate at the site of injury. Because these microparticles, have also been shown to carry GPIIb/IIIa thrombocyte receptors

they may also carry other receptors including GPIbα. The goal of this set of experiments is to examine whether or not zebrafish TMPs interact with zebrafish VWF in thrombosis thus participating in thrombus formation. Likewise we wish to explore whether the microparticles agglutinate *in vitro*.

Materials and Methods

Microparticle Collection

Plasma free microparticles were collected from 2 µl of citrated blood from wild type fish via centrifugation at 500 g for 10 min. The supernatant plasma was centrifuged at 20,000 g for 15 min. The resulting pellet was then washed with 1 ml PBS (pH 7.4) and centrifuged again at 20,000 g for 1 min. The resulting pellet was then suspended in 2 µl of PBS and kept as a stock for later use. Microparticles labeled with FITC were collected and prepared by the same method as explained above

Microparticle Rich Plasma

After collecting MPs, we also made a solution containing microparticles in plasma by using the 2 µl of blood collected in sodium citrate and centrifuged at 500 g for 10 min. After centrifugation the supernatant plasma was used for later experiments.

Microparticle Agglutination Assays With and Without Plasma

Plasma free microparticles were mixed with plasma rich microparticles, 1 µl of each and then mixed them separately with 2 µl of ristocetin and 7 µl of PBS. This was incubated for 15 to 30 minutes in order to mediate the agglutination reaction. An aliquot

was taken from the mixtures and observed under the microscope. Observation of the whole blood and blood free agglutination/aggregation assays were done using 1 µl collected from the assay which was examined under a cover slip using a Nikon E995 CoolPix digital camera.

Plasma Free Microparticle Agglutination with UL-VWF and the Effect of ADAMTS-13

One microliter of plasma free microparticles were mixed with 0.1 µl of 100 µg/ml ULVWF (a gift from Jing-Fei Dong, Baylor College of Medicine) and incubated for 5 min. In another reaction we added 0.1 µl 50 µg/ml ADAMTS-13 to a reaction mixture similar to the one described above.

After incubation an aliquot from these two reactions with and without ADAMTS-13 were taken and placed under a cover slip and observed under the microscope and images were taken using the Nikon CoolPix digital camera E995.

Effect of Stimate on Microparticles

Zebrafish larvae were injected with DiI and treated with Stimate as described in the previous chapter and subsequently images were taken as described above on samples with and without Stimate treatment.

In vivo Microparticle Agglutination During Injury

For examination of microparticles, adult fish were injected with DiI and a laser was used to induce an injury in the tail arteriole and the adherence of microparticles

was visualized using the Nikon Optiphot microscope or Nikon 80i eclipse microscope equipped with NIS Elements AR 2.30 software was used.

Results

Ristocetin Mediated Whole Blood Aggregation

Because TMPs are derived from thrombocytes, I expect them to retain the GPIbα receptor necessary for interaction of thrombocytes with VWF as well as other receptors such as GPIIb/IIIa, which have already been shown to be present. If TMPs do indeed retain this receptor then VWF mediated agglutination should occur when ristocetin is added to TMPs in the presence of VWF. To test whether microparticles are involved in ristocetin mediated agglutination, blood from the CD41-GFP transgenic zebrafish and induced agglutination with ristocetin. Results showed that microparticles were dispersed between thrombocytes along with other cells and microparticles that were of non-thrombocyte origin (Figure 5.1). These results suggested that ristocetin mediated agglutination may involve not only TMPs but non TMPs as well.

Agglutination of Total Microparticles

To test whether total microparticles agglutinate in the presence of ristocetin, total microparticles were prepared in plasma and tested whether they form aggregates/agglutinates in the presence of ristocetin. The microparticles did indeed agglutinate (Figure 5.2). Total microparticles were also labeled with FITC so that even smaller particles could be visualized. In order to test whether the agglutination is plasma/VWF dependent used plasma free and plasma rich microparticles were used in

agglutination reactions. The microparticles did not aggregate in the absence of plasma (Figure 5.3). These results suggested that the agglutination is VWF dependent.

