

# Analysis of Blood Coagulation in the Zebrafish

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**Abstract:** The zebrafish (*Danio rerio*) is a unique animal model in which saturation mutagenesis has been used to identify genes involved in vertebrate development. The relevance of the zebrafish as a genetic model for hemostasis depends, in large part, on the degree of similarity between the zebrafish and mammalian systems. The diminutive size of the zebrafish poses technical problems for analysis of coagulation. This study describes methods to obtain citrated whole blood and plasma from the zebrafish, analyze *in vitro* coagulation in small plasma volumes, obtain uniform dosing of zebrafish with oral anticoagulants, and demonstrate specific factor activities via chromogenic assays. Analysis of the zebrafish system demonstrates the presence of both the intrinsic and extrinsic pathways of coagulation, evidence for prothrombin, factor X, protein C, antithrombin, and heparin cofactor II activity, and a requirement for vitamin K dependent  $\gamma$ -carboxylation of zebrafish hemostatic proteins. Induction of a morphologically recognizable bleeding phenotype by warfarin treatment is also demonstrated. Characterization of zebrafish coagulation provides evidence that major hemostatic pathways are conserved between zebrafish and man. These similarities indicate that the zebrafish is a relevant genetic model for identification of novel genes involved in hemostasis and thrombosis.

**Keywords:** zebrafish, blood coagulation, animal-model, hemostasis, anticoagulation

## INTRODUCTION

Hemostasis is a vertebrate function of paramount importance for the response to vascular injury and the pathophysiology of human thrombotic disorders. Despite extensive biochemical and molecular characterization of plasma coagulation factors, significant questions remain regarding initiation and regulation of the coagulation response *in vivo*. The biochemical events leading to thrombosis are the same as those that occur during normal hemostasis, suggesting that dysregulation of this process is critical for development of disease. Progress in understanding the multigenic etiology of thrombosis is limited, in large part, by the ability to identify candidate genes (1-3). Thus, a genetic model for mammalian hemostasis and thrombosis is highly desirable.

The zebrafish is a unique and important animal model in which the power of saturation mutagenesis is applied to the study of vertebrate development (4). This approach allows the identification of genes in an “unbiased” manner,

as opposed to the gene by gene approach employed by knockout studies in the mouse (5). Theoretically, saturation mutagenesis can identify most of the genes that contribute significantly to a given pathway, which may result in identification of novel factors or previously unrecognized functions for known genes. Mutagenesis of the zebrafish genome has yielded important insights into genes involved in cardiac, vascular, and erythrocyte development, including models of disease such as congenital sideroblastic anemia and hepatoerythropoietic porphyria (4-7). Given the development of appropriate screening assays, the power of the zebrafish model can be harnessed for the study of other vertebrate functions such as hemostasis (8-10). For example, zebrafish larvae with defects in hemostasis (induced by copper chloride) can be identified by measuring the bleeding time following puncture of the caudal blood vessel (8). Thus, the zebrafish represents a novel and potentially powerful genetic model for vertebrate hemostasis.

The relevance of the zebrafish as a genetic model for hemostasis depends, in large part, on

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the degree of similarity between the zebrafish and mammalian systems. A number of important elements of vertebrate hematology first appear during the evolution of fish, including evidence for a similar coagulation system. Molecular evidence for fibrinogen and prothrombin, and biochemical evidence for factor X and protein C-like activities, have been demonstrated in various fish species (11-14). A marked increase in genomic complexity during the evolution of fish is suggested by the presence of multiple genome duplications, variation in ploidy, and existence of over 20,000 species (15). Given this remarkable diversity, specific characterization of the zebrafish system is critical to the development of a hemostatic model. Additionally, the diminutive size of the zebrafish poses technical problems for the study of its hemostatic system. To address these issues, development of appropriate methodology to characterize both the coagulation and thrombocyte segments of the zebrafish hemostatic system has been undertaken (16).

This study describes development of the required methodology to obtain citrated whole blood and plasma from the zebrafish, and using a combination of modified clotting and chromogenic assays, demonstrates the presence of both the intrinsic and extrinsic pathways of coagulation, and the natural anticoagulants protein C, antithrombin and heparin cofactor II. Additionally, the results demonstrate the ability to achieve uniform dosing with oral anticoagulants dissolved in the tank water, the effect of warfarin treatment on zebrafish coagulation, and a morphologically recognizable bleeding phenotype. Characterization of the zebrafish system provides evidence that the major coagulant and anticoagulant pathways are conserved between zebrafish and man. These similarities suggest that the zebrafish represents a relevant genetic model for identification of novel genes involved in hemostasis and thrombosis.

