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Zebrafish von Willebrand factor

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ABSTRACT

von Willebrand factor (vWF) is a large protein involved in primary hemostasis. A dysfunction in this protein or an insufficient production of the protein leads to improper platelet adhesion/aggregation, resulting in a bleeding phenotype known as von Willebrand disease (vWD). To gain a better understanding of vWF interactions *in vivo*, the use of zebrafish as a model is ideal because of the transparency of the embryos and larvae. In this article, we examined the presence and function of vWF in hemostasis of zebrafish utilizing a variety of molecular methods. Using RT-PCR and antibody staining, we have shown that vWF mRNA is present in thrombocytes. Through antibody staining, we demonstrated vWF is synthesized in blood vessels. The role of zebrafish vWF in hemostasis was established through knockdown methods using vWF morpholino (vWF MO) antisense oligonucleotides. Embryos injected with vWF MO at the one to four cell stages resulted in a bleeding phenotype. Injection of embryos with vWF MO also caused an increase in time to occlusion within arteries in larvae upon laser induced injury. We then used vWF-specific Vivo-morpholinos (VMO) to induce vWF knockdown in adult zebrafish by targeting the exon homologous to the human exon 28 of the vWF gene. The reduced ristocetin-mediated agglutination of thrombocytes in a plate tilting assay, using blood from adult zebrafish injected with VMO, provided evidence that vWF is involved in the hemostatic process. We also administered desmopressin acetate to larvae and adults which resulted in enhanced aggregation/agglutination of thrombocytes. Zebrafish genome database analysis revealed the presence of GPIbβ gene. It also revealed the exon of zebrafish vWF gene corresponding to exon 28 of human vWF gene is highly similar to the exon 28 of human vWF gene, except that it has an insertion that leads to a translated peptide sequence that separates the two A domains coded by this exon. This exon is also conserved in other fishes. In summary, we established that zebrafish vWF has a role similar to that of vWF found in humans, thus, making zebrafish a useful model for studying the cell biology of vWF *in vivo*.

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Introduction

von Willebrand factor (vWF) plays an integral role in primary hemostasis. In humans, von Willebrand factor is a large protein of 2813 amino acids encoded by the vWF gene, which is composed of 52 exons and is located on chromosome 12 [1]. The 2813 amino acid sequence includes a signal peptide of 22 residues, a propeptide of 741 residues, and a mature subunit of 2050 amino acids [1]. Synthesis of vWF occurs in endothelial cells as well as in megakaryocytes; vWF is stored in the Weibel-Palade bodies of endothelial cells and the α-granules of platelets, which are derived from megakaryocytes [2]. The role of vWF is to stabilize factor VIII, to act as an adhesive by binding to the subendothelium at the site of injury, and to promote platelet adhesion/aggregation via binding to the platelet receptor GpIbα [2]. The ability of vWF to form a stable hemostatic plug is imperative in arteries, due to the rates of blood flow within the vessels [3]. Evidence of the importance of vWF is apparent in individuals suffering from von Willebrand disease (vWD). vWD manifests as a qualitative or quantitative dysfunction, and is characterized by mucocutaneous bleeding and prolonged clotting time. The majority of the mutations in the vWF gene causing vWD is clustered within two A domains encoded by exon 28.

The study of vWD is important due to the fact that 1% of the population is afflicted with the disease [4]. To effectively study vWF and vWD, the employment of animal models is essential. Currently the models in use include murine, canine, rabbit and porcine, all of which have provided valuable information about vWF and vWD [5,6]. However, these models all present a challenge in that it is difficult to image vessel occlusion *in vivo* or to induce injury without being highly invasive. Additionally, observation of developing offspring is difficult due to the fact that all of these models develop in utero. Currently, another animal model, the zebrafish *Danio rerio*, is being used for studying hemostasis, but is not yet employed as a tool to study vWF and vWD [7,8]. Zebrafish have previously been shown to retain many of the clotting factors involved in both the intrinsic and extrinsic pathways found in humans as well as platelet-specific factors [9]. Evidence has also been presented for the presence of vWF and its

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conserved interaction with GPIbα receptor, found on human platelets [10]. Previously, by means of immunostaining, GPIbα was shown to be present on zebrafish thrombocytes, which are involved in forming vascular occlusion upon injury, similarly to human platelets [11]; this further solidifies that zebrafish make an appropriate model for the investigation of vWF and vWD.

