Effects of caplacizumab, a specific inhibitor of the A1 domain of von Willebrand factor binding with platelet membrane glycoprotein (GP) Ibα, on the length of platelet pseudopods supporting platelet adhesions on immobilized von Willebrand factor under blood flow condition

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Abstract A1 domain of von Willebrand factor (VWF) binding with platelet glycoprotein (GP) Ibα play crucial roles in platelet adhesion and subsequent passive shape changes in the platelets such as pseudopod formation under high wall shear rate conditions. However, the effects of specific inhibitors of VWF binding with GPIbα on the length of pseudopods supporting platelet adhesion on VWF are still to be elucidated. Here we measured the length of pseudopods in the presence of VWF-GPIbα inhibitor of caplacizumab. The length of pseudopods was 6.5 ± 0.2 μm (mean ± 95% confidential interval [CI]) and 6.9 ± 0.2 μm (mean ± 95% CI) at 100 and 200 nM of caplacizumab concentrations and was longer than those formed in its absence (5.2 ± 0.2 μm, p < 0.05). Our experiments also revealed that the surface area coverage by platelets in the presence of VWF-GPIbα inhibitor of caplacizumab at a concentration of 200 nM after 60-second blood perfusion was smaller than its absence (45.2 ± 7.5%, p < 0.05). Our results suggest that fewer numbers of VWF-GPIbα bonds generating larger binding force with a longer length of pseudopods, support the platelet adhesion on VWF in the presence of caplacizumab at a wall shear rate of 1,500 s⁻¹.

Keywords platelet, von Willebrand factor, glycoprotein Ibα, blood flow, caplacizumab, pseudopods

Introduction Platelet adhesion on immobilized von Willebrand factor (VWF) is mediated exclusively by the A1 domain of VWF binding with platelet membrane glycoprotein (GP) Ibα under the wall shear rate of 1,500 s⁻¹ [1–9]. Previous publications demonstrated that platelet adhesion is mediated mostly by fibrinogen under wall shear rates less than 630 s⁻¹, while VWF-mediated adhesion becomes dominant under shear rates higher than 630 s⁻¹ [6]. The VWF-mediated platelet adhesion was reported to occur even at 40,000 s⁻¹ [10]. According to these data, we have chosen the wall shear rate of 1,500 s⁻¹ in our experiments and designed our flow chamber to generate the wall shear rate without turbulence [5, 11, 12]. Indeed, platelet adhesion on VWF at the wall shear rate was grossly blocked by various inhibitors of GPIbα-VWF binding [6, 9, 10] or in blood with impaired functionality in molecules mediating the binding of VWF with GPIbα [7, 13, 14]. However, it is of note that a small number of platelet adhesion on VWF was still present even in the presence of specific inhibitor blocking VWF binding with GPIbα [6, 15]. Moreover, recent progress in the real-time imaging technic enabled the detection of the changes in platelets adhering on VWF both in the shape and the function under blood flow conditions [10, 16]. Here, we attempt to apply the ultra-fast confocal microscopy equipped with real-time imaging system [16, 17] to detect the length of pseudopods supporting platelet adhesion on VWF under dynamic flow conditions generating the wall shear rate of 1,500 s⁻¹.

Previous publications showed the heterogenous expression of GPIbα on platelets [18]. Molecular dynamic simulations revealed that a single molecular bond of VWF-GPIbα generates a binding force of 62.3 pN [19]. The fluid dynamic forces applied to platelets adhered on VWF with a cross-sectional diameter of 2–5 μm are approximately 200 pN at the wall shear rate of 1,500 s⁻¹. Thus, only several pairs of VWF-GPIbα bonds are enough to support platelet adhesion on VWF under 1,500 s⁻¹ despite single platelets

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expressing approximately 15,000 molecules of GPIba [20]. The whole single platelet adhesions on VWF are supported by VWF binding with their small GPIba-rich regions [18]. The fluid dynamic forces applied to the platelets result in the elongation of the part of platelets forming pseudopods to support their binding on VWF as previously described [10]. The number of GPIba-rich regions binding with VWF within a single platelet decreases when the GPIba binding function of VWF was blocked by its specific inhibitors. A previous publication suggested that the force generated by pseudopods supporting platelet adhesion is dependent on their length [21]. Taken together, it is conceivable that platelet adhered on VWF supported by smaller numbers of pseudopods, form longer pseudopods generating larger binding forces. In this study, we attempted to test the hypothesis that the length of pseudopods passively formed to support platelet adhesion on VWF under blood flow conditions becomes longer when the GPIba binding functions of VWF are blocked by the presence caplacizumab, a specific inhibitor of GPIba-VWF.