## MATERIALS AND METHODS

### *Materials*

Pooled normal human plasma (Citrol-I), rabbit brain thromboplastin (Dade Thromboplastin C Plus), and rabbit brain partial thromboplastin reagent containing ellagic acid (Dade Actin) were obtained from Baxter Diagnostics, Inc. (Deerfield, IL). Human prothrombin and factor Xa, were purchased from Enzyme Research (South Bend, IN). The purified factor X activator from Russell viper venom (RVV-Xa), Protac (purified protein C activator from *Agkistrodon contortrix* venom), and corn trypsin inhibitor were purchased from Hematologic Technologies (Essex Junction, VT). Human thrombin was purified from prothrombin as previously described (17). The chromogenic substrates for factor Xa (S-2765, Z-D-Arg-Gly-Arg-pNA) S-2337, thrombin (S-2238, H-D-Phe-pipecolyl-Arg-pNA), activated protein C (S-2366 pyro-Glu-Pro-Arg-pNA), and the CoaTest Factor X kit (including the substrate S-2337, Bz-Ile-Glu(piperidyl)-Gly-Arg-pNA), were purchased from DiaPharma (Franklin, OH). Recombinant hirudin, unfractionated porcine heparin, bovine mucosa dermatan sulfate, and sodium warfarin were purchased from Sigma Chemical (St. Louis, MO). Dermatan sulfate was treated with nitrous acid to remove contaminating heparin (18). Hypaque sodium radiographic dye was from Nycomed Inc. (New York, NY). All other chemicals were at least reagent grade and purchased from major suppliers.

### *Zebrafish Aquaculture*

Zebrafish were purchased from Scientific Hatcheries (Huntington Beach CA) and grown as previously described (8). Briefly, zebrafish were kept in 20 gallon tanks with deionized water containing 60 mg of Instant Ocean (Lone Star, San Antonio, TX) per liter, which is constantly recirculated through biological filters and treated

with UV light. Young adult fish (approximately 3 cm in length) were used for these studies.

### *Collection of Zebrafish Blood*

Blood was harvested from adult zebrafish by making an incision in the region of the dorsal aorta and inferior vena cava, just posterior to the dorsal fin (avoiding the gastrointestinal tract) (16). Zebrafish skin was punctured by a small dissecting scissors in a partially open position, with closure of the blades resulting in a superior lateral incision (~ 0.3-0.5 cm). Blood welling up from this incision was rapidly collected by micropipet tip (~ 1-5  $\mu$ l per fish) into citrate buffer. Blood from multiple fish was collected directly into an eppendorf tube containing 10  $\mu$ L of citrate buffer (150 mM NaCl, 3.8% sodium citrate, 20 mM HEPES, pH 7.4, and 0.1% PEG-8000), and brought to a final volume of approximately 70  $\mu$ L (~20 adult fish per tube). Pooled, citrated plasma for the modified clotting assays was obtained by centrifugation of the blood at 1000 g for 3 min.

### *Preparation of Zebrafish Thromboplastin*

A species specific tissue thromboplastin reagent was prepared from zebrafish muscle. Approximately 40 zebrafish bodies were filleted from the gill region posteriorly with removal of muscle mass from the head, internal organs, scales and fins. The muscle tissue was suspended in 0.1 M NaCl, 50 mM Tris, pH 7.5 on ice and homogenized with a Brinkman polytron rotor (setting = 5) for 10-15 sec times three, avoiding heating or foaming. This homogenate was stirred at 37 C for 30 min, and then centrifuged at 11,000 g for 15 min. The supernatant was harvested, EDTA added to 20 mM, and centrifuged at 48,000 g for 60 min. The resulting pellet was resuspended in 0.1 M NaCl, 50 mM Tris, pH 7.5, and 20 mM EDTA, and the high speed centrifugation repeated. Following the second wash step, the pellet was resuspended in the same buffer without EDTA, and centrifuged again at

48,000 g for 60 min. The final pellet was resuspended in a minimal volume of buffer without EDTA (1.5-3 ml), aliquoted, and stored at -25 C for use in the modified prothrombin time.