In addition to retaining proteins and pathways involved in the clotting process found in humans, zebrafish also provide the advantage of transparent eggs, embryos, and larvae throughout development. This transparency enables investigators to observe development as well as formation of vasculature [10]. The convenience of this model being transparent throughout development, coupled with a variety of genetic and screening tools, provides rapid investigation of dysfunctional proteins involved in the clotting process, disease, and development [11,12]. In this paper, we will provide evidence that vWF function is conserved and aids in the clotting process in zebrafish, just as in humans; and therefore, zebrafish should make a useful model for the study of cell biology of vWF function *in vivo*.

Materials and methods

Zebrafish aquaculture

The following methods of zebrafish aquaculture were conducted similarly to those previously described [13]. Briefly, adult zebrafish, larvae, and embryos were kept at 28 °C in deionized water, supplemented with instant ocean, in a circulating water system. Embryos were collected as previously described.

RT-PCR using Zebrafish thrombocytes and Whole larvae and PCR using Zebrafish genomic DNA

Thrombocytes were collected from adult zebrafish blood by individually suctioning thrombocytes under the microscope using a microinjection needle. 500 thrombocytes were used for isolating RNA using Absolutely RNA miniprep kit (Stratagene, Inc.; Santa Clara, CA). Total RNA from whole larvae was prepared using the above kit, then used for RT-PCR amplification of vWF mRNA with the following primers: forward primers: 5′-TGAGTGGAGATATAACACCTGTGC-3′ (F1), 5′-CAGTAACTGGTTTAACCTCCACACT-3′ (F2), 5′-CTGTTGACGG-CAAGTGCTAA-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′-GTTTTCA-CAAATGTTTTCAAGTCCT-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.

Immunostaining of whole larvae

Whole larvae were fixed in 4% paraformaldehyde for 6 h at 4 °C, then washed with 0.1 M phosphate buffer (pH of 7.3) for 5 min. The larvae were then washed in distilled water for 5 min, incubated at −20 °C for 7 min in acetone, and washed in distilled water for 5 min followed by a 5 min 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 h. After blocking, the larvae were incubated overnight at 4 °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 h with a change to fresh solution every 30 min. For visualization, the larvae were incubated for 4 h at 20 °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).

Immunostaining of thrombocytes

A blood smear was made using whole blood from adult zebrafish and allowed to dry for 10 min. The slide was immersed in 70% cold ethanol for 10 min. Then, the slides were rinsed three times in phosphate buffered saline (PBS) and incubated in vWF-Ab 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 h. After incubation, the slides were rinsed as described earlier and then incubated with FITC conjugated anti-rabbit IgG (Jackson Immuno Research; West Grove, PA) which was diluted 20 times in 1×PBS for 1 h. Once the second incubation was complete the slides were rinsed with 1×PBS three times; then, the slides were subjected to one final rinse in double distilled water.

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 one to four cell stages using the picospritzer III (Parker Precision Fluidics; Hollis, NH) [14]. A standard control MO 5′-CCTCTTACCT-CAGTTACAATTTATA-3′ was also injected into the embryos at the one to four cell stages. Adult zebrafish were injected intravenously with either 5 μl of 0.5 mM vWF Vivo-morpholino (VMO), or control VMO designed with the same sequence as the vWF MO, or control MO, respectively (Gene Tools; Philomath, OR) as previously described [15].

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 recorded using a Nikon Optiphot microscope as described previously [12].

Injection of vivo-morpholino and agglutination assay

Blood was collected from injected fish and used in the ristocetin-mediated thrombocyte agglutination assay. The assay was performed as previously described [9]. In order to induce the secretion of vWF, we used 1.5 mg/ml Stimate (Desmopressin acetate) nasal spray (gift from Shelly Crary, UT Southwestern Medical School) by first spraying it into an Eppendorf centrifuge tube and then diluting the Stimate five fold with distilled water. After the dilution, 5 μl of the diluted Stimate was placed on the gills and the fish were returned to water for 30 min, after which time blood was collected and the above agglutination assay was performed.

Effect of morpholino on time to occlusion (TTO) and Stimate effect on TTO

TTO was measured in seconds on 5–6 day post fertilization (dpf) larvae generated after injection with the MO at the one to four cell stages by induction of an injury to the artery, using a nitrogen laser light pumped through coumarin 440 dye using a MicroPoint Laser

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