Original Article

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Functional characterization of tissue factor in von Willebrand factor-dependent thrombus formation under whole blood flow conditions

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Running head: *TF/VWF in flow-dependent thrombus formation*

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Abstract

Von Willebrand factor (VWF) plays an important role in mediating platelet adhesion and aggregation under high shear rate conditions. Such platelet aggregates are strengthened by fibrin-network formation triggered by tissue factor (TF). However, little is known about the role of TF in VWF-dependent thrombus formation under blood flow conditions. We evaluated TF in thrombus formation on immobilized VWF under whole blood flow conditions in an *in vitro* perfusion chamber system. Surface-immobilized TF amplified intra-thrombus fibrin generation significantly under both low and high shear flow conditions, while TF in sample blood showed no appreciable effects. Further, immobilized TF enhanced VWF-dependent platelet adhesion and aggregation significantly under high shear rates. Neutrophil cathepsin G and elastase increased significantly intra-thrombus fibrin deposition on immobilized VWF-TF complex, suggesting the involvement of leukocyte inflammatory responses in VWF/TF-dependent mural thrombogenesis under these flow conditions. These results reveal a functional link between VWF and TF under whole blood flow conditions, in which surface-immobilized TF and VWF mutually contribute to mural thrombus formation, which is essential for normal hemostasis. By contrast, TF circulating in blood may be involved in systemic hypercoagulability, as seen in sepsis caused by severe microbial infection, in which neutrophil inflammatory responses may be active.

***Key Words**:* blood flow, fibrin, thrombus formation, tissue factor, von Willebrand factor

Introduction

Mural thrombi to arrest bleeding at the injured vessel wall sites is formed by the collaborative functions of platelet adhesion/aggregation and blood clotting mechanisms [1, 2]. This hemostatic system is precisely regulated to guarantee normal blood circulation crucial for maintenance of life, but can also trigger pathological arterial thrombosis in conditions such as brain stroke or myocardial infarction [2-5].

Recent flow studies clearly have indicated that blood rheological conditions seriously affect the mechanisms of platelet adhesion and aggregation where von Willebrand factor (VWF) plays a determinant role under high shear stress conditions [6-8]. Indeed, such mechanisms under high flow differ significantly from the classic theory established by static platelet functional assays. In this context, blood clotting mechanisms have also been studied recently under flow conditions [9-14]. To clarify the mechanisms of flow-dependent thrombin or fibrin generation, comprehensive thrombus formation including platelet thrombogenesis and blood clotting has been analyzed under whole blood flow conditions. In this regard, most recent studies analyzed fibrin generation on surface-immobilized collagen in the presence of tissue factor (TF), a critical initiator or amplifier of blood coagulation, using a microfluidic device [9, 13, 15-17]. Although TF plays a pivotal role in the in vivo coagulation process, its functional relevance and mechanism of action have not yet been fully elucidated under whole blood flow conditions.

Thus, we studied TF-dependent fibrin generation under physiologic whole blood flow conditions with various shear rates. To highlight or focus on the flow effects in TF-mediated blood coagulation, we employed surface-immobilized VWF, although a collagen-coated surface may be more relevant physiologically as an experimental thrombogenic surface. Indeed, the VWF function in mural thrombus formation is

absolutely dependent upon shear rate alteration [6-8, 18], more suitable for clarifying the flow-dependent properties of TF.

Since there has been considerable interest in thrombotic potentials of soluble TF circulating in blood [16, 17], the purpose of the present study is to characterize two distinct forms of TF, surface-immobilized TF and mobile TF in blood on thrombus formation on VWF-surface under whole blood flow conditions by use of an in vitro flow chamber system. We also evaluated effects of neutrophil proteases, cathepsin G and elastase, on VWF/TF-dependent thrombus formation, clarifying a functional link between VWF and TF in mural thrombus formation and/or inflammation under flow conditions which are most relevant for in vivo thrombosis and hemostasis.

Materials and Methods

Blood collection

The present study was approved by the institutional review board of Nara Medical University. Blood was gathered via venipuncture with an 18-gauge needle from five healthy volunteers, who had not taken any medications in the preceding 2 weeks, and immediately anti-coagulated by 1/10th volume of 3.8% sodium citrate. Sample whole blood was then treated with corn trypsin inhibitor (50 μg/ml; CTI, Haematologic of Technologies Inc., VT, USA) to minimize the contact activation of blood and was recalcified with 8 mM $CaCl_2$ just prior to the perfusion experiment to initiate blood coagulation, as described [19].

Preparation of "immobilized TF" on VWF-coated glass surfaces

Human VWF with highest molecular weight multimer was purified from cryoprecipitate as described elsewhere [19-21]. Recombinant human tissue factor (rhTF; Dade Innovin) was purchased from Siemens Healthcare (Marburg, Germany). Glass coverslips were reacted with a sample mixture of purified VWF (constant concentration of 100 μg/ml) and various concentrations of rhTF (0 as a control, and 1, 3, 10, 30 100, and 300 pM) for 2 h at room temperature, as previously described [19]. After washing out of non-adherent proteins, the amount of rhTF immobilized to the glass surfaces with VWF was measured by ELISA-based assay, as described [19], using an anti-TF monoclonal antibody (American Diagnostica, Stanford, CT) to which peroxidase was conjugated with a Labeling Kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan). In brief, a rubber ring (5 mm diameter) was put on a glass coverslip coated with rhTF. A peroxidase-conjugated anti-TF monoclonal antibody was reacted then to the inner zone of the rubber ring. The end reactant with an enzyme activity was aspirated and transferred to an ELISA plate, and the enzyme activity was measured at an optical

density of 492 nm with an ELISA reader.