### *Modified Prothrombin, Activated Partial Thromboplastin, and Russell Viper Venom Coagulation Times*

Modified coagulation assays were performed as follows: 10  $\mu$ l citrated zebrafish plasma was diluted 1:2 in phosphate buffered saline without calcium (PBS), followed by addition of 20  $\mu$ l of a specific clotting reagent at 37 C. This reaction was recalcified with 10  $\mu$ l of 78 mM CaCl<sub>2</sub>, the entire volume (50  $\mu$ l) immediately added to a polystyrene cuvette containing 200  $\mu$ l of clotting buffer (150 mM NaCl, 10 mM imidazole-HCl, pH 7.4 and 6.6% PEG-8000) and 2 mg/ml human fibrinogen (final concentration), and the clotting time determined by fibrometer (BBL fibrosystem). The modified prothrombin time (PT) was performed using either rabbit brain thromboplastin (Dade Thromboplastin C Plus) or zebrafish thromboplastin (see above) as the clotting reagent. The modified activated partial thromboplastin time (APTT) was performed by incubating with rabbit brain partial thromboplastin (Dade Actin) for 10 min at 37 C prior to recalcification. The modified Russell viper venom time (RVV-T) was performed by addition of 20  $\mu$ l of 15.6  $\mu$ g/ml purified factor X activator from Russell viper venom (RVV-Xa) as the clotting reagent. Negative controls were performed by replacing the clotting reagent with PBS. To evaluate for the presence of heparin cofactor activity, unfractionated heparin was added to the dilute zebrafish plasma prior to recalcification in the modified RVV-T and APTT. To evaluate for the presence of activated protein C activity, Protac (the purified protein C activator from *Agkistrodon contortrix* venom) was added to the dilute zebrafish plasma and incubated for 10 min at 37 C, prior to incubation with the clotting

reagent and recalcification in the modified RVV-T and APTT.

### *Radiographic Examination of the Zebrafish Gastrointestinal Tract*

Adult zebrafish were incubated in tank water containing 10% Hypaque sodium radiographic dye for 15 and 60 min. These zebrafish were then anesthetized, washed with deionized water, mounted, and radiographs were obtained with a Faxitron X-ray machine using Kodak X-OMAT AR film at 20 kvp and 6 sec exposure in the middle shelf.

### *Warfarin Treatment of Zebrafish*

Sodium warfarin (Sigma) was dissolved in water and diluted into aquaculture tanks at a final concentration of 0, 50, 500, or 5000 ng/ml. Approximately 40-50 adult zebrafish were incubated for 4 days at each warfarin concentration with changes in the tank water every 48 hrs, followed by harvesting of pooled plasma from each treatment group for analysis of coagulation times.

### *Chromogenic Assay for Factor X Activity*

Factor X chromogenic activity was determined using the CoaTest Factor X kit, with the protocol modified as described. Normal human plasma was diluted 1:14 in 50 mM Tris, pH 7.8 and 20 mg/ml polybrene to represent 100% activity, and a standard curve was prepared by making additional triplicate dilutions (75, 50, 25, and 12.5% activity). To determine factor X chromogenic activity, citrated plasma was collected from control and warfarin treated zebrafish (5000 ng/ml for 4 days), and prediluted 1:28 in 50 mM Tris, pH 7.8, and 20 mg/ml polybrene. Dilute plasma (50  $\mu$ l) and 1.5 mM S-2337 (50  $\mu$ l) were incubated for 30 sec at 37 C in a 96 well plate, followed by addition of 50  $\mu$ l RVV-Xa (43.3  $\mu$ g/ml) in 50 mM CaCl<sub>2</sub>. The rate of chromogenic substrate (S-2337) cleavage was

determined by the change in absorbance at 405 nm over 2 min ( $\Delta A_{405}/\text{min}$ ) in a  $V_{\text{max}}$  kinetic microtiter plate reader (Molecular Devices Corp., Menlo Park, CA). Zebrafish factor X activity was quantitated by comparing the rate of substrate cleavage in triplicate to the standard curve, following subtraction of background cleavage by plasma in the absence of RVV-Xa.

### *Chromogenic Assay for Protein C Activity*

Normal human plasma was diluted 1:5 in 50 mM Tris, pH 8.0 to represent 100% activity, and a standard curve was prepared from duplicate serial dilutions. Citrated plasma was collected from control and warfarin treated zebrafish (5000 ng/ml for 4 days), and diluted 1:10 in the assay. Diluted plasma samples (88  $\mu$ l) were incubated with 500 ng/ml Protac (purified protein C activator from *Agkistrodon contortrix* venom) and 500 nM hirudin for 15 min at 37 C at a 100  $\mu$ l final volume in a 96 well plate. Following incubation, 1.0 mM S-2266 (100  $\mu$ l) in 0.2 M NaCl, 50 mM Tris, pH 8.0 was added, and the rate of chromogenic substrate cleavage ( $\Delta A_{405}/\text{min}$ ) determined over 2 min as described above. Activated protein C activity in zebrafish plasma was quantitated by comparing the rate of substrate cleavage from triplicate determinations to the standard curve, after subtracting background cleavage by plasma in the absence of Protac.

