Analysis of Platelet Adhesion to a Collagen-Coated Surface Under Flow Conditions: The Involvement of Glycoprotein VI in the Platelet Adhesion

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Platelet adhesion to the exposed surface of the extracellular matrix in flowing blood is the first and critical reaction for in vivo thrombus formation. However, the mechanism of this in vivo platelet adhesion has yet to be studied extensively. One of the reasons for this is the lack of a practical assay method for assessing platelet adhesion under flow conditions. We have devised an assay method (the fluorescent adhesion assay) that is based on the technique originally reported by Hubbell and McIntire (Biomaterials 7:354, 1986) with some modifications to make it more amenable for assaying small samples and have developed an analysis method to quantify the extent of platelet adhesion and aggregation from fluorescence images by using a computer-assisted image analysis system. In our assay, platelet adhesion, expressed as the percentage of the area covered by adhered platelets, was found to increase biphasically as a function of time. In the first phase, platelets interacted with the coated collagen, transiently stopping on the surface; we called this reaction the temporary arrest. In the second phase, platelets adhered much more rapidly and permanently on the surface, and this adhesion was dependent on the shear rate; platelets formed aggregates in this phase. We used our assay to analyze the effects of platelet aggregation inhibitors on platelet adhesion. All three examined inhibitors, EDTA (10 mmol/L), antiglycoprotein (GP) IIb/IIIa, and GRGDS peptide (1 mmol/L), inhibited the second phase adhesion in flowing blood. Furthermore, GPVI-deficient platelets also showed defective second-phase adhesion under the same conditions. These results suggested that GPIIb/IIIa activation and GPVI contribute to the reaction inducing the second phase. The second-phase adhesion has been extensively investigated, and the consensus is that this reaction is mainly attributable to the platelet-platelet interaction. In this report, we were able to detect an earlier reaction, the temporary arrest. This temporary arrest would reflect the fast and weak interaction between platelet GPIb/IX and collagen-von Willebrand factor complexes on the collagen-coated surface.

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Although platelet adhesion to the subendothelium is the first reaction in the thrombus-forming system of blood vessels, the components involved and the precise mechanisms of their interactions in adhesion under physiologic conditions are yet not well understood. Although a number of methods have been devised to monitor the platelet adhesion that occurs under flow conditions, the lack of a facile standard assay has been a major obstacle to delineating the steps involved in adhesion. Furthermore, because the various assay systems have not been tested simultaneously with the same blood samples, the seemingly disparate results obtained from different laboratories cannot be resolved.

The classical method for measuring platelet adhesion under the flow condition was invented by Baumgartner and his group, who used a piece of inverted denuded aorta as the adhesive surface. They showed that both von Willebrand factor from the plasma and platelet surface glycoprotein (GP) Ib are involved in the initial adhesion of platelets to the subendothelium, especially at a high flow rate. Later, newer assays used flat thin flow over a surface coated by purified collagen, which is a more controlled flow condition that yielded more reproducible and quantitative data. There are two types of assays using flat thin flow, with the main difference between them being how the adherent platelets are monitored. In the method developed by Sakariassen et al., the platelets are fixed and stained after they adhere to the collagen surface. In the other method, originally reported by Hubbell and McIntire,6,7 the platelets are prelabeled with the fluorescent dyes mepacrine before the adhesion reaction, and then the adherent and aggregated platelets are visualized by fluorescent microscopy. Both these methods require a relatively large blood sample.

We have devised an assay for measuring platelet adhesion based on the flat thin flow method of Hubbell and McIntire with modifications that substantially decrease the sample volume requirement. Both our present method (the fluorescent adhesion assay) and that of Hubbell and McIntire have several advantages over other assays: they allow the continuous recording of data throughout the whole time period of interest, and the data obtained can be analyzed at a later time, so that a particular reaction can be isolated from the data on the entire adhesion process. Furthermore, the small volume of our perfusion chamber allows platelet adhesion to be measured over a much longer time span and also makes it possible to measure the adhesion of patients' platelets, neither of which was practical in the original method due to the large volume of blood required. Platelet adhesion in vivo is a complex phenomenon in which platelets come in contact with and adhere to a collagen-coated surface and, at the same time, the components involved and the precise mechanisms of their interactions in adhesion under physiologic conditions are yet not well understood. Although a number of methods have been devised to monitor the platelet adhesion that occurs under flow conditions, the lack of a facile standard assay has been a major obstacle to delineating the steps involved in adhesion. Furthermore, because the various assay systems have not been tested simultaneously with the same blood samples, the seemingly disparate results obtained from different laboratories cannot be resolved.