In vitro perfusion studies

In the flow studies to evaluate platelet adhesion and aggregation, citrated whole blood was incubated with 1 μM of DiOC6 fluorescence (Molecular Probes Inc., Eugene, OR, USA) for 10 min at 37ºC to label platelets, allowing observation of platelet adhesion and aggregation on the surface by confocal laser scanning microscopy (CLSM), as previously described [19, 21-26]. Just prior to perfusion, 8 mM of $CaCl_2$ was mixed to sample blood to start blood coagulation reactions. Whole blood containing DiOC6-labeled platelets was applied over the VWF-surface in the presence or absence of immobilized TF under flow conditions with a low (250 s^{-1}) or high (1500 s^{-1}) shear rate, as described [19, 21-26]. Fluorescent images by CLSM were used to calculate the platelet surface coverage (percentage of the area covered by platelets) as well as the total thrombus volumes in defined areas using an image-analyzing application (Image Pro Plus version 4.5; Planetron, Tokyo, Japan), as previously described in detail [19, 23, 25].

For evaluation of flow-dependent fibrin generation, sample blood was perfused without fluorescence-labelling of platelets, as described elsewhere [12, 19, 21]. Thrombi formed on a coverslip were then fixed and incubated with 200 μL of a mixed solution of mouse anti-fibrin monoclonal antibody (15 μg/mL; REF350-Ab which detects fibrin specifically and cannot recognize the intact fibrinogen, Sekisui Diagnostics, Stanford, USA) and rabbit anti-fibrinogen polyclonal antibody that totally recognizes fibrinogen (15 μg/mL; Dako Cytomation, Kyoto, Japan) for 90 min at 37°C. Sample thrombi on a coverslip were then stained with 200 μL of a mixed solution of Cy3-anti-mouse IgG (5.0 μg/mL; Sigma-Aldrich Co., Tokyo, Japan) and FITC-anti-rabbit IgG (5.7 μg/mL; Biosource, Camarillo, CA, USA) for 90 min at 37°C as secondary antibodies and observed by CLSM. The fibrin deposition level within thrombi was assessed by the

"fibrin/fibrinogen" ratio, defined as the fluorescence intensity of fibrin relative to fibrinogen. Three-dimensional images of thrombi were created by the computed image-evaluating system equipped with CLSM, as described [12, 21, 25]. In some experiments, various concentrations of rhTF was added to the sample blood immediately before perfusion. In some specified flow experiments, effects of neutrophil cathepsin G (MP Biomedicals, Tokyo, Japan) or elastase (Calbiochem, Darmstadt, Germany) on intra-thrombus fibrin generation were evaluated with or without each corresponding inhibitor (cathepsin G inhibitor: ab142181 or elastase inhibitor: ab142154, Abcam Japan, Tokyo, Japan).

Evaluation of flow-path occlusion time

In the in vitro perfusion experiment to evaluate flow-dependent fibrin generation, thrombotic flow-path occlusion occurred at some point during perfusion because the flow-path in the chamber became gradually filled with coagula or fibrin clot generated in the perfused blood. In general, the average flow-path occlusion time (the duration defined from the start of perfusion to flow cessation) was assumed to be 10–12 min in our flow experimental system. Thus, most of the perfusion experiments in this study, which required up to 5-min perfusion, could be completed successfully. However, occasionally 5-min perfusions could not be completed due to much earlier stoppage of sample blood flow in experiments in which TF was added to the blood. Thus, we decided to assess flow-path occlusion times to evaluate the thrombogenic potential of TF added in blood in this type of experiment.

Statistical analysis

All data were expressed as averages ± standard deviation (SD). Statistical differences between two groups of data were evaluated by Student's t-test. P values < 0.05 denote statistical significance.

Results

VWF-coated glass surfaces containing varying concentrations of TF (immobilized TF)

The surface-immobilized TF on the VWF-coated glass surface, measured by the ELISA-based assay, increased as a function of the amount of rhTF added to a coating sample solution, reaching a plateau at rhTF concentrations greater than 100 pM (Fig. 1). As a result, a variety of VWF-coated glass plates with immobilized TF were prepared (see Fig. 1; control without immobilized TF, and three species of plates, (A), (B), and (C)).

Effects of immobilized TF or TF added in sample blood on flow-dependent fibrin generation in thrombus formation on VWF-surfaces under high and low shear rate conditions

Immobilized TF augmented the flow-dependent fibrin generation within thrombi in a concentration-dependent and saturated manner under both high and low shear rate conditions (Fig. 2). Fluorescent 3D images also visually confirmed that immobilized TF enhanced intra-thrombus fibrin deposition on the VWF surface (Fig. 2). In contrast, no appreciable or reproducible effects on intra-thrombus fibrin generation were confirmed under either high or low shear rate conditions when TF was added to sample blood (results not shown). Formation of coagula or fibrin clot that prevents constant and continuous perfusion was markedly amplified by the addition of TF in the perfused sample blood, perhaps representing the limitation of the present experimental approach.

Thus, we employed another experimental approach to evaluate coagulation potentials of TF added in sample blood, the evaluation of flow-path occlusion time (Fig. 3). Indeed, the dose-dependent shortening of flow-path occlusion time by TF added in blood clearly supported the above interpretation, while immobilized TF had no such

effect (Fig. 3).