### *Chromogenic Assay for Heparin and Dermatan Sulfate Cofactor Activity*

The presence of heparin or dermatan sulfate cofactor activity was determined by the ability of dilute plasma to inhibit either human thrombin (15 nM), or factor Xa (19 nM), respectively. Normal human plasma was diluted 1:30 in buffer containing 0.2 M NaCl, 50 mM Tris, pH 8.0, 7.5 mM EDTA, 1% PEG-8000, and either unfractionated heparin (3 U/ml) or dermatan sulfate (625  $\mu$ g/ml), to represent 100% activity. Standard curves for both heparin and dermatan

sulfate were prepared in a 96 well plate by duplicate serial dilutions. Zebrafish plasma was diluted 1:120 for determination of heparin cofactor activity, and 1:30 for dermatan sulfate cofactor activity. To determine plasma cofactor activity, either 120 nM thrombin (25  $\mu$ l) or 150 nM factor Xa (25  $\mu$ l) in TS/PEG buffer (0.15 M NaCl, 20 mM Tris, pH 7.4, 0.1% PEG-8000) was incubated with 100  $\mu$ l dilute plasma at room temperature for exactly 45 sec. To determine residual thrombin activity, 75  $\mu$ l of the chromogenic substrate S-2238 (800  $\mu$ M) in TS/PEG buffer with 0.5  $\mu$ g/ml polybrene was added, and the rate of substrate cleavage ( $\Delta A_{405}/\text{min}$ ) determined over 2 min as described above. To determine residual factor Xa activity, 75  $\mu$ l of the chromogenic substrate S-2765 (800  $\mu$ M) in 0.15 M NaCl, 20 mM HEPES, pH 7.4, 2 mM  $\text{CaCl}_2$ , 0.1% PEG-8000, and 0.5  $\mu$ g/ml polybrene was added and the rate of substrate cleavage similarly determined. Standard curves plotted the residual rate of substrate cleavage versus the plasma dilution expressed as % activity for each condition (thrombin or factor Xa, heparin or dermatan sulfate). Heparin or dermatan sulfate cofactor activity in zebrafish plasma was quantitated by comparing the rate of residual substrate cleavage from triplicate determinations to the standard curve for that condition.

## RESULTS

### *Demonstration of Procoagulant Activity in Zebrafish Plasma*

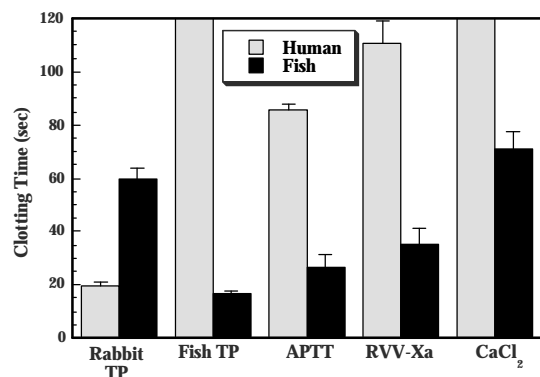
To assay for the presence of the extrinsic, intrinsic and common pathways of coagulation, zebrafish and normal human plasmas were compared in modified prothrombin (PT), activated partial thromboplastin (APTT), and Russell viper venom (RVV-T) times (see methods). To control for baseline stimulation of zebrafish coagulation during blood collection, all clotting times were compared to recalcified

plasma in the absence of clotting reagents. The results of the modified coagulation times were reproducible between independent batches of pooled, citrated zebrafish plasma (data not shown). The modified prothrombin time (PT) was performed by incubating an equal volume of plasma diluted 1:2 in PBS with either rabbit thromboplastin, or zebrafish thromboplastin prepared from muscle (see methods). Human plasma demonstrated rapid clotting when exposed to rabbit thromboplastin, but did not form clot in response to zebrafish thromboplastin. In contrast, zebrafish plasma demonstrated only modest shortening of the clotting time (relative to recalcified controls) in response to the rabbit thromboplastin, but rapidly formed clot in response to zebrafish thromboplastin (Fig. 1).

The modified activated partial thromboplastin time (APTT) was performed in similar fashion to the PT, except that an equal volume of a rabbit partial thromboplastin reagent (Dade Actin) was incubated with the dilute plasma for 10 minutes at 37 C prior to recalcification. Human plasma demonstrated a relatively prolonged clotting time under these conditions, but was significantly shorter than recalcified human controls which failed to clot (> 250 sec). Zebrafish plasma incubated with the partial thromboplastin reagent demonstrated significantly shorter clotting times than human plasma or recalcified zebrafish controls under the same conditions (Fig. 1).

Addition of phospholipid alone (rabbit brain cephalin) did not significantly reduce the clotting time relative to recalcified zebrafish controls (data not shown). Addition of 20  $\mu$ g/ml corn trypsin inhibitor (a human factor XIIa inhibitor) to zebrafish plasma prolonged the APTT, with no significant effect on the PT (data not shown).