It is of note that the pseudopods passively formed by fluid dynamic forces as described above [10] are not the same as those formed by platelet activation-dependent shape changes. It is also important to note that both mechanisms of pseudopods could not be discriminated either by their length or their shape. Thus, the length of pseudopods we measure in our experiments includes both passively and actively formed ones.

Method

1. Sample Preparation

Our study protocol for drawing blood from healthy adult volunteers was approved by the Institutional Review Board (IRB) of the Tokai University School of Medicine (19R281). Before drawing blood specimens, written informed consent was obtained from all the participants. All 4 participants did not take any medications known to influence platelet function. Written informed consent was obtained from all the participants. All 4 participants did not take any medications known to influence platelet function. All 4 participants did not take any medications known to influence platelet function. All 4 participants did not take any medications known to influence platelet function. All 4 participants did not take any medications known to influence platelet function. All 4 participants did not take any medications known to influence platelet function.

The blood specimens containing fluorescently labeled platelets were exposed to the immobilized human von Willebrand factor (VWF: gift from Dr. Soejima in The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) -covered glass plate at a constant wall shear rate of 1,500 s$^{-1}$ controlled by the rectangular flow chamber [12, 25]. The experimental details are published previously [16]. The shape of platelet adhering on VWF under wall shear rate of 1,500 s$^{-1}$ was assessed by our imaging system equipped with the ultra-fast confocal microscopy imaging system as published previously [16]. The focus of the confocal microscopy was settled 0.5 μm above VWF covered glass plate. The newly adhering platelet on VWF within 1 second after start adhesion was targeted in our study.

2. Flow Chamber and Imaging System

To visualize the shape of the platelets adhering to VWF under the blood flow condition, the focus of the confocal microscope was set at 0.5 μm above VWF. The confocal microscopic images were sequentially obtained every 0.017 seconds [26]. The typical dynamic behavior of single platelet adhering to VWF is illustrated in Fig. 1 panel A. Flowing platelets express glycoprotein (GP) Ibα to capture VWF (shown as 1 in the panel A). Then, the GPIba on the flowing platelet binds to VWF expressed on the vessel wall (shown as 2 in the panel A). Driven by the effect of the detaching force from the fluid dynamic on the adhered platelet, region(s) of platelets including GPIba bound with VWF is elongated as shown in 3 of panel A of the Fig. 1. The platelet rotates to stably adhere on VWF as shown in 4 of panel A of the Fig. 1. These processes occur dynamically within a second. For measuring the length of pseudopods, the platelets in condition shown in 4 of panel A were targeted.

Panel B and C of Fig. 1 summarize the methods for the measurement of pseudopods length supporting the platelet adhesion to VWF. The length of pseudopods was defined as the distance between the start to the end of the pseudopod as shown in panel B. Representative example of a single platelet adhered to the VWF with the pseudopod is shown in panel C. The length of the pseudopod was measured using a simple Python program. The pseudopods were manually traced using the program. The measurement of the program was calibrated by measuring the ruler of 100 μm for 10 times. The relationship between the actual length and measured pixels as position co-ordinates was 10 μm = 61.787 ± 7.743 pixels, ($n = 10$).