Effects of immobilized TF on VWF-dependent platelet adhesion and aggregation, and on comprehensive mural thrombus formation on VWF-coated surfaces under high and low shear rate conditions

Surface-immobilized TF significantly augmented VWF-dependent platelet adhesion and aggregation, as judged by the platelet surface coverage of thrombi, under the high shear rate condition, while no appreciable effects were confirmed under the low shear rate condition (Fig. 4). Thus, immobilized TF significantly increased thrombus volume generated on VWF surfaces under high and low shear rate conditions, which reflects comprehensive mural thrombus formation involving platelet aggregation and intra-thrombus fibrin deposition (Fig. 5). No enhancing effects of TF added in sample blood were confirmed on platelet adhesion and aggregation under either high or low shear rate conditions (results not shown).

Neutrophil cathepsin G and elastase on flow-dependent fibrin generation in thrombus formation on VWF-surfaces in the presence or absence of immobilized TF under high shear rate condition

In order to identify any functional association between inflammation and VWF/TF-dependent thrombus formation, we evaluated two neutrophil proteases, cathepsin G and elastase, in this experimental system. As a result, elastase significantly augmented the flow-dependent fibrin generation within thrombi formed on VWF-surfaces under the high shear rate condition (Fig. 6). In addition, significant augmentation by cathepsin G was confirmed also when examined in the presence of immobilized TF (Fig. 6). These effects of cathepsin G or elastase were clearly abolished by each corresponding inhibitor (Fig. 6), suggesting that the inflammatory responses of neutrophils are critically involved in VWF/TF-dependent thrombus formation.

Discussion

Mural thrombus formation is the fundamentals for both physiologic hemostasis and pathological intravascular thrombosis. This crucial event results from the interaction, under blood flow conditions, between platelet functions and fibrin clot generation; these processes may be closely related and may up-regulate each other, thus comprising a crucial human defense mechanism. The adhesive protein VWF plays a central role in triggering platelet adhesion and aggregation in the earliest phase of primary hemostasis [18, 27]. Meanwhile, TF actively initiates blood coagulation mechanisms on activated cell surfaces, such as those of injured endothelial cells or stimulated platelets, at local sites of thrombosis and hemostasis. Indeed, alteration or disruption of endothelial cell layers immediately results in the activation or release of both VWF and TF, which play a representative role in primary and secondary hemostasis, respectively. The present study sought to determine the relevant role of TF in thrombus formation under flow conditions, and employed immobilized VWF as an experimental thrombogenic surface. This experimental approach may also be appropriate for assessing the flow-dependent properties of TF, since VWF is the thrombogenic adhesive protein which is most sensitive to blood rheological conditions [18, 27-29].

We investigated the roles of two distinct forms of TF in thrombus formation on VWF-coated surfaces under flow conditions with two different shear rates. First, TF which was immobilized on the experimental VWF surface significantly enhanced intra-thrombus fibrin generation in a concentration-dependent manner under both high and low shear rate conditions (Figs. 1 and 2). This flow-dependent fibrin generation promoted by immobilized TF is crucial for stable thrombus formation and physiologic hemostasis. Indeed, native human TF in vivo, not a soluble protein in blood, which is expressed on the membrane of stimulated cells such as endothelial cells or leukocytes,

therefore may be comparable to the immobilized TF in the present experiment. Thus, our results may recapitulate the in vivo process of normal hemostasis where immobilized TF plays a critical role in mural thrombus formation under physiologic blood flow conditions.

In contrast, no reproducible effects on intra-thrombus fibrin deposition were confirmed under either high or low shear rate conditions when TF was added to sample blood (results not shown). In this regard, the dose-dependent shortening of flow-path occlusion time clearly indicated that TF added in sample blood potentially amplified the formation of fibrin clot and coagula in the perfused sample blood, resulting in earlier flow arrest in the chamber (Fig. 3). This fibrin clot generation may not be directly involved in the mural thrombus formation which is crucial for normal hemostasis, but may contribute instead to thrombotic vessel occlusion. These observations might reflect the pathological thrombotic events often seen in clinical settings, such as disseminated intravascular coagulation (DIC) [30]. TF added in blood sample in the present experiment may be equivalent to microparticles or truncated soluble TF molecules flowing in the blood stream, which are derived from TF-bearing cells such as monocytes or macrophages [30-33].

In addition to increasing intra-thrombus fibrin generation, the immobilized TF enhanced platelet functions under flow conditions. Consistent with previous studies on collagen-coated surface [9, 34], platelet adhesion and aggregation on the VWF-coated surface were significantly up-regulated by immobilized TF under the high shear rate condition, as judged by platelet surface coverage (Fig. 4). In this regard, enhanced thrombin generation by immobilized TF could directly contribute to activating platelets on the VWF-coated surface through protease-activated receptor-1 and platelet glycoprotein Ib receptor [35-37]. Such platelet activation could make platelet surfaces into an active state for blood coagulation, such as membrane exposure of phosphatidylserine or enhanced expression of P-selectin, providing the reaction field for

thrombin generation [37, 38]. Indeed, membrane expression of P-selectin can assemble TF-complexes efficiently on platelet surfaces. These platelet activation processes in turn result in enhanced thrombin and fibrin generation on the VWF-surface [37, 38]. Thus, amplified platelet activation together with fibrin generation by immobilized TF resulted in a significant increase in the total thrombus volume generated on the VWF surface (Fig. 5). Further, these properties of immobilized TF were prominent under the high shear rate condition where VWF is functionally relevant, suggesting a functional association between VWF and TF in mural thrombus formation under whole blood flow conditions.