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time, also interact with each other. In this study, computer-aided image analysis enabled us to extract data corresponding to specific interactions from the complex platelet adhesion data to quantify total platelet adhesion, temporary adhesion, and platelet aggregation.

The fluorescent adhesion assay was used to determine the effects of several reagents known to inhibit platelet aggregation: EDTA, RGD peptide, and a monoclonal antibody (MoAb) against GPIIb/IIIa. These reagents completely inhibited platelet aggregation and partially inhibited platelet adhesion. These data suggest that platelet aggregation, ie, the activation of GPIIb/IIIa, participates in the adhesion of platelets under flow conditions. We also analyzed the adhesion of GPVI-deficient platelets under flow conditions. The defect in the GPVI-deficient platelets was similar to the adhesion impairment observed in normal platelets assayed in the presence of these three inhibitors. These results suggest that GPVII also contributes to the platelet adhesion to the collagen surface under flow conditions.

MATERIALS AND METHODS

Blood samples and other materials. Whole blood was drawn from the cubital vein of healthy volunteers into 0.1 vol of 3.8% sodium citrate. The whole blood was directly used for the parallel-post perfusion assay (fixing adhesion assay). Before use in our flow adhesion assay (the fluorescent adhesion assay), the whole blood was first incubated with 5 μmol/L mepacrine (Sigma, St Louis, MO) for 30 to 90 minutes at room temperature. Mepacrine-loaded blood was also incubated for 30 to 60 minutes with 10 mmol/L EDTA or 1 mmol/L gly-arg-gly-aspartic (GRGDS) peptide, which was synthesized in our laboratory using a model 431A peptide synthesizer (Applied Biosystems, Foster City, CA) and the FastMoc method according to the company's protocol. For use as a negative control, GRGDS peptide was also synthesized. Both peptides were purified by reverse-phase high-performance liquid chromatography, and the structures were confirmed by protein sequencing analysis using a model 473A protein sequencer (Applied Biosystems). Blood was also preincubated with 10 μg/mL anti-GPIIb/IIIa MoAb, AP2 or P2. AP2 was previously characterized, and P2 was purchased from Immunotech S.A. (Marseille, France). Both MoAbs showed a similar effect on adhesion.