4. Measurement of the Coverage of VWF Surface by Platelets

The experiments were conducted with the method described in section 3. Similary to the measurements of pseudopods,
platelet images were obtained at 0.5 μm above the VWF using confocal microscopy. The surface area coverage by platelets was calculated after binarization of the images at the threshold of 16% using ImageJ software. (NIH, the USA)

5. The Effects of Caplacizumab on the Length of Pseudopods and the Surface Area Coverage by Platelets

The nanobody of the specific monoclonal antibody against A1 domain of VWF, known as caplacizumab, inhibits the binding of VWF with platelet glycoprotein (GP) Ibα [27, 28]. For the experiment, caplacizumab was obtained from Creative Biolabs Co. (NY, USA) Various concentrations of caplacizumab was added to the blood specimens to achieve final concentrations between 100 to 800 nM at least 15 min before starting blood perfusion experiments. The doses used in our experiments were within plasma concentrations of caplacizumab achieved within 24 hours after 10 mg single subcutaneous injections in healthy volunteers [29]. It is of note that GPIbα binding capacities of VWF were less than 10% in these volunteers receiving 10 mg subcutaneous administrations of caplacizumab for 24 hours [29]. Thus, the GPIbα binding capacity of VWF in the presence of 100 to 800 nM of caplacizumab in our experiments should be less than 10% as compared to the control. Both the length of pseudopods and the surface area coverage by platelets were measured as described above.

6. Statistical analysis

The length of platelet pseudopods and the surface area coverage by platelet are expressed as mean ± 95% confidence interval (95% CI) unless otherwise specified. The differences were seemed significant when the 95% CI did not cross each other.

Results

1. Length of Pseudopods

Fig. 2 and Table 1 show the length of pseudopods of platelets adhering to the VWF at a wall shear rate of 1,500 s⁻¹. The experiments were conducted with 3 (control and caplacizumab at a final concentration of 100 nM) and 4 (caplacizumab at a final concentration of 200 nM) blood specimens from different donors. The length of pseudopods was 5.2 ± 0.2 μm (mean ± 95% CI: n = 638 platelets) in the control. They were 6.5 ± 0.2 μm (n = 1597 platelets) and 6.9 ± 0.2 μm (n = 1774 platelets), respectively in the presence of 100 nM and 200 nM caplacizumab. The pseudopods supporting platelet adhesion became longer in the presence of caplacizumab in both concentrations (p < 0.05) as compared to the control condition. (Fig. 2) The longer pseudopods formed on platelet adhering to VWF were also
observed by the frequency distribution (Fig. 3). Apparently, the distributions of pseudopods with length more than 5 μm increased in the presence of caplacizumab both at 100 and 200 nM. (Fig. 3)

2. Surface Area Coverage by Platelets

The supplemental movie 1 and movie 2 represent the platelet adhesion dynamics in presence of caplacizumab at a concentration of 200 nM and in the control condition. Substantial amounts of platelets initially adhered and flew away. Fig. 4 and Table 2 show the time-dependent change in the surface area coverages by platelets in control or in the presence of 200, 400 and 800 nM of caplacizumab. The surface area coverage by platelets became lower in the presence of caplacizumab as compared to control. The difference between control and 200, 400, 800 nM of caplacizumab was apparently largest at 60 seconds of blood perfusion. The surface area coverage by platelet at 60 seconds in control blood perfusion (45.2 ± 7.5%) was larger than in the presence of 200, 400, 800 nM caplacizumab (26.1 ± 6.4%, 24.7 ± 7.2%, and 26.0 ± 3.3%, respectively).

Table 1: Experimental Details

<table>
<thead>
<tr>
<th>Biological experiments</th>
<th>Control</th>
<th>Caplacizumab (100 nM)</th>
<th>Caplacizumab (200 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Pseudopods for Measuring Their Length</td>
<td>638</td>
<td>1597</td>
<td>1774</td>
</tr>
<tr>
<td>Average Length of Pseudopods Measured</td>
<td>5.24</td>
<td>6.54</td>
<td>6.86</td>
</tr>
<tr>
<td>95%CI of the Length of Pseudopods Measured</td>
<td>0.21</td>
<td>0.18</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Figure 2: Lengths of Pseudopods Formed in Platelets Adhering on VWF at a Wall Shear Rate of 1,500 s⁻¹.