Interestingly, both neutrophil cathepsin G and elastase were found to enhance fibrin generation within mural thrombi formed on the VWF-coated surface in the presence of immobilized TF under a high shear rate condition (Fig. 6), while no appreciable effects of these neutrophil proteases were confirmed on platelet adhesion and aggregation. The mechanism by which these neutrophil proteases modestly upregulate intra-thrombus fibrin generation is presently unknown. Both cathepsin G and elastase are serine proteases which are released from azurophilic granules of neutrophils upon exposure to various inflammatory stimuli. Proteolysis by these serine proteases may be associated with the activation of clotting factors or inactivation of anti-clotting factors [39-41]. In fact, a previous study reported that cathepsin G inactivated tissue factor pathway inhibitor by partial proteolysis, resulting in enhanced blood clotting [39]. Further, previous studies by us and others indicated that VWF could be involved in neutrophil recruitment at local thrombogenic sites [42-44]. Altogether, our results suggest that the inflammatory responses of neutrophils may play a role in VWF/TF-dependent thrombus formation under whole blood flow conditions [45, 46].

In conclusion, we investigated the thrombogenic potential of immobilized TF or TF in blood on VWF-dependent thrombus formation under whole blood flow conditions. We demonstrated that VWF/TF-dependent thrombus formation was closely

related to neutrophil inflammatory responses. Our results provide insights into the interplay between coagulation and inflammation, a dynamic which may contribute to the uncontrollable soluble TF activity involved in severe hypercoagulable conditions such as septic DIC.

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Conflicts of interest

Mitsuhiko Sugimoto, Masaaki Doi and Hideto Matsui belong to the Department of Regulatory medicine for Thrombosis, Nara Medical University, which was endowed by the Bayer Pharmaceutical Company, Japan. Other authors declare that they have no conflict of interest.

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Legends for Figures

Fig. 1. Preparation of VWF-coated glass surfaces containing varying concentrations of immobilized TF.

A glass plate was coated with a mixture containing a constant concentration of purified VWF (100 μg/ml) and varying concentrations of recombinant human TF (rhTF; 0, 1, 3, 10, 30, 100, 300 pM). The amount of rhTF immobilized on the glass surfaces with VWF was quantified by an ELISA-based assay using a peroxidase-conjugated anti-TF monoclonal antibody. Each data point represents mean ± SD in five independent experiments. Note that the amount of immobilized TF as determined by enzyme activity at an optical density of 492 nm increased as a function of recombinant TF added to the glass surface, reaching a plateau at TF concentrations greater than 100 pM. Thus, various VWF-coated glass plates with immobilized TF (three species of plates, (A), (B), and (C), as indicated in the figure) were prepared.

Fig. 2. Functional evaluation of TF bound to VWF immobilized on a glass surface.

Citrated whole blood from healthy volunteers was perfused over a VWF-coated glass surface with or without immobilized TF under a high (1500 s^{-1}) or low (250 s^{-1}) shear rate. Just prior to perfusion, $CaCl_2$ was added to the sample blood (8 mM) to initiate blood coagulation responses. Thrombi generated on VWF-coated glass surfaces at 5-min perfusion in the presence or absence of immobilized TF were fixed, double-stained (FITC-fibrinogen: green and Cy3-fibrin: red) and viewed by CLSM. **Upper panels:** Bars represent the mean (+ SD) fibrin/fibrinogen ratio in 25 examined areas (each 133 x 100 mm) (five areas randomly selected from five independent perfusions of blood from five individual donors). Note that the intra-thrombus fibrin generation, as a function of immobilized TF (indicated as (A), (B) or (C); see Fig. 1), significantly (*; $P < 0.05$) increased as compared to control thrombi generated without immobilized TF under both

high and low shear rates. **Lower panels:** The 3D images of thrombi, corresponding to upper panels (control thrombi and thrombi with immobilized TF; surface B) under both high and low shear rates, are representative of five independent flow experiments (original magnifications: X 600). Merged 3D images, obtained by superimposing two images of the identical area, indicate that immobilized TF enhances intra-thrombus fibrin deposition under both high and low shear rate conditions.

Fig. 3. Effects of immobilized TF or TF added in sample blood on flow-path occlusion time under a high (1500 s^{-1}) shear rate.

Experimental conditions are basically same as those described in the Fig. 2 legend, except that varying concentrations of TF (0 as a control, and 0.1, 0.3, 1.0 pM) were added to sample whole blood just prior to perfusion (left panel). In order to evaluate the flow-path occlusion time, blood perfusion was continued until the sample blood flow stopped because the generated coagula occluded the flow-path in the chamber. Bars represent mean (+SD) in three independent perfusions using blood from three individual donors. TF added in sample blood significantly (*; $P < 0.05$) shortened the flow-path occlusion time in a dose-dependent manner (left panel), while immobilized TF did not (right panel; see Fig. 1 for 3 species of plates, (A), (B), (C)).

Fig. 4. Time course of platelet adhesion and aggregation on VWF-coated surfaces in the presence or absence of immobilized TF under high or low shear rate conditions.

Experimental conditions were basically same as those described in the Fig. 2 legend, except that whole blood containing DiOC6-labeled platelets was perfused over a VWF-coated glass plate with (plate B: see Fig.1) or without immobilized TF under a high (1500 s^{-1}) or low (250 s^{-1}) shear rate. The process of platelet adhesion and aggregation was evaluated by the surface coverage of thrombi generated at the time points indicated

in the figure. Each data point represents mean ± SD in three independent perfusions using blood from three individual donors. Note that immobilized TF significantly (*; $P < 0.05$) enhanced platelet functions under the high shear rate condition.