Perfusion studies: fluorescent adhesion assay. The perfusion chamber was constructed from a polycarbonate distributor, a thin glass, a glass cover slide coated with collagen, and an aluminum base plate, essentially according to the design developed by Hubbell and McIntire. We used a 15- or 20-μm-thick sheet of aluminum foil instead of a 0.2-mm-thick silastic gasket (used in the original design); this decreased the slit height of the chamber and thereby dramatically reduced the blood volume required for the assay. The glass cover slide (50 × 24 mm, 0.3-mm-thick; Takahashi Giken Glass Co, Tokyo, Japan) was coated with type III bovine collagen (0.1 mg/mL; Koken, Tokyo, Japan) in cold HEPES-Tyrode buffer (pH 7.4; 136 mmol/L NaCl, 2.7 mmol/L KCl, 0.42 mmol/L NaH₂PO₄, 12 mmol/L NaHCO₃, 5.5 mmol/L glucose, and 5 mmol/L HEPES) for 1 hour at room temperature. Whole blood loaded with mepacrine was passed through the flow chamber at a controlled flow rate using a syringe pump (model STC-525; Tenno Co, Tokyo, Japan). Shear rates were calculated from the equation obtained by Muggli et al, in which a 1.2-cm slit width and a 0.0015-cm slit height gave a shear rate of 800 sec⁻¹ at a flow rate of 1.3 mL/h. Platelet adhesion and aggregation in the flow chamber were monitored with an inverted-stage microscope (Diaphot-TMD; Nikon, Tokyo, Japan) equipped with an epifluorescence illumination attachment. An ND32 filter was inserted in the excitation light path to reduce the light strength. The fluorescent image was recorded to a video tape recorder with an Argus-50 fluorescence measuring system (Hamamatsu Photonics, Hamamatsu, Japan) that includes a silicon-intensified target (SIT) camera (C2400-08), an image processor (C4015-50), and a computer. The video signals were captured periodically, at 5-second intervals, by a computer while processing background subtraction and rolling averaging of the captured signals using Argus-50 software. The video camera recorded the signals at the rate of 30 frames per second, and then the data were transferred to a computer that averaged more than 64 frames to yield one fluorescence image. Therefore, each image would cover the events occurring in a time period of about 2 seconds. The signals thus obtained represent platelet signals from which noise and moving signals were extracted during processing. These data were further processed to obtain the area occupancy as an indicator of platelet adhesion. The percentages of area occupancy were plotted as a function of time, and the velocity of the area occupancy was obtained from the slope of these plots, which was each curve fitted by linear regression analysis using the program Prism (GraphPad Software, Inc, San Diego, CA). The amount of platelets showing temporary arrest at a particular time was calculated as follows: From each fluorescence image, which was obtained at 5-second intervals, was subtracted the next fluorescence image (ie, the image after 5 more seconds). The fluorescence image thus obtained is the image of platelets that moved within the next 5 seconds. We calculated the area covered by this fluorescence and expressed it as the percentage of area coverage.

As an indicator of platelet aggregation, we calculated the total fluorescence intensities of each platelet aggregate, which was performed by the particle analysis program of the Argus software. This program identified the areas of platelet aggregates as particles and calculated the total intensity in each of these particles. The signals of single platelets and aggregates were differentiated from those due to background by only including particles with diameters of 1 μm or more in the calculations. The average of these signals was calculated, and each one was normalized by dividing it by the average of the total intensities of particles having the size of single platelets. This number was expressed in arbitrary units. To calculate the value with which to normalize the data, particles with diameters of 1 to 2 μm were taken to be single platelets. Because single platelets have weaker fluorescence signals because most of them only transiently stop (ie, temporary arrest), as contrasted with aggregates that are stably attached to the collagen-coated surface, the normalized values would give a reasonable estimate of the number of platelets in an aggregate, but cannot give the exact number.

In the experiment to determine platelet movement in the early stage of blood flow, the fluorescent images were captured at 1-second intervals, with each image corresponding to the averaging of 4 frames (ie, each image covering a time period of about 0.1 seconds). The positions of the platelets were identified in each captured frame, and the movements of adhered platelets were traced. The distance of movement was calculated and plotted as a function of time.

Perfusion studies: fixing adhesion assay. The details of this method were published previously. Plastic coverslips (Miles Scientific, Naperville, IL) were coated with equine tendon collagen (Cromwell Corp, Haverstown, PA) that was sprayed as a solution from an airbrush. Two coated coverslips were inserted into each flat-plate perfusion chamber. Whole blood was circulated through the perfusion chamber at a shear rate of 800 sec⁻¹ by using a Cole-Palmer peristaltic pump (model 7016-20; Masterflex, Chicago, IL). Three different blood aliquots (15 to 20 mL) were circulated for 2, 5, or 10 minutes. Then the two coverslips were removed from the chamber, rinsed with 10 mmol/L phosphate-buffered saline (pH 7.4), fixed with 0.5% glutaraldehyde solution, and stained with 0.02%
toluidine blue solution. The amount of adhered platelets was determined by a light microscope connected to an image analyzer through a video camera, and the extent of adhesion was expressed as a percentage of the total surface of the coverslip screened (% surface coverage).  