Agglutination of Total Microparticles by UL-VWF

Because UL-VWF is more reactive than constitutively released VWF, I was curious as to whether or not UL-VWF in the presence of ADAMTS-13 could agglutinate microparticles; therefore, we tested whether UL-VWF will induce microparticle agglutination. To determine whether agglutination is abolished in the presence of ADAMTS-13 plasma free microparticles were incubated with UL-VWF in the presence and absence of ADAMTS-13. I found that UL-VWF induced agglutination in the absence of ristocetin, and in presence of ADAMTS-13 such agglutination was not observed (not shown). The agglutination in these experiments appeared just like the results in Figure 5.3; with the UL-VWF resembling the left panel, forming an agglutinate and the ADAMTS-13 treated sample resembling the right panel, absence of agglutinate formation.

Because Stimate treatment induces the release of UL-VWF which is more reactive, there should be a decrease in TTA, similar to what was seen with thrombocytes in the previous chapter. The decrease in TTA indicates that UL-VWF is indeed stored. UL-VWF is more reactive due to the increased amount of multimerization compared to VWF found in normal circulation. Due to the increase multimerization there is also an increase in the amount of GPIbα binding sites available for interaction with thrombocytes. A decrease in TTA should also occur when exogenous UL-VWF is added to the TTA. Due to our observations it appears that UL-

VWF plays a role in microparticle agglutination. To further examine the effect of Stimate on such agglutination *in vivo* observations were performed after Stimate treatment.

Effect on Stimate on Microparticle Agglutination

To test whether microparticles agglutinate *in vivo,* larvae were either injected with DiI alone or co-injected with Stimate and DiI, for visualization of microparticle formation. Clusters of microparticles formed in the vein; however, no such clusters were found in the artery (Figure 5.4).

In vivo Microparticle Agglutination During Injury

To test whether microparticle agglutination occurs during injury, adult fish were injected with DiI and injury was induced by laser in the tail arterial and agglutination was recorded in real time (Figure 5.5). The images showed even before the thrombocytes coming to the injury site, a string of microparticles were recruited. These results suggest that most likely thrombocyte microparticles do play a role in initiation of thrombus formation.

Discussion

Our results with the first agglutination assay, using whole blood from CD41-GFP zebrafish in the presence of ristocetin showed agglutination of thrombocytes and their microparticles as well as other non-thrombocyte microparticles, suggesting that microparticles do indeed play a role in agglutination. These results also suggested that for agglutination thrombocyte microparticles are not exclusively needed. Indeed this

result prompted us to explore whether microparticles themselves have the ability to agglutinate in the presence of VWF. These results for the first time document that thrombocyte microparticles alone were capable of forming an agglutinate in the absence of other cells but only in the presence of plasma. Since this is ristocetin dependent agglutination the inferance can be that it is VWF dependent. We could also deduce that the interaction occurring in this agglutination is between VWF and the GPIbα receptor, which is likely retained during microparticle formation from thrombocytes. However, this experiment could not unequivocally demonstrate that VWF is involved in the agglutination. Through these experiments, I was able to confirm that microparticles were interacting with VWF by using human UL-VWF in a separate agglutination assay in the absence of ristocetin. Because agglutination occurred in the presence of UL-VWF and in absence of plasma, this provided evidence that agglutination of microparticles is a VWF dependent event.

This experiment was also able to confirm the interaction of VWF with microparticles by demonstrating that agglutination does not occur in the presence of ADAMTS-13 even though UL-VWF is present. ADAMTS-13 in humans is a disintegrin metalloproteinase involved in cleaving UL-VWF in the A2 domain so that the more reactive form is not released into circulation causing the probability of spontaneously interacting with platelets [21]. A defect in ADAMTS-13 in humans results in retention of UL-VWF in plasma and UL-VWF is not cleaved and thereby can lead to thrombotic thrombocytopenic purpura, characterized by bleeding due to sequestration of platelets in thrombi; on the other end of the spectrum a bleeding disorder can occur if there is an increased proteolysis of UL-VWF by ADAMTS-13, which is seen in vWD 2A [3; 36]

There was in fact a decrease in agglutinate formation when ADAMTS-13 was added to the solution containing only microparticles and UL-VWF indicating that the proteolytic effect of ADAMTS-13 on UL-VWF has an impact on MP interaction with VWF.

According to previous experiments, it appears that UL-VWF may be stored and released when zebrafish are treated with Stimate, as seen with the decrease in TTA in the previous chapter. This also indicates that microparticles are likely to play a role in thrombus formation *in vivo*. As previously mentioned, an overactive ADAMTS-13 can result in a form of vWD where UL-VWF is over processed reducing the multimers in circulation thus reducing the ability to form a proper clot. Here again, MPs may play an important role.