The modified Russell viper venom time was also performed in similar fashion to the PT, except that an equal volume of purified factor X activator from this venom (RVV-Xa) was incubated with the dilute plasma. Human plasma demonstrated very prolonged clotting times (over 100 sec) under these conditions. In contrast,



**Figure 1.** Comparison of the modified prothrombin (PT), partial thromboplastin (APTT), and Russell viper venom (RVV-T) coagulation times for pooled, citrated human and zebrafish plasma. The modified PT was performed with both rabbit brain (Rabbit TP) and zebrafish thromboplastin (Fish TP), the modified APTT with a rabbit partial thromboplastin reagent (APTT), and the RVV-T with the purified factor X activator from Russell viper venom (RVV-Xa). Clotting times for human (diagonal hatched bar) and zebrafish plasma (black bar) were expressed as the mean  $\pm$  S.D. (error bars). Recalcified plasma controls (CaCl<sub>2</sub>) are provided for comparison.

zebrafish plasma demonstrated significant shortening of the clotting time relative to both human plasma and recalcified zebrafish controls (Fig. 1). Likewise, incubation of RVV-Xa with dilute zebrafish plasma generates cleavage of the factor X chromogenic substrate (S-2337) at approximately 200% of the activity of normal human plasma (Fig. 4). No significant cleavage of the chromogenic substrate was observed by RVV-Xa in the absence of plasma, and this chromogenic activity was not inhibited by excess hirudin.

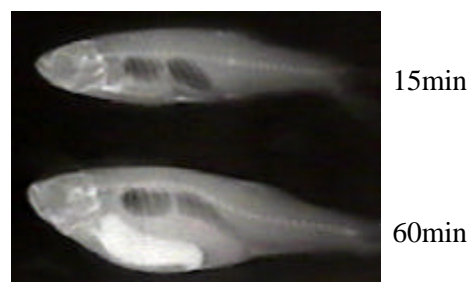
#### *Demonstration of Radiographic Dye Uptake into the Gastrointestinal Tract of Adult Zebrafish*

Oral ingestion of drugs dissolved in the tank water could potentially provide reliable and consistent dosing of the diminutive zebrafish. The feasibility of administering oral anticoagulants to the zebrafish in this manner was assessed by the ability of the zebrafish to ingest a radiographic dye dissolved in the tank water. Adult zebrafish were incubated in tank water containing 10%

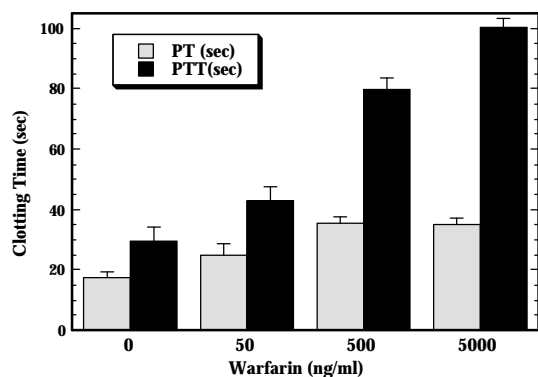
Hypaque sodium for 15 and 60 min, rinsed extensively with water, and radiographically examined. Significant uptake of the radiographic dye into the gastrointestinal tract was evident, suggesting that zebrafish ingest sufficient amounts of water to allow dosing with water soluble oral medications in a standard manner (Fig. 2).

#### *Dose Dependent Oral Uptake of Warfarin and Anticoagulant Effects on Zebrafish Plasma*

A dose response was performed to characterize the effect of warfarin dissolved directly in the tank water on zebrafish coagulation. Forty to fifty adult zebrafish were incubated for 4 days in tanks containing 0, 50, 500, or 5000 ng/ml sodium warfarin in solution. Tank water was changed every other day. At the end of the incubation period, plasma was harvested and pooled for each group of zebrafish (see methods) and a modified PT and APTT performed as described. Exposure of the zebrafish to warfarin resulted in a dose dependent prolongation of the both the PT and APTT (Fig. 3). To correlate the effects of warfarin on the clotting assays with individual factor activities, chromogenic assays for both factor X (see above) and protein C-like (see below) activity were performed on plasma from untreated and warfarin-exposed (5000 ng/ml for 4 days) zebrafish. Plasma from the warfarin



**Figure 2.** Oral uptake of radiographic dye from the tank water. Zebrafish were incubated in tank water containing 10% Hypaque dye for 15 min (above) and 60 min (below). Fish were then anesthetized, rinsed extensively with water, and radiographs were obtained.