Blood from the GPVI-deficient donor. Blood samples were obtained from the individual described in our previous report who had GPVI-deficient platelets. Using two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography of the radiolabeled platelets, we had found that this person had normal levels of GPla/IIa and GPVI. Before using her platelets in the present adhesion studies, we verified that the platelets had a normal level of GPVI by flow cytometry (data not shown). Blood from this individual and control blood were taken at the same time and transferred to the lab. The adhesion assays were started about 5 hours after the blood was taken.

RESULTS

Platelet adhesion onto a collagen-coated surface. When the mepacrine-loaded blood was passed through the perfusion chamber at different flow rates, the fluorescent platelets were found to adhere to the collagen-coated glass surface and form aggregates (Fig 1). Sixty-four frames were averaged to produce each of these fluorescence images. Thus, the fluorescence images, like those in Fig 1, would show adhered platelets that did not move in the flow chamber for about a 2-second period. In Fig 1, the independent fluorescent granules corresponding to single platelets were observed to adhere on the surface at the early stage of flow. After that, aggregates of platelets were observed to form, and their sizes and fluorescence strengths became larger as the flow time increased. The fluorescence patterns of adhesion were similar in the late stage (Fig 1D through F). In contrast, the three patterns obtained during the early flowing time (Fig 1A through C) were different from each other. This indicates that most of the platelets adhering during the early flow time only transiently stopped, moving again within the next 5 seconds.

As a parameter to measure the extent of platelet adhesion, we calculated the percentage of area covered by fluorescence. As indicated in Fig 2, which is typical of the patterns we observed, the area coverage increases biphasically with the increase in flowing time. Because the second phase of platelet adhesion increased almost linearly with time, each curve was analyzed by linear regression analysis to obtain the slope. This slope was defined as the coverage velocity (percentage per second), which serves as the indicator of adhesion in our assay system. As can be seen in Fig 2, the coverage rate increases with the shear rate. The first phase of the adhesion, which is less prominent in some cases, showed lower coverage velocities than did those of the second phase adhesion. The first phase of adhesion was noted to correspond to the time when the platelets adhered as single cells to the collagen-coated surface, as shown in Fig 1A through C.

Temporary arrest of platelets on the collagen-coated surface. To analyze the mechanisms of these two phases of platelet adhesion, we processed the fluorescence images using two different methods. This showed that there are really two different types of adhesion under blood flow, each with defining characteristics. The first method calculated the fluorescence images that moved during the next 5 seconds. This was performed by subtracting the image obtained each 5 seconds by the next one (the images 5 seconds later), and the results were expressed as the percentage of the area covered by platelets. These values are much lower than those of total area coverage (Fig 3). However, in the early stage, the coverage by temporarily adherent platelets was a significant portion of the total coverage, especially under lower flow rates. These results indicate that the adhesion during the early phase of blood flow is mainly composed of temporary adhesion. This was designated as temporary arrest. In this stage, platelets adhere on the collagen surface for a moment and then move to another area or flow away, being carried off by the flowing blood.

Measurement of platelet aggregates. Next, we calculated the extent of platelet aggregate formation. In the later flowing times, platelets adhered to the surface as aggregates (Fig 1). These platelets adhered to each other both horizontally and vertically. The adherent single platelets or platelet aggregates were recognized as fluorescent particles by the image analysis software, and the total fluorescence intensities of each of the particles were calculated. As an indicator of the aggregate formation, we calculated the average of the total fluorescence intensities of the particles. These averages were normalized and expressed in terms of arbitrary units (details given in the Materials and Methods). The average of particle intensity increased linearly with time in a manner similar to the total coverage area. These results suggest that the second phase of platelet adhesion also includes platelet aggregation, ie, platelet-platelet interactions. As shown in Fig 3, the average intensities at lower shear rates tended to be larger than those at higher shear rates.