This interaction between microparticles and VWF was further examined when by an *in vivo* arterial thrombosis experiment in adult zebrafish. In this *in vivo* experiment the TMPs seem to attach to the site of injury before thrombocytes within the artery. It is not clear why TMPs might attach before thrombocytes; this could perhaps be due to the smaller size of TMPs. The smaller size might permit them to interact and stick more readily to VWF or UL-VWF that is already attached and extended at the sit of injury exposing the GPIbα binding site. Having made these observations demonstration of microparticle adhering to the endothelium where UL-VWF is released was required. Therefore, our demonstration that spontaneous sticking of MPs to the vessel wall could occur in zebrafish when larvae were treated with Stimate providing evidence for possible VWF mediated agglutination. Stimate treatment also resulted in spontaneous TMP clustering in the vein while no clusters were seen in the artery. This may be due to the lower rate of blood flow in veins as opposed to arteries. While this sounds counter

to what was previously mentioned regarding VWF importance at high blood flow; in this observation no injury was induced therefore VWF did not anchor and extend exposing binding sites. Another contributing factor to spontaneous accumulation of MPs in the vein versus the artery during Stimate treatment could be due to the observation by Cho *et. al* that in human veins appear to have more VWF than arteries [149].

Conclusions

This set of experiments showed that TMPs accumulate and agglutinate in the presence of ristocetin using whole blood suggesting that they partly behave like zebrafish thrombocytes from which they are derived, with respect to ristocetin mediated agglutination. They also deduced that TMPs retain the GPIbα binding receptor that interacts with VWF by using the ristocetin induced agglutination assay, and showed that TMPs seem to be more reactive than thrombocytes. In support of this interaction we showed that TMPs initiate the clotting process at the site of injury. It was also interesting to see that not only TMPs but non-TMPs can interact with human UL-VWF, and that the interaction can be negated by treating the UL-VWF with ADAMTS-13. These studies also emphasize the utility of human factors in examining defects in TMPs and thrombocytes in zebrafish.

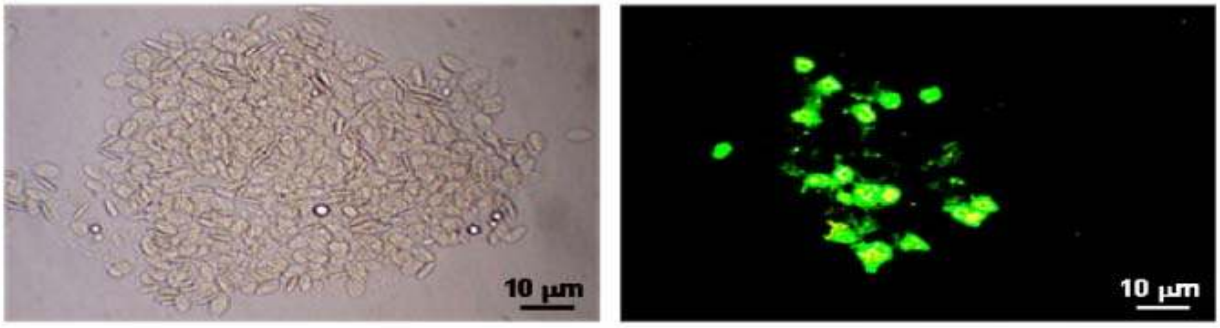

Figure 5.1: Ristocetin-mediated agglutination of whole blood and TMPs. Left panel is brightfield of agglutinate/aggregate under microscope on microscope slide. Right panel is CD41-GFP labeled thrombocytes and TMPs.