Fig. 5. Effects of immobilized TF on thrombus formation under high (1500 s^{-1}) or low (250 s^{-1}) shear rate conditions.

Experimental conditions are the same as those described in the Fig. 4 legend. **Upper panels:** Bars represent mean (+ SD) total thrombus volume in 15 defined areas (each 133 x 100 mm) (five areas randomly selected from three independent perfusions of blood from three individual donors). Note that thrombus generation is significantly (*; $P < 0.05$) enhanced in the presence of immobilized TF under both high and low shear rate conditions. **Lower panels:** The findings in the upper panels are supported visually by 3D images of thrombi, corresponding to 5 min after perfusion under both high and low shear rates; images are representative of three independent flow experiments (original magnifications: X 600).

Fig. 6. Effects of neutrophil cathepsin G and elastase on fibrin generation within thrombi generated on VWF-coated surfaces with or without immobilized TF under a high shear rate condition.

Experimental conditions are basically same as those described in the Fig. 2 legend, except that cathepsin G (0.37 μM) or elastase (0.38 μM) was added to sample blood just prior to perfusion. In specified experiments, molar excess of each corresponding inhibitor, cathepsin G inhibitor (100 μM) or elastase inhibitor (100 μM), was added also to the sample blood, respectively. Thrombi generated on glass surfaces coated with VWF in the absence (left two bars in the figure) or presence (right four bars; plate (B) as indicated in the Fig. 1) of immobilized TF at 5 min after perfusion were evaluated. Bars represent mean (+ SD) fibrin/fibrinogen ratios in 15 defined areas (each 133 x 100

mm) (five areas randomly selected from three independent perfusions of blood from three individual donors). Note that both cathepsin G (upper panel) and elastase (bottom panel) augmented the generation of intra-thrombus fibrin within thrombi which formed on VWF-coated surfaces under the high shear rate condition. Immobilized TF significantly (*; $P < 0.05$) enhanced this cathepsin G activity, and effects of these neutrophil proteases on the VWF-TF-surface were completely abolished by the corresponding inhibitors.

Figure 1

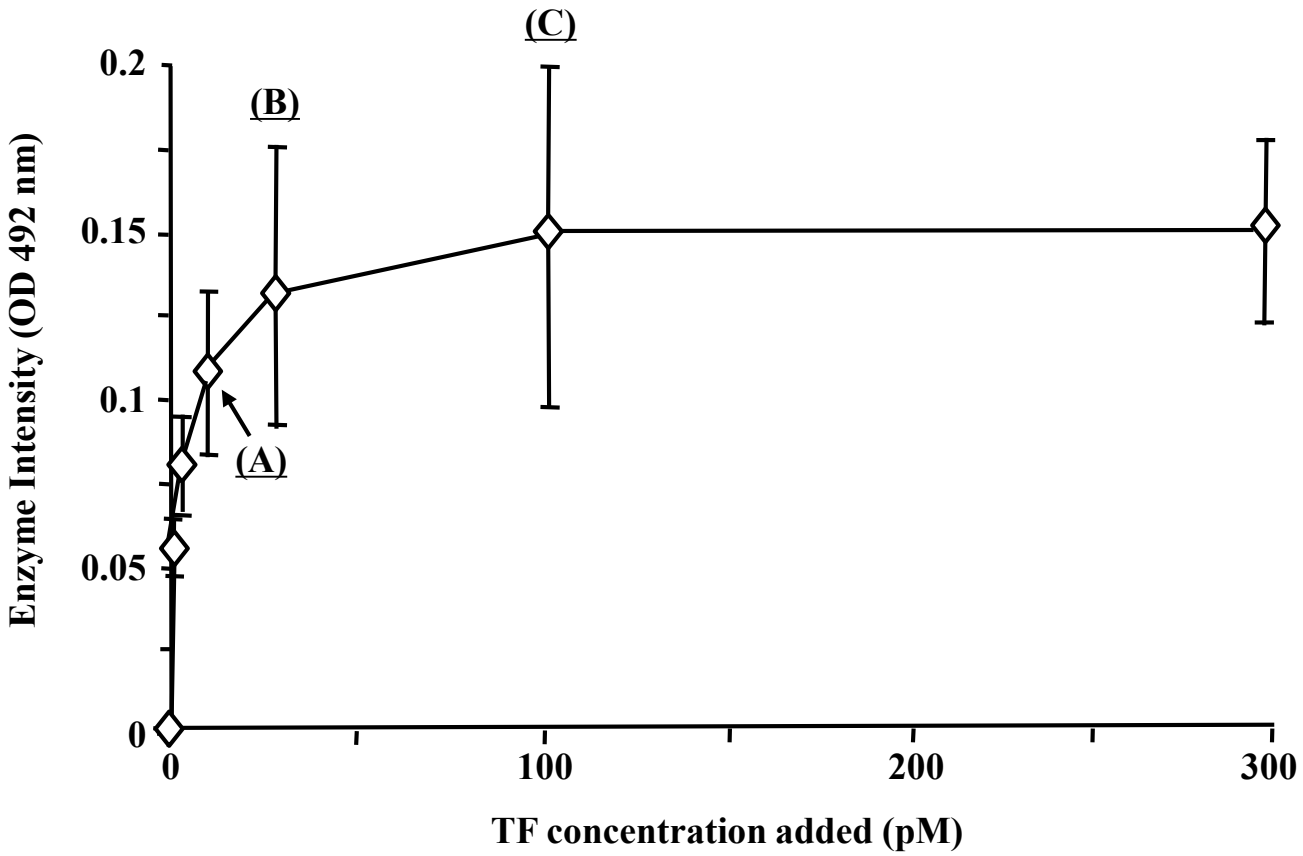
(C)
(B)
(A)
0.2
0.15
0.1
0.05
0
Enzyme Intensity (OD 492 nm)
0
100
200
300
TF concentration added (pM)