Measurement of platelet movement in the early stage of blood flow. The fluorescence images were captured each second for 17 seconds, averaging 4 frames to yield each image, and the movements of each single fluorescent platelet were traced during the early stage of blood flow. In the later stage of adhesion, most of the platelets, even single cells, did not move under these conditions. However, in the earlier stage, in which temporary arrest was mainly observed, as described above, most of the adhered platelets were single cells (not aggregated), and there were many moving platelets. The movements of eight platelets that transiently stopped and moved again through the 17-second observation period were traced and summarized in Fig 4. Results were not shown for platelets that showed no movement during the observation period. Figure 4 shows that platelets move and then remain at the same place very randomly, with this behavior being observed as temporary arrest. Among the cells, the speeds of movement are also very different. The arrows in Fig 4 indicate the platelets that flowed away during the observation period, whereas the other cells remained at their positions when the observation was stopped. When mepacrine-labeled blood was perfused over a bovine albumin-coated surface, no platelets, moving or arrested, were detected on the surface (data not shown). This indicates that the platelet movement on the collagen-coated surface is due to the specific interaction between platelets and the coated collagen.
The effects of reagents that inhibit platelet aggregation. Platelet adhesions were also measured in the presence of three different reagents that inhibit platelet aggregation. In the presence of 10 mmol/L EDTA, 1 mmol/L GRGDS peptide, or the anti-GPIIb/IIIa MoAb P2 (10 μg/mL), most of the platelets adhered as single cells under the shear rate of 800 sec⁻¹ (Fig 5A through D). The fluorescence data were treated as those in Fig 3 (in Fig 6, note that the range of the scale is about 10-fold that in Fig 3). Figure 6 shows that, in all of the experiments, temporary arrest was similar to the total area coverage, and the average of the total intensity increased only slightly. These results suggest that most of the platelets adhered temporarily in the presence of these inhibitors, and some portion of the platelets became permanently adhered to the surface, remaining at the same positions even after longer flowing times. However, in the presence of 10 μg/mL of the MoAb P2, the average intensity increased slightly but significantly, suggesting that the platelets can start to aggregate under these conditions because the antibody produces incomplete inhibition at this concen-
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The total area coverage suggests a biphasic adhesion in the presence of GRGDS peptide (Fig 6), although the extent of the second phase is small and did not include platelet aggregation (Fig 5D). GRGES peptide had no effect on platelet adhesion under these conditions (data not shown).

Platelet adhesion in the presence of these reagents was compared with that of normal platelets without any inhibitor. Figure 7, which shows the total area coverage under the shear rate of 200 sec⁻¹, indicates that the level of platelet adhesion in the presence of each of these inhibitors is at the level of the first phase adhesion of normal platelets.

Platelet adhesion of GPVI-deficient platelets. Blood from the GPVI-deficient donor was subjected to the adhesion assay. A typical fluorescence image is shown in Fig 5F, and the area coverage curves of these platelets are given in Fig 8. In Fig 8, the anti-GPⅡb/Ⅲa MoAb AP-2 (10 μg/mL) was added to the GPVI-deficient or control blood. The adhesion of GPVI-deficient platelets decreased significantly under all the tested shear rates: 400, 800, 1,600 (data not shown), and 3,200 sec⁻¹. The platelet adhesion level of the GPVI-deficient platelets was similar to that of the control platelets in the presence of anti-GPⅡb/Ⅲa antibody. The fluorescence microscopic photo (Fig 5F) shows that the GPVI-deficient platelets adhered to the collagen-coated surface independently and did not form aggregates. This phenomenon is similar to the adhesion of normal platelets in the presence of the aggregation inhibitors (Fig 5B through D). These results suggest that the defective adhesion of GPVI-deficient platelets to the collagen-coated surface under blood flow is responsible for the lack of collagen-induced aggregation in these platelets.