Figure 3: Frequency Distributions of the Length of Pseudopods Forming in Platelets Adhering on VWF

The numbers of the measured values of the length of pseudopods in platelet adhering on VWF were 638, 1597, and 1774 in control and 100 nM and 200 nM caplacizumab group, respectively. All results were summarized as frequency distributions in each 1 μm interval of the length of pseudopods. The frequencies of the measured values were expressed as mean and 95% confidential interval in each class.
Discussion

Our results show that a single platelet adhesion on immobilized VWF was supported by pseudopods starting from the region of the platelet expressing GPIbα binding with VWF at the wall with the shear rate of 1,500 s⁻¹. Our results also show that the length of pseudopods supporting platelet adhesion becomes longer in the presence of caplacizumab, a specific inhibitor of the binding of the A1 domain of VWF with GPIbα [30]. The caplacizumab used in our experiments should have the ability to inhibit VWF binding with GPIbα in our experimental conditions because the surface area covered by platelets on VWF after 30 seconds or longer blood perfusion was markedly inhibited by caplacizumab. The inhibiting effects of caplacizumab on platelet adhesion to VWF in our experiments were in agreement with the results published previously using other VWF-GPIbα inhibitors such as monoclonal antibody against GPIbα [2] or using blood specimens obtained from patients with deficiency in VWF binding with GPIbα [31, 32]. Our experimental results show that the length of pseudopods become longer to support platelet adhesion on VWF even when the majority of platelet adhesion was inhibited by the effects of GPIbα-VWF inhibition by caplacizumab.

There are approximately 15,000 GPIbα molecules expressed on the surface of a single platelet [20, 33, 34]. Previous biochemical experiments using atomic force microscopy [35] and optical tweezers [36] along with the molecular dynamic simulation calculation [19] revealed that a single pair of VWF-GPIbα generates approximately 50–100 pN of binding force. Previously published kinetic Monte Carlo simulation suggested that the adhesion force between a platelet and vessel wall was less than 250 pN [21]. Accordingly, platelet adhesion to VWF could be supported by only several pairs of VWF-GPIbα bonds [37]. It is important to note that the distributions of GPIbα on platelet surface was heterogeneous [18]. Thus, the region(s) of platelet with higher densities of GPIbα tend to bind more likely to bind with VWF under blood flow conditions [38]. Based on these results, we are proposing a model of longer pseudopods supporting platelet adhesion on VWF in the presence and absence of caplacizumab as shown in Fig. 5. Briefly, the GPIbα-rich regions of a single platelet capture VWF. Then, the shapes of the adhering platelets are changed passively by the effects of fluid dynamic force applied to the body of platelet to form pseudopods. As pre-

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**Table 2** Time-Dependent Changes in the Surface Area Coverage by Platelets on VWF.

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Control</th>
<th>Caplacizumab (200 nM)</th>
<th>Caplacizumab (400 nM)</th>
<th>Caplacizumab (800 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0</td>
<td>5.2 ± 4.8</td>
<td>9.0 ± 6.6</td>
<td>4.0 ± 1.8</td>
<td>5.2 ± 1.76</td>
</tr>
<tr>
<td>15.0</td>
<td>9.5 ± 8.0</td>
<td>11.6 ± 7.0</td>
<td>9.0 ± 4.0</td>
<td>9.7 ± 3.2</td>
</tr>
<tr>
<td>30.0</td>
<td>21.7 ± 9.4</td>
<td>19.7 ± 4.7</td>
<td>19.3 ± 4.2</td>
<td>17.9 ± 3.3</td>
</tr>
<tr>
<td>60.0</td>
<td>45.2 ± 7.5</td>
<td>26.1 ± 6.4</td>
<td>24.7 ± 7.2</td>
<td>26.0 ± 3.3</td>
</tr>
<tr>
<td>90.0</td>
<td>47.8 ± 10.3</td>
<td>32.2 ± 2.4</td>
<td>25.5 ± 7.9</td>
<td>24.5 ± 4.2</td>
</tr>
<tr>
<td>111.0</td>
<td>44.5 ± 7.1</td>
<td>34.1 ± 4.0</td>
<td>25.1 ± 4.7</td>
<td>25.8 ± 4.0</td>
</tr>
</tbody>
</table>

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**Figure 4** Surface Area Coverage by Platelets

The mean surface area coverages are indicated by dots and the error bar represents 95% CI.
Previously reported, the adhesion force generated by pseudopods becomes larger when their length become longer [39]. The platelet adhesion on VWF were supported by several relatively shorter pseudopods generating relatively smaller binding forces in control condition. However, there are only a few VWF molecules available to bind with GPIbα in the presence of caplacizumab. Accordingly, platelet adhesion is supported by smaller number of longer pseudopods generating larger binding force in the presence of caplacizumab.