Figure 2

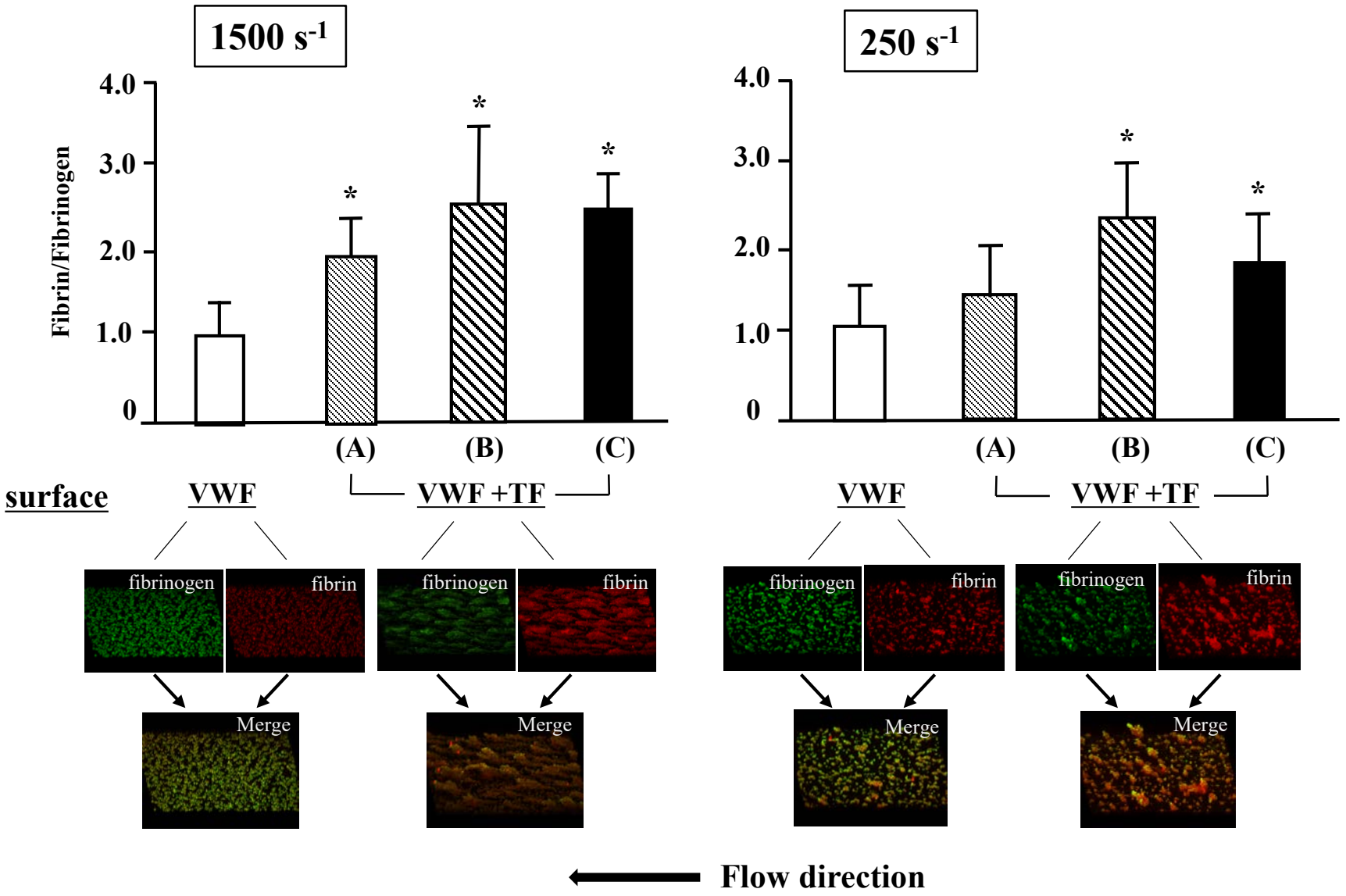
1500 s-1
250 s-1
Fibrin/Fibrinogen
4.0
3.0
2.0
1.0
0
*
(A)
(B)
(C)
surface
VWF
VWF +TF
fibrinogen
fibrin
Merge
Flow direction