The adhesion of GPVI-deficient platelets under blood flow was also measured by the fixing adhesion assay. After histologic processing of the coverslips, the surface covered by platelets was analyzed in individual microscope fields by image analysis and was expressed as the percentage of the total surface screened. Values of the percentage of area coverage obtained in the experiments using control blood were 18.67% ± 1.3%, 23.97% ± 1.9%, and 34.7% ± 5.2% after perfusions of 2, 5, and 10 minutes, respectively. When the coverslips were perfused with blood aliquots from the GPVI-deficient donor, the percentage of area coverage (19%, 24.4%, and 30.2% after 2, 5, and 10 minutes, respectively) did not differ significantly from those observed with control
The method for monitoring in vitro platelet adhesion to extracellular matrix proteins under flow conditions has been undergoing development since 1973, when Baumgartner et al \cite{1} first showed platelet adhesion to denuded aorta in flowing blood. These methods for measuring platelet adhesion under flow conditions are valuable for analyzing the mechanism of in vivo thrombus formation because they provide conditions more closely approximating the physiologic environment in which platelet adhesion actually takes place. In contrast, adhesion assays performed under static conditions are far less likely to reflect what is happening in vivo. We have devised the fluorescent adhesion assay, a method to measure platelet adhesion under flow conditions that uses a flow cell based on the design of Hubbell and McIntire, \cite{2,3} but modified to dramatically reduce the blood volume required. Using computer analysis of the fluorescence image data obtained by our system, we developed methods to quantify three distinct reactions involved in platelet adhesion under flow conditions.

For the flow cell, we used a 15-\(\mu\)m slit height for flat flow instead of the 0.2-mm slit height used by many researchers. This substantial decrease in blood volume resulting from the slit height reduction made it possible to measure platelet adhesion under many different conditions and made it practical to use valuable antibodies at an effective concentration for inhibiting platelet adhesion. As little as 4 mL of blood was sufficient to perform experiments under 5 different shear rates. However, such a small slit height gave rise to a high background and a high level of noises that mainly come from the fluorescent leukocytes and platelets flowing near the coated surface. This problem was solved by computer software that subtracted the appropriate background level and then applied a averaging procedure on the raw data continuously captured on video tape. We also encountered another problem, ie, a strong light beam, such as that used in the epifluorescence microscope, would affect platelet adhesion. Ross et al \cite{4} reported that there was higher platelet accumulation on the area of the surface exposed to extended illumination, and we also noticed a similar phenomenon. Because decreasing the slit height would exacerbate this effect, we used an ND32 filter to reduce the light strength and increased the sensitivity of the SIT camera. To verify that these changes solved the problem, we usually compared the adhesion pattern in the illuminated area with that in a nonilluminated area after finishing the assay about 3 minutes later and confirmed that there was no effect of illumination on platelet adhesion.

Another advantage of the fluorescent adhesion method is its real time measurement of platelet adhesion. When we plotted the time course of area coverage by fluorescence, the percentage of area coverage increased biphasically, and the increase in the second phase was almost a linear function of time (Fig 2). This is the first report showing that the adhesion of platelets is biphasic under flow conditions. The first phase of reaction mainly consisted of the phenomenon of temporary arrest, and the second phase represented the main portion of the adhesion when the data are expressed as the area coverage. Therefore, we calculated the velocity of area coverage of the second phase and took this as an indicator of platelet adhesion. The values obtained from five to seven experiments at different shear rates (Table 1) showed that the platelets from normal individuals show adhesion falling with a characteristic range for each shear rate. Thus, the area coverage velocity can serve as a parameter for quantitating platelet adhesion. Although we attempted to calculate the velocity of area coverage in the first phase, the values obtained were not reproducible because all the values were very low and only a small number of data points could be measured unless measurements were started much earlier, which is technically not feasible.

Hubbell and McIntire originally presented the platelet adhesion as three-dimensional pictures, \cite{5} and then Wagner and Hubbell \cite{6} calculated the total number of adhered platelets as an indicator of platelet adhesion. In other studies from McIntire’s group, \cite{7,8,9} the number of platelets in each aggregate was also calculated. These calculations of platelet numbers would indicate the extent of platelet aggregation rather than the platelet adhesion under flow conditions. As an indicator of platelet aggregation, we calculated the average of the total intensity of the aggregates. This value would repre-
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Fig 5. Fluorescence images of adhered platelets. Fluorescence images under the different conditions were captured at 60 seconds after the start of flow. (A) Normal platelets. (B) Platelet adhesion in the presence of 10 mmol/L EDTA. (C) Platelet adhesion in the presence of MoAb P2 (10 μg/mL). (D) Platelet adhesion in the presence of 1 mmol/L GRGDS peptide. (E) Adhesion of control platelets in the experiment on the GPVI-deficient platelets. (F) Adhesion of GPVI-deficient platelets. In all the experiments, the shear rate was 800 sec⁻¹.