It is of note that the model proposed in Fig. 5 is hypothetical. However, our experimental results did not contradict with the proposed mechanism.

There are substantial number of publications demonstrating platelet shape changes after biological activation of platelets such as those occurring under high wall shear rate condition [40, 41]. These activation-induced shape changes include pseudopod formation [40]. However, in our experiments, the influences of biological activation of platelet were excluded by focusing on platelets immediately after the start of adhesion to VWF. Indeed, we have previously shown that at least 4 seconds are necessary before intracellular calcium ion concentration increases ([Ca\(^{2+}\)]\(\text{I}\)) in platelets after their adhesion to VWF [16]. The rise in [Ca\(^{2+}\)]\(\text{I}\) is the initial events occurring prior to other events such as active form of conformational changes in platelet membrane GPIIb/IIIa [17] or shape changes induced by actin polymerization [42]. The platelets targeted in our study only include the ones adhered on VWF within 1 second after the start of adhesion. Thus, the effects of activation-dependent platelet shape changes including pseudopod formation could be excluded in our experiments. As shown in Fig. 3, there are substantial heterogeneity within the length of pseudopods formed on platelet adhering to VWF. The potential inclusion of pseudopods induced by platelet

Figure 5 Potential Mechanism for the Elongation of Pseudopods Supporting Platelet Adhesion to VWF under High Wall Shear Rate Conditions in the Presence of Caplacizumab.

Individual platelets are shown as red ellipses (platelets). GPIbα on platelets are distributed heterogeneously as published previously [18]. The regions where GPIbα molecule are present densely, are shown as green particles (GPIbα-rich regions). The presence of A1 domain of von Willebrand factor expressed on the vessel wall were shown as blue rectangles (VWF).

Panel A represents the condition of platelet adhesion on VWF in the absence of caplacizumab. Platelet adhesion on the vessel wall is supported by 2 pseudopods representing VWF binding with GPIbα (pseudopod (1) and pseudopod (2)). As previously published, the supporting forces for platelet adhesion by pseudopods dependent on the length of pseudopods [39]. Platelet adhesion on VWF is mediated by accumulations of forces generated by the 2 pseudopods (pseudopod (1) and pseudopod (2)).

Panel B represents the condition of platelet adhesion on VWF in the presence of specific inhibitor of VWF binding with GPIbα namely caplacizumab shown as red semi-circles. Since the binding capacity of VWF with GPIbα were blocked on the majority of VWF, there are only a small number of VWF available for GPIbα binding. In this condition, only small numbers of VWF-GPIbα bonds support platelet adhesion on VWF. The length of pseudopods become longer to generate greater amount of binding force.
activation occurring before platelet adhesion could not be excluded. However, our main results are not influenced by this methodological limitation.

It is of note that there are several other methodological limitations for our experimental results to support the model of platelet adhesion proposed in Fig. 5. First, the sizes of platelets are not fully homogenous. Typically, the diameter of platelet body varies from 2 to 5 μm. The fluid dynamic force applied to platelet adhering to VWF is influenced by the cross-sectional area of the bodies of platelets exposed to the blood flow. Thus, even though our experiments were conducted in condition with constant wall shear rate of 1,500 s⁻¹, the forces applied to individual adhering platelet may vary substantially. Despite the fact that VWF-mediated platelet adhesion occurs at any wall shear rate higher than 600 s⁻¹ [6], our experiments were conducted under a single wall shear rate of 1,500 s⁻¹. There is also potential heterogeneity in the shape of platelet regions including GPIbα to bind with VWF. These heterogeneities influence the fluid forces applied to individual platelets adhering on VWF resulting in heterogeneities in the length of pseudopods. Despite these methodological limitations, our conclusion that the length of pseudopods supporting platelet adhesion became longer in the presence of VWF-GPIbα inhibitor of caplacizumab is not influenced.

In conclusion, we show here the longer passively formed platelet pseudopods in platelets adhering to VWF in the presence of caplacizumab. Our results are in agreement with the hypothesis that the platelet adhesions on VWF are supported by smaller numbers of longer pseudopods generating larger force to support platelet adhesion on VWF in the presence of VWF-GPIbα inhibitors.

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