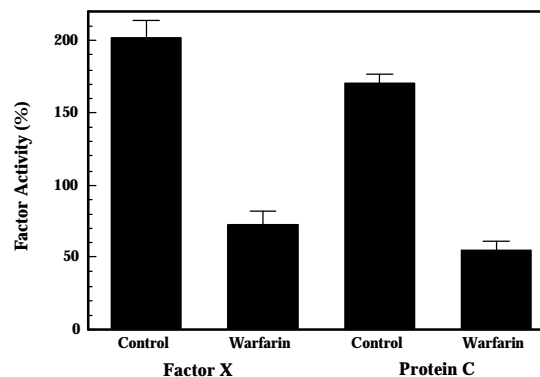


**Figure 3.** The effect of warfarin treatment on zebrafish coagulation. Groups of approximately 40 to 50 zebrafish were treated with warfarin dissolved in the tank water at 0, 50, 500, or 5000 ng/ml for 4 days. Pooled, citrated zebrafish plasma was collected from each treatment group as described (see methods). The modified PT (diagonal hatched bars) and PTT (black bar) were performed for each treatment group with rabbit thromboplastin or Dade Actin, respectively. Clotting times were expressed as the mean  $\pm$  S.D. (error bars).

treated zebrafish demonstrated factor X and protein C chromogenic activities of approximately one-third the level of untreated fish (Fig. 4).

#### *Demonstration of Physiologic Anticoagulant Activity (Protein C, Antithrombin, and Heparin Cofactor II-like Activity) in the Zebrafish*

The presence of natural anticoagulant activities in zebrafish plasma was evaluated by both clotting and chromogenic assays. Protac (the purified protein C activator) was incubated with zebrafish plasma for 10 min at 37 C prior to performing the modified RVV-T or APTT. Preincubation of zebrafish plasma with Protac resulted in dose and time dependent prolongation of the clotting time in both the modified RVV-T (Table 1) and the APTT (data not shown). Similarly, incubation of Protac (500 ng/ml) with dilute zebrafish plasma for 15 min at 37 C results in markedly increased cleavage of the chromogenic substrate for activated protein C (S-2366), which is not inhibited by the presence of hirudin (Fig. 4). Zebrafish plasma demonstrates elevated levels of protein C-like



**Figure 4.** Effect of warfarin treatment on factor X and protein C-like chromogenic activity in dilute zebrafish plasma. Pooled, citrated plasma from untreated controls, and zebrafish treated with warfarin (5000 ng/ml) for 4 days, were collected for analysis. Factor X-like activity was determined by incubating equal volumes of dilute zebrafish plasma, RVV-Xa (43.3  $\mu$ g/ml), and the chromogenic substrate S-2337 (1.5 mM) at 37 C. The rate of chromogenic substrate cleavage was determined over 2 min ( $\Delta A_{405}/\text{min}$ ) in a  $V_{\text{max}}$  microtiter plate reader, and the activity quantitated by comparison to a standard curve prepared with normal human plasma (see methods). Protein C-like activity was determined by incubating dilute zebrafish plasma with Protac (500 ng/ml) for 15 min at 37 C, followed by addition of the chromogenic substrate S-2366 (500  $\mu$ M). The rate of chromogenic substrate cleavage was determined over 2 min ( $\Delta A_{405}/\text{min}$ ), and the activity quantitated by comparison to a standard curve prepared with normal human plasma (see methods).

chromogenic activity (170%) relative to human plasma, which is significantly reduced in the warfarin treated fish. No significant cleavage of substrate was observed with Protac in the absence of plasma.

Zebrafish plasma was also examined for the presence of heparin and dermatan sulfate cofactor activity, which would suggest the presence of antithrombin-like inhibitors. Addition of unfractionated heparin to zebrafish plasma prolongs the modified RVV-T (Table 1) and APTT (data not shown) in a dose dependent fashion. To further evaluate this cofactor activity, the ability of dilute zebrafish plasma to inhibit human thrombin or factor Xa was examined in the presence of either unfractionated heparin or dermatan sulfate. Briefly, zebrafish plasma was diluted in buffer containing either heparin or factor Xa, and residual enzyme activity

**Table 1.** Dose Dependent Prolongation of the Modified Russell Viper Venom Time (RVV-T) in Zebrafish Plasma by Unfractionated Heparin and Protac (Purified Protein C Activator).