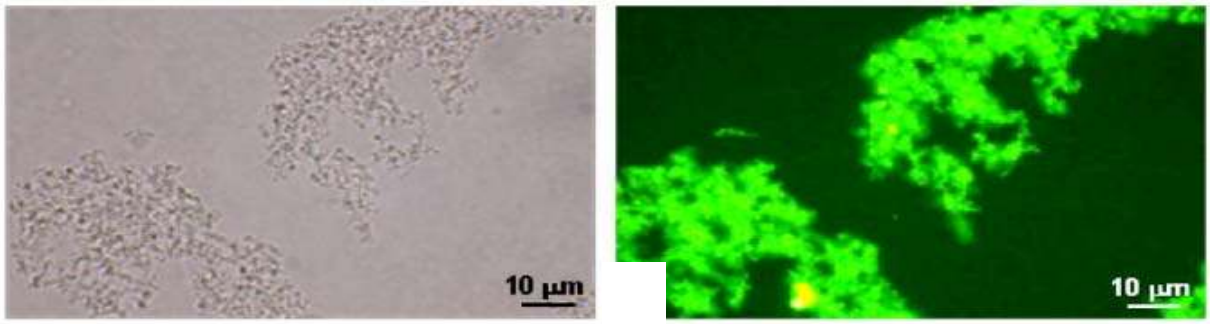

Figure 5.2: Ristocetin mediated agglutination of total MP. Left panel shows brightfield of aggregate/agglutinate. Right panel shows aggregate/agglutinate under fluorescence.

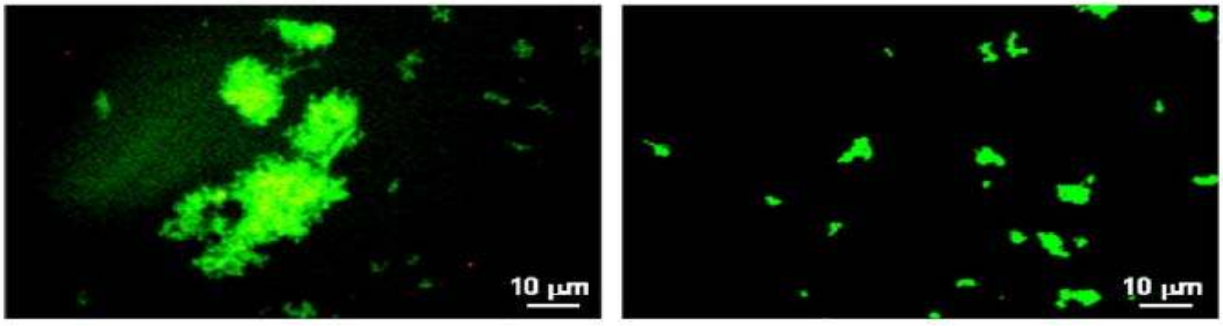

Figure 5.3: Total MPs with or without plasma. In the presence of ristocetin. Left panel shows the effect of plasma on the ability of total MPs to form agglutinates/aggregates. Right panel is in the absence of plasma. TMPs were collected from FITC labeled whole blood.