sent the extent of the aggregation as a whole, although it shows a wide deviation because new platelet depositions were always observed when the blood was flowing. The average of the total intensity of the aggregates increased almost linearly with time, indicating growth of the platelet aggregates under flow conditions. Under a lower shear stress, platelet aggregates tended to have higher average fluorescence intensities than those at higher shear stress. Also, the average fluorescence densities of the aggregates under the lower shear rates was higher than those at the higher shear rates (data not shown). These results suggest that aggregates formed under lower shear stress are vertically larger. A reasonable scenario for this might be that, under a low shear stress, platelets are able to form higher stacks, as opposed to covering a larger area, whereas under a high shear rate, such high stacks of platelets would be quickly destroyed by the rapidly flowing blood. In theory, this tendency would be magnified in our flow method, because the narrow slit height used would increase the actual shear rate over the stack of platelets more than the conventional flow method that uses a 10-fold larger slit height. However, the actual increase of shear rate would not be very great, because we observed that, upon the formation of large aggregates, the flow direction of the main stream of blood became modified so that it flowed around the sides of the platelet aggregate (data not shown).

One of the new findings in this report is the observation that platelets temporarily adhered on the collagen-coated surface. This phenomenon, the temporary arrest, was first noticed by observing the movement of many fluorescent platelets over the coated surface and further evidenced by the diverse fluorescence image patterns in the early phase of adhesion (Fig 1). To analyze this temporary arrest, at each
The platelet adhesion was measured in the presence of platelet aggregation inhibitors. The three inhibitors (EDTA, anti-GPIIb/IIIa antibody, and RGD peptide) each showed a similar effect. They all inhibited aggregate formation under blood flow (Figs 5, 6, and 7). The inhibition of platelet adhesion and aggregation under flow by anti-GPIIb/IIIa antibodies was reported by other investigators. Because all these substances inhibit the activity of GPIIb/IIIa, although the actions of EDTA and RGD peptide are not restricted to only GPIIb/IIIa, GPIIb/IIIa would be activated and mainly be involved in the formation of aggregates in the course of adhesion under flow conditions. The presence of the activated form of GPIIb/IIIa on the surface of adhered platelets was shown by immunoelectronmicroscopy. These inhibitors also reduced the platelet adhesion expressed as the decrease of total area coverage of platelets, which suggests that the activation of GPIIb/IIIa would also be related to the formation of stably arrested platelets under flow conditions. Figures 6 and 7 indicate that these reagents inhibited only the second phase adhesion of platelets. Therefore, the second

time point of the assay, we calculated the fluorescent platelets that did not stay in the same place for the next 5 seconds. The values for the temporary arrest, which were each expressed as a percentage of the area coverage, were small, but some low level of temporary arrest was seen throughout the entire assay period. After an increase in the early phase of adhesion, it decreased slightly and thereafter remained at a constant level (Fig 3). The nature of the temporary arrest was further analyzed by tracing the movement of each adhering platelet (Fig 4). Platelets were shown to adhere to and then move off the surface randomly, and this short stay on the surface would be measured as temporary arrest. A similar phenomenon of cell movement under flow conditions has been observed as the rolling of leukocytes on endothelial cells. We could not directly observe the rolling of platelets under our flow conditions because platelets are too small to allow a direct observation of rolling. Because the rolling of leukocytes was reported to be a more continuous movement than the stop-and-go movement detected in the present study, the movement of platelets under our flow conditions may not be analogous to rolling.