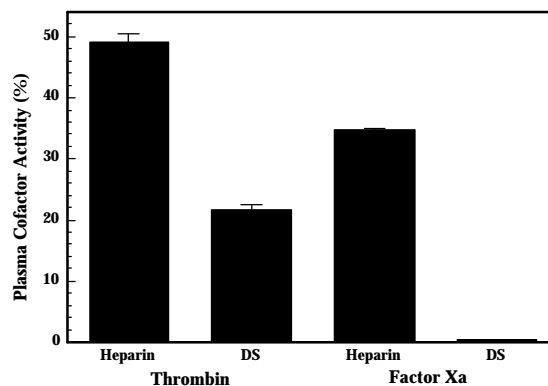
Amount (µg/ml)	RVV-T (sec)
Heparin	
0	35.0
1	37.4
5	116.4
10	160.0
Protac	
0	34.4
1.0	40.4
2.5	47.9
10.0	81.9

*Note.* Unfractionated heparin was added to diluted zebrafish plasma immediately before recalcification and determination of the clotting time (see methods). Protac was incubated with diluted zebrafish plasma for 10 min at 37 C, followed by addition of RVV-Xa and recalcification. Unfractionated heparin and Protac are expressed as final concentrations in the fibrometer (250 µl).

dermatan sulfate, incubated with human thrombin assessed by addition of the appropriate chromogenic substrate (see methods). In the presence of heparin, addition of dilute zebrafish plasma resulted in significant inhibition of both human thrombin and factor Xa, consistent with antithrombin-like activity (Fig. 5). Controls demonstrated that this inhibition was dependent on the presence of both zebrafish plasma and heparin. In the presence of dermatan sulfate, addition of dilute zebrafish plasma resulted in significant inhibition of human thrombin, but no discernible inhibition of human factor Xa, consistent with heparin cofactor II-like activity (Fig. 5). Controls demonstrated that the inhibition of human thrombin was dependent on the presence of both zebrafish plasma and dermatan sulfate.

#### *Demonstration of Spontaneous Morphologically Recognizable Bleeding in Warfarin Treated Zebrafish*

Morphological identification of zebrafish with hemostatic defects would facilitate phenotypic selection following large scale



**Figure 5.** Chromogenic assays for heparin and dermatan sulfate cofactor activity in zebrafish plasma. The ability of zebrafish plasma to inhibit human thrombin or factor Xa was determined by diluting the plasma in buffer containing either 3 U/ml unfractionated heparin or 625 µg/ml dermatan sulfate. Thrombin (15 nM) or factor Xa (19 nM) was incubated with the dilute plasma for exactly 45 sec at room temperature, followed by addition of either 300 µM S-2238 or S-2765 to determine residual thrombin or factor Xa activity, respectively. Plasma heparin or dermatan sulfate cofactor activity was quantitated by determining the rate of chromogenic substrate cleavage over 2 min ( $\Delta A_{405}/\text{min}$ ), and comparison to a standard curve prepared with normal human plasma (see methods).

saturation mutagenesis. Large adult zebrafish (>0.5 gm in weight) continuously exposed to warfarin (0.5 mg/ml) for up to 10-14 days developed spontaneous bleeding visible within posterior portion of the body and the tail fin of the adult fish (Fig. 6). Although this spontaneous bleeding was not observed in smaller zebrafish (< 0.5 gm) exposed to warfarin for shorter time periods, anesthetized zebrafish exposed to physical or chemical injury also demonstrate visible hemorrhage in the tail and fin regions (data not shown).

## DISCUSSION

The results of these investigations have established that the major coagulant and anticoagulant pathways in zebrafish plasma demonstrate remarkable overall similarity to the mammalian system. The response of zebrafish plasma to both rabbit and zebrafish thromboplastin is consistent with the presence of



**Figure 6.** Spontaneous bleeding in warfarin treated zebrafish. Larger zebrafish (> 0.5 mg) were incubated for prolonged periods (greater than two weeks) with warfarin (5000 ng/ml) and observed for evidence of visible bleeding. As demonstrated above, reddish discoloration of the tail and fin regions consistent with bleeding was morphologically evident with prolonged warfarin exposure.

an extrinsic pathway of coagulation (Fig. 1). The markedly increased potency of zebrafish thromboplastin suggests species specificity in the putative tissue factor-factor VIIa interaction, consistent with differences noted between lamprey and human tissue sources (19). Likewise, the response of zebrafish plasma to the partial thromboplastin reagent (Dade Actin) suggests the presence of an intrinsic pathway of coagulation (Fig. 1). The markedly increased potency of this reagent compared to phospholipid alone (rabbit brain cephalin) suggests that the contact activator (ellagic acid) is critical to the response. The selective inhibition of the APTT by corn trypsin inhibitor (a human factor XIIIa inhibitor), similarly suggests that the response to the partial thromboplastin reagent depends on the presence of a contact activation system. Thus, both contact activation system and intrinsic pathway of coagulation appear to be present in zebrafish plasma. The response to RVV-Xa in the modified clotting assays, and the

generation of chromogenic activity versus a factor X substrate (S-2337), suggests the presence of factor X-like activity in zebrafish plasma. The presence of factor X-like activity, along with the previously demonstrated expression of prothrombin (9) in the zebrafish, suggests a common pathway of coagulation similar to the mammalian system.