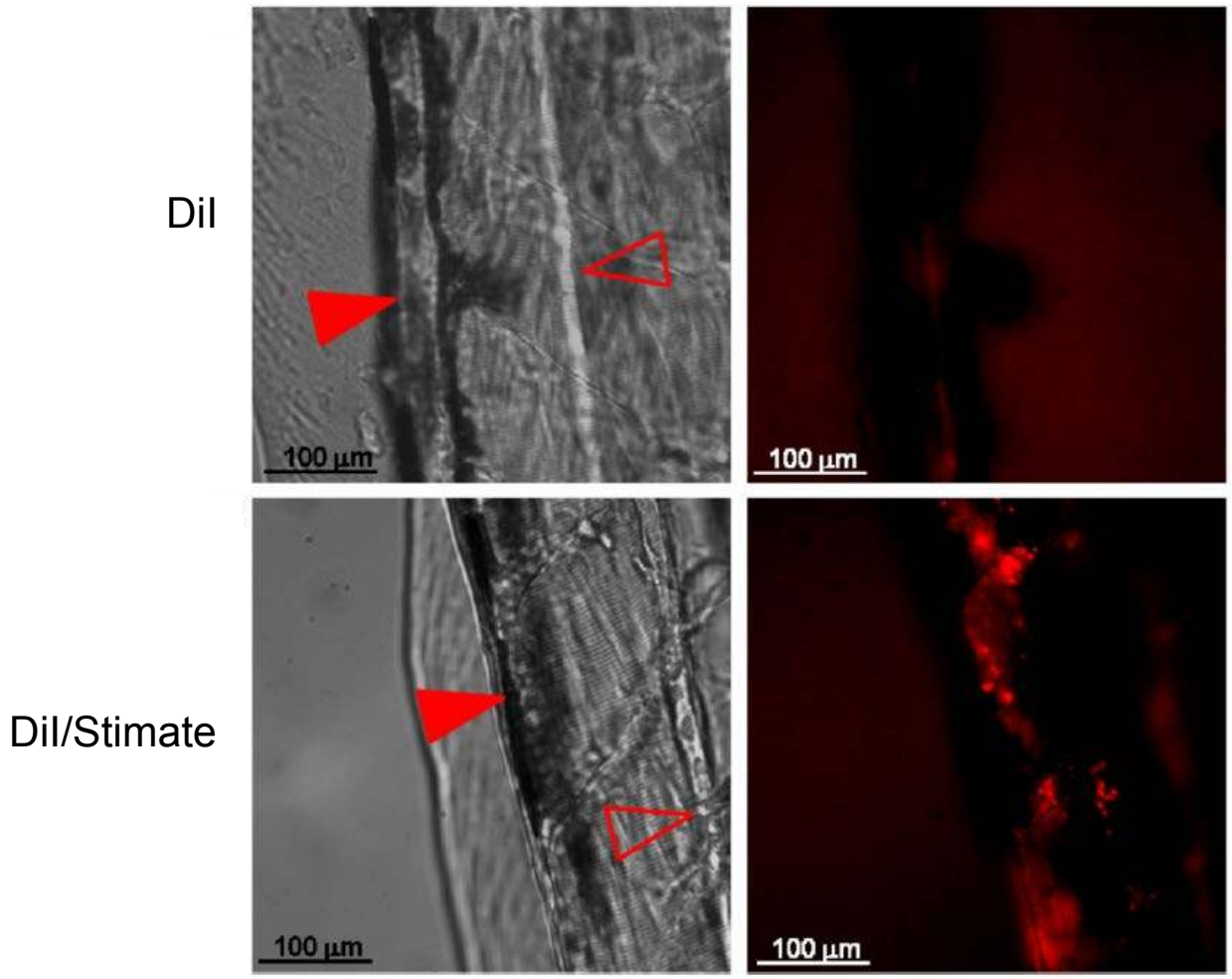

Figure 5.4: Effect of Stimate treatment on TMPs *in vivo*. Left panels both show brightfield images of vessels and right panel shows the fluorescent images of the vessels. The top panel is of larvae injected with DiI only. Bottom panel shows larvae injected with DiI and Stimate together. Arrows point to the vessels, solid arrows point to the vein, and open arrows points to the artery.