Figure 3

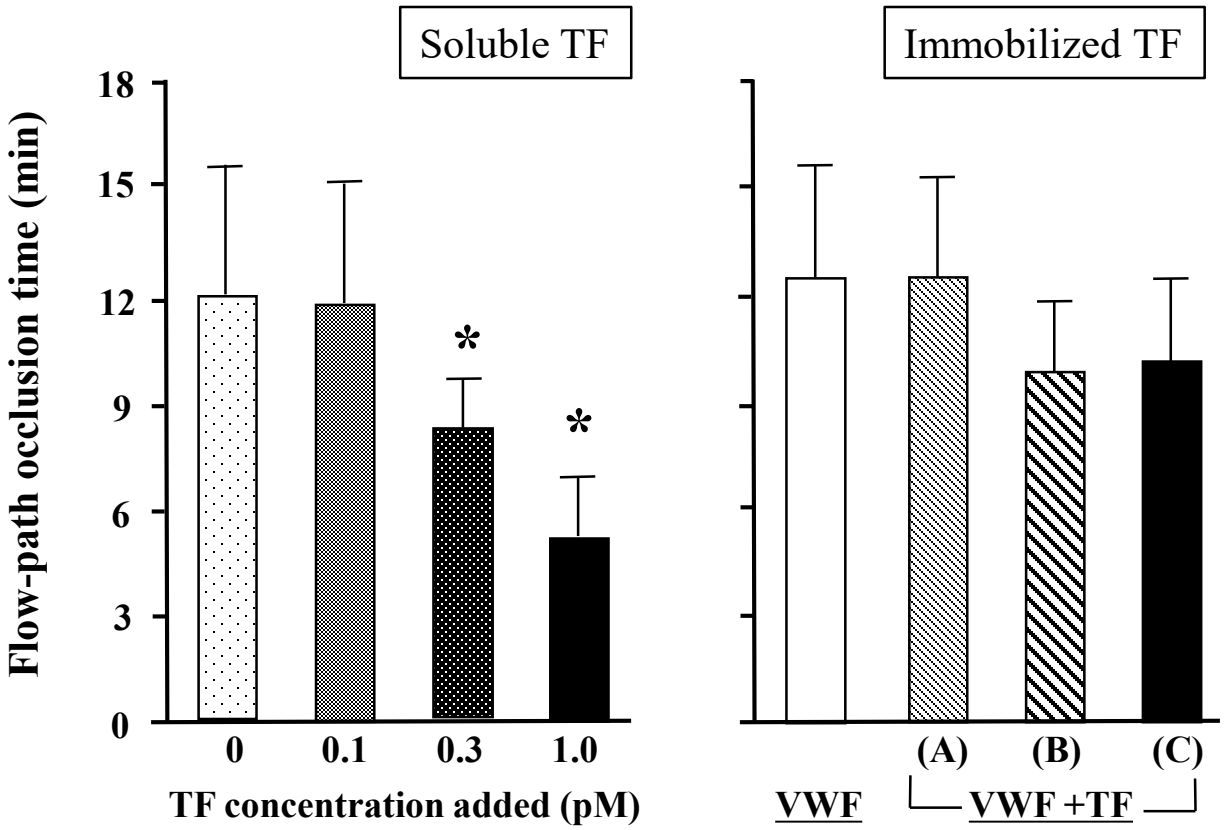
Soluble TF
Immobilized TF
Flow-path occlusion time (min)
18
15
12
9
6
3
0
*
*
0
0.1
0.3
1.0
TF concentration added (pM)
(A)
(B)
(C)
VWF
VWF +TF

Figure 4

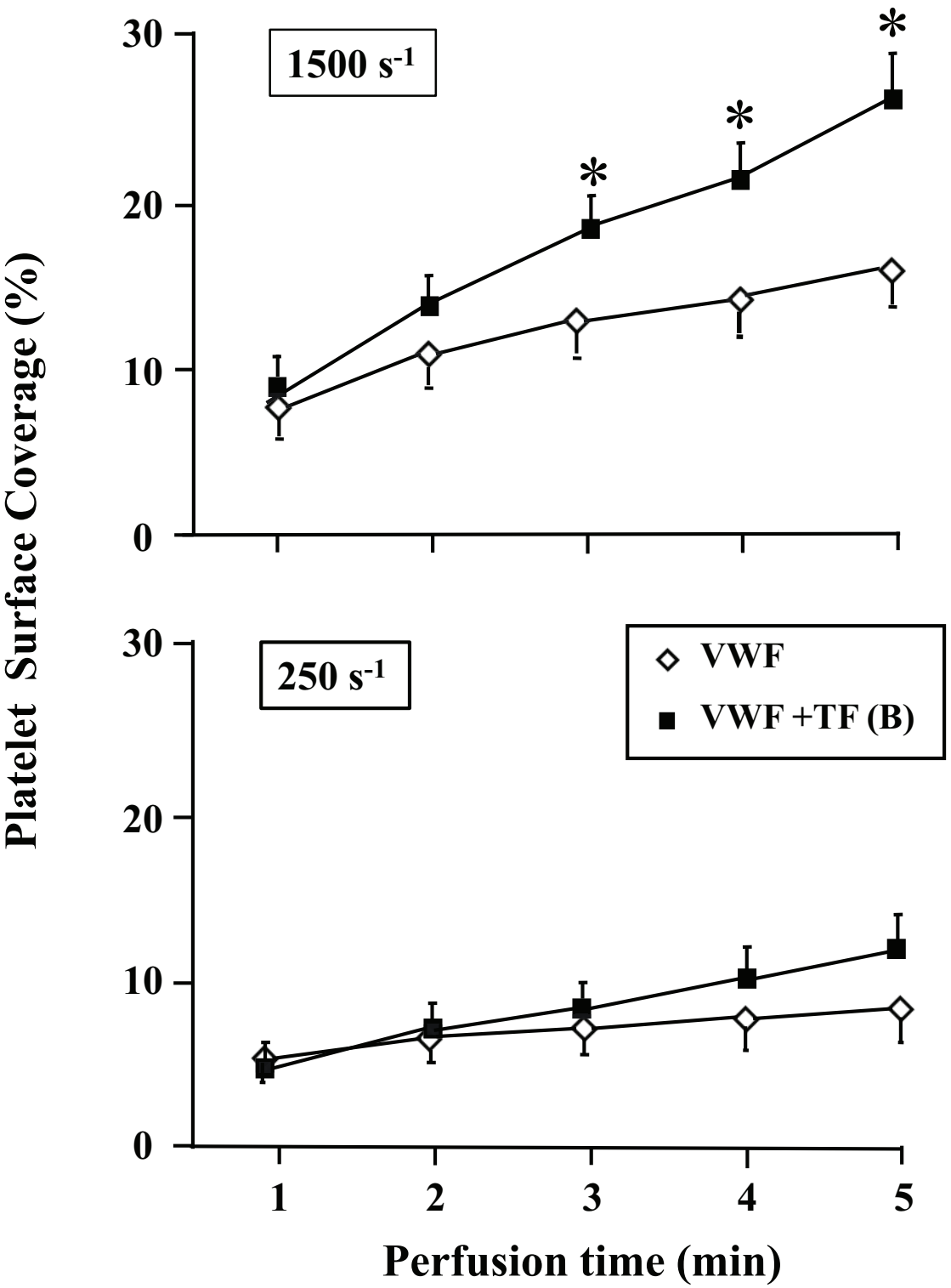
1500 s-1
250 s-1
VWF
VWF +TF (B)
Platelet Surface Coverage (%)
Perfusion time (min)
0
10
20
30
1
2
3
4
5
*
*
*