In addition to coagulant pathways, major anticoagulant pathways also appear intact in zebrafish plasma. Preincubation of zebrafish plasma with Protac results in dose dependent prolongation of the RVV-T and APTT (Table 1), and generation of chromogenic activity versus a protein C substrate (S-2336) (Fig. 4), suggesting the presence of activated protein C-like activity. Whether the mechanism for protein C prolongation of the clotting times (inactivation of the factor Va and factor VIIIa) is similar in zebrafish plasma awaits direct demonstration of these cofactor activities. Addition of unfractionated heparin to zebrafish plasma results

in dose dependent prolongation of both the modified RVV-T and APTT (Table 1), indicating the presence of “heparin cofactor” activity. Inhibition of both human thrombin and factor Xa in the presence of dilute zebrafish plasma and heparin is consistent with antithrombin III-like activity (Fig. 5). The modest reduction in activity observed for zebrafish compared to human plasma may represent decreased levels of the antithrombin activity, or cross-species incompatibilities between the human protease and zebrafish antithrombin. Likewise, selective inhibition of human thrombin in the presence of dilute zebrafish plasma and dermatan sulfate is consistent with the presence of heparin cofactor II-like activity (Fig. 5). The reduced dermatan sulfate cofactor activity relative to human plasma may again represent either decreased levels of heparin cofactor II-activity in zebrafish plasma, or cross-species incompatibilities between human thrombin and zebrafish heparin cofactor II. Thus, evidence for the physiologic anticoagulants protein C, antithrombin III, and heparin cofactor II exists in the zebrafish.

The feasibility of oral drug dosing directly from the tank water is demonstrated by the rapid uptake of radiographic dye into the gastrointestinal tract of the zebrafish (Fig. 2). The dose dependent response of the PT and APTT to 4 days of warfarin treatment confirms the ability to achieve uniform dosing of anticoagulants by the oral route (Fig. 3). Prolongation of the PT and APTT by warfarin treatment is consistent with a requirement for vitamin K dependent  $\gamma$ -carboxylation of zebrafish hemostatic protein(s). Similarly, the reduction in both factor X and protein C-like chromogenic activity is consistent with the expected vitamin K dependent  $\gamma$ -carboxylation of these enzymes. The conservation of glutamic acid residues within the Gla domain of the predicted protein sequence for hagfish prothrombin further suggests that a similar pathway for vitamin K dependent  $\gamma$ -carboxylation exists in fish (20). Prolongation of clotting times and reduction in factor X and

protein C-like activity have been reported in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) directly fed with warfarin pellets, however, direct uptake of the drug from water has not been previously observed (13-14). The ability to achieve uniform, reproducible dosing of the zebrafish with water soluble oral compounds suggests the feasibility of drug selection strategies for identification of mutants, and a potential discovery system for novel oral anticoagulants.

The diminutive size of the zebrafish required the development of novel methods to obtain plasma and analyze the coagulation system. Collection of blood by micropipet through an incision in the region of the dorsal aorta proved to be a reliable method to obtain pooled zebrafish plasma. This method is verified by the reproducibility of the modified coagulation assays (Fig. 1) and thrombocyte function assays performed on independent batches of pooled, citrated plasma or whole blood, respectively (16). These investigations were also facilitated by the preparation of a species specific thromboplastin reagent, and *in vitro* coagulation assays that allowed detection of thrombin generation from small volumes (10  $\mu$ l) of zebrafish plasma. The ability to visually detect drug or injury induced hemorrhage suggests the potential for morphologic identification of zebrafish hemostatic mutants (Fig. 6). Additionally, the radiographic technique developed to visualize the uptake of dye into the gastrointestinal tract, may also be useful for identification of mutant zebrafish with defects in bony development. Thus, methods developed here may facilitate further analysis of this important animal model.

In summary, characterization of the zebrafish coagulation system demonstrates that major mammalian coagulant and anticoagulant pathways appear to be conserved, despite the significant evolutionary distance. Investigation of the morphology and function of the zebrafish thrombocyte has demonstrated similar parallels

to the mammalian platelet. Jagadeeswaran (in press) The significant degree of functional homology between the zebrafish and human hemostatic systems strongly suggests that the zebrafish is a relevant genetic model for mammalian hemostasis. The potential application of this powerful vertebrate model for identification of novel genes in a multigenic disorder such as thrombosis is particularly appealing.

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