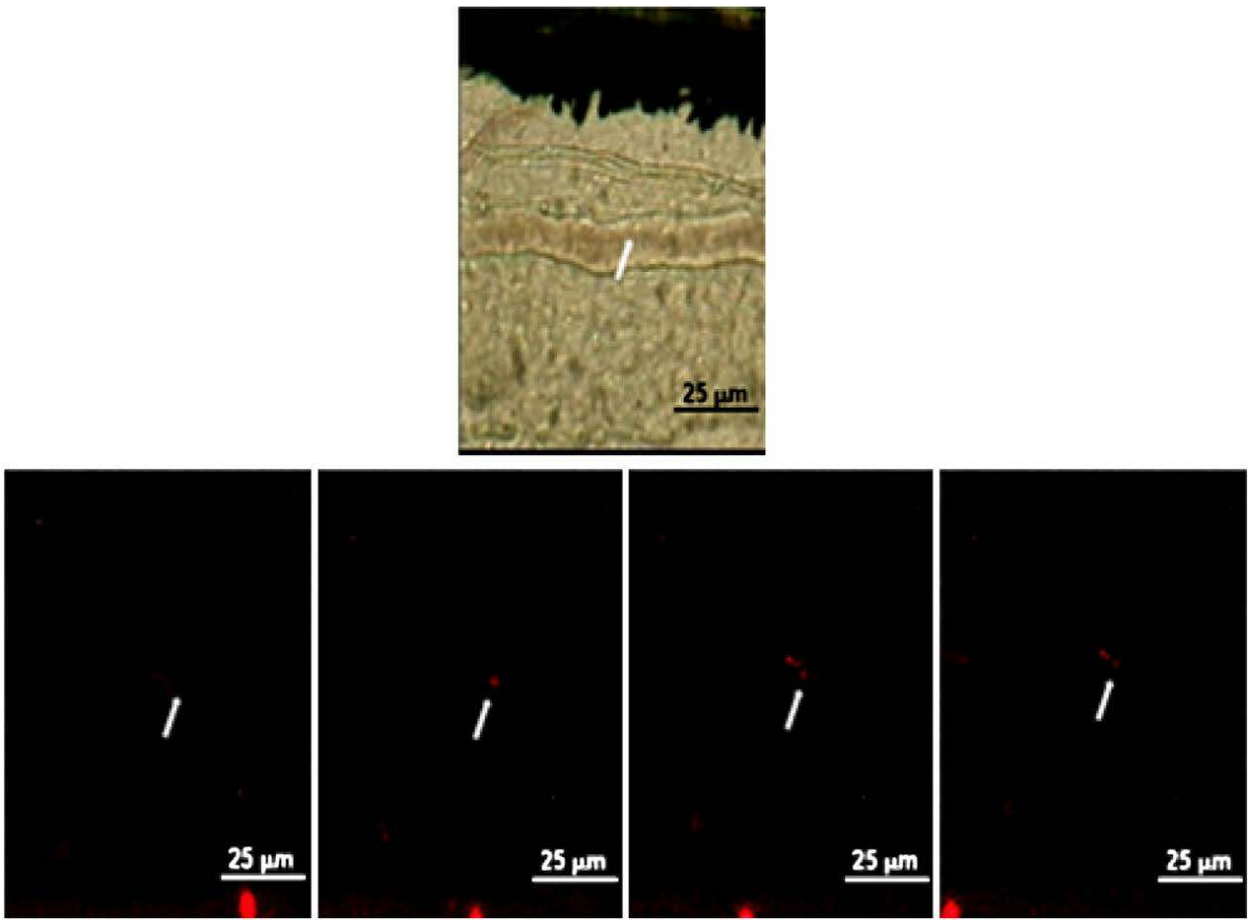

Figure 5.5: Thrombocyte microparticle accumulation at the site of laser induced arterial injury. Top panel shows a brightfield image of the tail artery in an adult zebrafish; the black are of the panel is melanin pigment. The lower images are time lapse images in sequential order from left to right of thrombocyte microparticle accumulation at the site of injury, indicated by the arrow. The images were taken one every second. In the lower portion of the pictures a thrombocyte can be seen flowing a in a different vessel. The thrombocyte is significantly larger than the thrombocyte microparticles at is approximately 5 microns.

CHAPTER 6

GENERAL CONCLUSIONS AND PERSPECTIVES

Discussion

The research herein has provided information regarding VWF in zebrafish, which was not previously known. Using bioinformatics the VWF gene was located and assigned to chromosome 18, which had not been annotated at the time the research project was initiated. Not only did this work show using bioinformatic tools that the gene is in fact present, and that synteny is also conserved, i also amplified the VWF sequence homologous to exon 28 in human VWF. In addition to this, bioinformatics also showed that there is some homology in the gene sequence for exon 28 as well as homology in the protein sequence. This is important because this is the site for VWF interaction with GPIbα found on thrombocytes and platelets. The fact this region is conserved providing some initial evidence that VWF function in hemostasis is conserved. In addition to this conserved sequence we also found that the zebrafish GPIbβ amino acid sequence shows homology and has conserved an important Cysteine122, involved in the interaction of GPIbβ with the GPIbα subunit. In addition to this I also amplified a region of zebrafish VWF showing that not only is the gene present, but mRNA is also being produced indicating that the gene is transcriptionally active.

During this work the zebrafish VWF sequence was annotated on Ensembl. However, I found that there was a discrepancy between the two sequences in the region encoding GPIbα binding, which is encoded by exon 28 in human VWF. The intron proposed by Ensembl was not present; however, this finding provided evidence

that any annotations in a database should be carefully examined and all possibilities for discrepancies in a sequence should be taken into consideration. This variance in sequences could be due to an error in the annotation or the use of a different strain of zebrafish. The differences in sequences led us to examine the GPIbα binding region in other fish for the presence of introns. The importance of the GPIbα binding region in hemostasis is evident even though it is split into two and sometimes three exons in other fish; the region is conserved indicating it may be essential for thrombus formation. All of the information collected using the bioinformatic tools provided evidence that VWF function in hemostasis is likely conserved in zebrafish.

These experiments for the first time were able to show that VWF is synthesized in thrombocytes and that VWF mRNA is also being translated to protein. This indicates that VWF may be stored and released upon activation of thrombocytes similar to human platelets.