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Fig 8. Area coverage of GPVI-deficient platelets. Area coverages of control (■) and GPVI-deficient (□) platelets plotted as a function of time under three different shear rates. Linear regression curves were also calculated. (△) The area coverage of control platelets in the presence of 10 μg/mL monoclonal anti-GPllb/llla antibody. (□) The area coverage of GPVI-deficient platelets in the presence of anti-GPllb/llla MoAb.

Because GPVI was reported to be involved in the platelet adhesion to a collagen-coated surface under static conditions, we analyzed the adhesion of GPVI-deficient platelets with the fluorescence adhesion method to determine the involvement of this glycoprotein under flow conditions. In this experiment, the adhesion of control platelets was lower than that of freshly obtained cells, probably due to the longer time of transport (about 5 hours). The adhesion level of the GPVI-deficient platelets was similar to that of the control sample tested in the presence of the anti-GPllb/llla antibody (Fig 7). The same GPVI-deficient sample was subjected to the flow adhesion assay with a fixing method (Fig 8). In this assay, the aggregate formation on the collagen surface was inhibited in the GPVI-deficient platelets, but the extent of platelet adhesion was similar to that of the control platelets. We do not have an exact explanation for this discrepancy, but it might be due to differences in the condition of the collagen coated on the cover slip surface. In the fixing adhesion method, the suspension of equine type I collagen fiber was sprayed over plastic cover slips and dried before use. On the other hand, in the fluorescent adhesion assay, bovine type III collagen was coated by adsorption on the glass cover slide, without becoming dry at any time during the experiment. The high concentration of collagen yielded by the spray method may have assisted collagen adhesion under flow conditions. The present results suggested that GPVI is involved in platelet adhesion under flow conditions, especially in the process involved in the formation of platelet aggregates.

As to the mechanism of platelet adhesion onto the collagen-coated surface under the flow condition, GPIb/IX on the platelet surface and von Willebrand factor in the plasma were reported to be involved in this process, especially under a high shear rate (> 1,500 sec⁻¹). Also, the contribution of GPllb/llla, especially in the activated form, to platelet adhesion was shown. The activated GPllb/llla would bind to two ligands in the plasma. One is fibrinogen and the binding with fibrinogen would form platelet aggregates. The other ligand is von Willebrand factor, which stimulates the adhesion on the collagen surface. These previous observations and the cumulative results of our present study enable us to form an hypothesis about the events involved in platelet adhesion under the flow condition. In the first reaction, platelets would interact with the coated collagen, probably through a GPIb/IX-von Willebrand factor interaction. This fast and rather weak interaction would reduce the movement of platelets in the blood flow and sometimes result in a transient stoppage of the movement (temporary arrest). Recently, Savage et al published a report describing their observations of platelets moving on a von Willebrand factor-coated surface under the flow condition using an apparatus similar to our system described here. Because the temporary
Fig 9. Micrographs of adhered control (A) and GPVI-deficient (B) platelets (fixing adhesion method). Control and GPVI-deficient blood was circulated through the perfusion chamber for 2 minutes, and the adhered platelets were fixed and observed by microscopy.
arrest that we observed would correspond to the platelet movement on von Willebrand factor, their results would further support our hypothesis of the involvement of the von Willebrand factor-GP Ib interaction in the phenomenon of temporary arrest. Some of the temporarily arrested platelets would remain for a longer period on the surface and could then be activated by the interaction with von Willebrand factor. The activation of platelet GP Ib/IIa by von Willebrand factor has been reported before.\textsuperscript{29,30} However, GP VI and probably GP Ia/IIa\textsuperscript{30,31} would also be involved in this activation. This is reasonable because the mechanism of immobilizing platelets on the collagen fibers and the subsequent activation of platelets would be a complex reaction and many platelet proteins would be involved in the reaction. Because the area coverage rate of platelets in the second phase was much faster than that in the first phase, activated platelets would have modified surface structures that make them more adhesive towards other platelets, resulting in increased aggregability. The mechanistic details of platelet activation under flow conditions remain to be explored.

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Analysis of platelet adhesion to a collagen-coated surface under flow conditions: the involvement of glycoprotein VI in the platelet adhesion

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