Figure 5

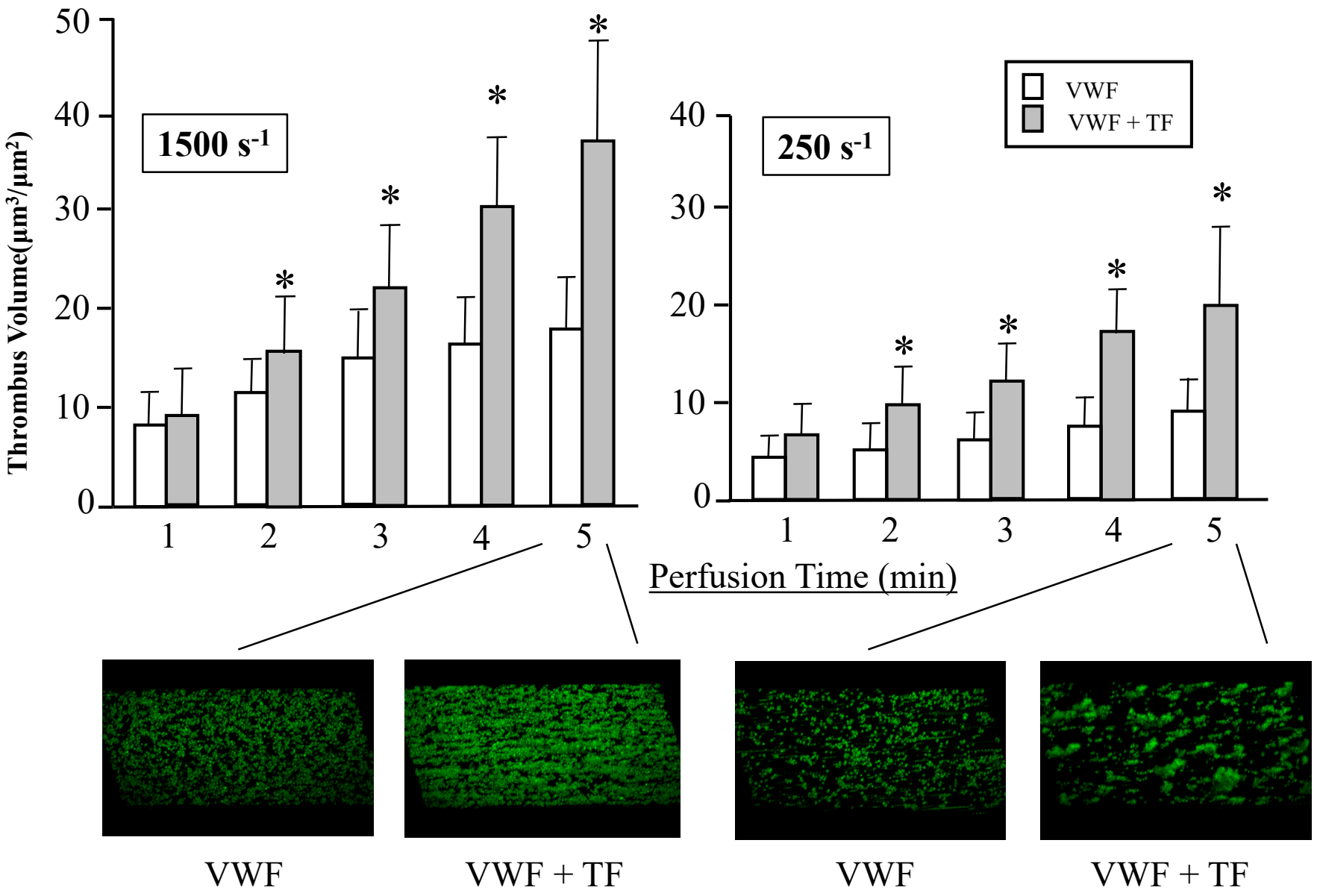
Figure 5
Thrombus Volume(μm³/μm²)
1500 s-1
250 s-1
VWF
VWF + TF
Perfusion Time (min)
VWF
VWF + TF
VWF
VWF + TF

Figure 6

Figure 6