In addition to the above VWF was also found in the endothelium of arteries and veins. The presence of VWF in the endothelium of veins and arteries resembles human VWF expression in the vascular endothelium. The location of VWF in the vasculature leads to the notion it may be synthesized there; however, it has yet to be seen whether or not zebrafish VWF is secreted constitutively as well as stored.

To confirm that VWF functions in hemostasis knockdown technology was employed. Using knockdown technology a vWD like phenotype was developed thus confirming that zebrafish can be utilized as a model for studies of vWD. Since, in this model there will be reduced amounts of VWF in circulation; this type of model is similar to human type 1 vWD, where there is a reduction in VWF. In addition to this the use of

VMO injection into adults provided basis for biochemical studies which would be difficult with the larvae.

I found that MPs seem to have conserved the GPIbα receptor and can interact with human UL-VWF, in the absence of ristocetin. Interestingly in mouse platelet studies GPIbα does not interact with human VWF [150]. Thus, in this context the zebrafish model appears to be advantageous over the mouse model. Furthermore, this interaction can occur in the absence of ristocetin suggesting that UL-VWF is indeed more reactive than VWF in plasma. In addition to this after Stimate treatment microparticles began to agglutinate in the absence of an induced injury. This is consistent with the thought that an UL-VWF form is stored within storage granules of zebrafish endothelial cells and may be responsible for the agglutination of MPs *in vivo*. Evidence was also provided that TMPs form a long stringy agglutinate *in vivo* at the site of injury in adult arteriole. This again favors the model that UL-VWF may be responsible for such extended stringy TMPs. Our work has shown that TMPs are important in initiation of thrombus formation and this has not been previously shown *in vivo* even in the platelet field.

Fish thrombocytes express a GPIbα like receptor that could interact with human VWF as demonstrated in our system. Therefore this model system can be utilized to rapidly screen for bleeding phenotypes caused by VWF or GPIbα mutations. The system can also be used to select compounds to regulate GPIbα-VWF interactions, especially under prothrombotic conditions.

Conclusion

All of the experiments performed, collectively provide evidence that VWF is present in zebrafish and its function in thrombus formation and possibly in its interaction with FVIII has been conserved. It is because of the conserved function of VWF that now I can say there is now a vWD/VWF model for the research community within a powerful animal model for the investigation of the disease and protein. The continued study of vWD is of great importance due to the prevalence of the disease affecting up to 1% of the population making it one of the most common bleeding disorders [38].

Zebrafish will prove to be a powerful model for the investigation of VWF, vWD and microparticle function. The ease with which zebrafish can be bred, manipulated and screened make this organism a valuable tool in the field of hemostasis. While a significant amount of information was provided in this complete set of experiments, there is much more that needs to be learned about VWF, and microparticles in humans and zebrafish.

Future Directions

Here it has been established that zebrafish can be a powerful model for the study of vWD; however, there is still work that can be done to further characterize zebrafish VWF as well as other proteins that interact with VWF such as ADAMTS-13 and P-selectin. Some future directions of study include:

1. Determining whether or not zebrafish VWF does form multimers.
2. Identification of zebrafish ADAMTS-13.
3. Studying VWF proteolysis by the zinc metalloprotease ADAMTS-13 and pathological triggers for thrombotic thrombocytopenia purpura.

4. Development of a type 2 vWD model using ENU mutagenesis.
5. Possibly creating a transgenic VWF-GFP fish to observe movement of the protein *in vivo*.
6. Demonstrating whether UL-VWF is responsible for microparticle agglutination *in vivo*.
7. Using the zebrafish model to identify modifier genes.

While there is still information to be learned about zebrafish VWF, it is clear that this model will continue to provide researchers with a powerful tool for determining mechanisms of vWD and microparticle related disorders. These experiments are paving the way for future research in field of hemostasis.

*The results of this research resulted in two publications: M. Carrillo, S. Kim, S.K. Rajpurohit, V. Kulkarni, and P. Jagadeeswaran, Zebrafish von Willebrand factor. Blood Cells Mol Dis 45 326-33

S. Kim, M. Carrillo, U.P. Radhakrishnan, and P. Jagadeeswaran, Role of zebrafish thrombocyte and non-thrombocyte microparticles in hemostasis. Blood Cells Mol Dis 48 188-96.

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