The Differential Involvement of von Willebrand Factor, Fibrinogen and Fibronectin in Acute Experimental Thrombosis in Rat Cerebral and Mesenteric Microvessels

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Abstract: Arterial thrombi are primarily composed of platelets. Platelets are bound to injured endothelial cells, sub-endothelial matrices, and other platelets by a range of adhesive proteins. Some of these reactions are governed by shear-forces. The role of adhesive proteins in the pathogenesis of arterial thrombosis is not fully understood. The aim of this study was to examine the involvement of von Willebrand factor (vWF), fibrinogen (Fg), and fibronectin (FN) in the formation of microvascular thrombi *in vivo* using a helium-neon laser-induced thrombosis method. Transmission electron microscopy demonstrated that laser irradiation resulted in platelet-rich thrombosis in arterioles and venules, and revealed that this occurred in the absence of

endothelial denudation. The mean wall shear rates in mesenteric arterioles and venules were 641 ± 40 and $280 \pm 20 \text{ s}^{-1}$, respectively. Shear rates increased approximately fivefold in arterioles and tenfold in venules during the formation of occlusive thrombi. Antibody to vWF inhibited thrombosis in arterioles and venules. Antibodies to Fg and FN inhibited thrombosis in venules but not in arterioles. These results confirm that vWF, Fg, and FN were involved in thrombogenesis *in vivo* and demonstrated that significantly higher shear rates were required for the reactions involving vWF than those involving either Fg or FN. [Japanese Journal of Physiology, 47, 431–441, 1997]

Key words: thrombosis, platelet aggregation *in vivo*, adhesive protein, von Willebrand factor, fibrinogen.

Thrombosis in arteries may develop in narrowed blood vessels in association with stenosis and/or spasm where high shear-forces are produced. Under these circumstances, thrombi are primarily composed of platelets which are activated locally, adhere to injured endothelial cells or subendothelial structures, and aggregate to extend the thrombus.

Several animal models have been adopted to inves-

tigate thrombosis in large blood vessels [1–7] and microvessels [8–11]. The helium-neon (He-Ne) laser-induced model is a straightforward technique with high reproducibility which produces platelet-rich thrombi in mesenteric and cerebral arterioles and venules as shown by light and transmission electron microscopy [8, 9]. This method has been shown, therefore, to be potentially useful to study mechanisms of acute

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platelet-rich thrombus formation.

Studies of platelet reactions *in vitro*, together with investigations of patients and animals with von Willebrand's disease, have shown that platelets are bound to subendothelial matrices and to each other by mechanisms utilising adhesive glycoproteins such as vWF [12–16], Fg [17–19], and FN [20–23]. Moreover, the precise role of these ligands is governed by localised haemodynamics [24–32].

The aim of this study was to examine the morphological features of laser-induced thrombi using transmission electron microscopy with special reference to the damage of endothelial cells, and to identify possible roles for vWF, Fg, and FN in shear-related platelet-rich thrombosis in microvessels *in vivo*.

MATERIALS AND METHODS

Materials

Animals. Male Wistar/ST rats (SLC Co. Ltd., Japan) of 240–330 g (8–9 weeks) were used. They were maintained in compliance with the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences," published by the Physiological Society of Japan.

Antisera. Polyclonal antiserum to rat vWF was obtained by immunising rabbits with purified rat vWF, prepared as previously described [33]. The antiserum was diluted threefold with saline prior to use. Another polyclonal antibody to human vWF (Dako, USA) was also used in preparing the agarose gel containing anti-vWF antibody. Anti-rat Fg antibody (goat IgG fraction to rat Fg, 5.45 mg IgG/ml, Organon Teknika Corporation, USA) was injected without dilution. Anti-rat FN (rabbit antiserum to rat FN, UCB-Bioproducts, USA) was diluted fivefold with saline.

Methods

Transmission electron microscopy. Mesenteries were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1% OsO₄. They were dehydrated through a graded acetone series and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed under a JEM 2000EX electron microscope.

Administration of antibodies to adhesive proteins. Antiserum or IgG fractions were injected through canulae into the femoral vein (0.77 ml/kg), followed by Evans blue. Normal rabbit serum diluted with saline (at the same dilution factor as antisera) or saline were substituted for antisera or IgG and used as controls.

Formation of thrombi in pial and mesenteric microvessels

Pial blood vessel preparation. Closed cranial

windows were created using the technique described by Sasaki *et al.* [34]. Briefly, animals were anaesthetised with intraperitoneal Nembutal (60 mg/kg), respired artificially with a mixture of air and oxygen (4:1), and held in a stereotaxic frame on the stage of an operating microscope. The left parietal cortex was exposed by craniectomy in the centre of the left parietal bone using a hand drill. The dura mater was removed under the operating microscope and the exposed pial surface was superfused with artificial cerebrospinal fluid (CSF; pH 7.35) warmed to 37°C and continuously gassed with 6% O₂, 10% CO₂, and N₂. Polyethylene tubing (PE50, Nippon Becton-Dickinson, Tokyo, Japan) was used to superfuse the CSF, and a cover glass 14 mm in diameter was placed over the window resting on dental resin. The pressure in the intracranial window was maintained at 5 mm mercury to avoid brain herniation. Arterioles and venules with an inner diameter of 30–40 µm were selected for study.

Mesenteric blood vessel preparation. Rat mesenteric blood vessels were utilised as previously described [35]. In outline, animals were anaesthetised with intramuscular Nembutal (60 mg/kg) and supported on a specially designed microscope stage. An intestinal loop was exposed surgically and secured flat on the operating stage. Arterioles (ID: 20–25 µm) and venules (ID: 30–35 µm) were used for the antibody experiments.

He-Ne laser-induced thrombosis. Thrombi were induced following the procedure of Kovacs *et al.* [8] with slight modification [35]. Evans blue (21 mg/kg for pial blood vessels and 14 mg/kg for mesenteric blood vessels) was injected into the animals via a femoral vein, and blood vessels were irradiated on the microscope stage at the focal plane with the He-Ne laser at a power of 21 mW for venules, 10 mW for pial arterioles, and 16 mW for mesenteric arterioles. The blood vessels were observed on a video monitor, and repeatedly irradiated with the laser beam for 5 s at 30 s intervals. The number of pulses necessary to completely occlude the blood vessels was used as an index of the effects of the various agents on thrombosis. The thrombi were continuously monitored for 50 or 60 min.

Estimation of vWF, Fg, and FN in plasma after intravenous administration of antibodies. Antibody to vWF, Fg, or FN was injected into the femoral vein (0.77 ml/kg). Blood was collected from the jugular vein (blood:3.2% citrate=9:1) just before injection and 10, 30, and 50 min after injection. Platelet poor plasma was prepared by centrifugation (3,000 rpm, 15 min, 4°C) and the content of adhesive proteins in the plasma was estimated by the Laurell method. The

concentrations of adhesive proteins after the administration of antibody were compared with those before injection.

Inhibition of collagen-induced platelet aggregation by antibodies to vWF, Fg, or FN. This was measured by the whole-blood aggregation method using a Chronolog C-540 aggregometer. Briefly, 5 μ l of antibody to rat vWF, Fg, or FN diluted as shown in MATERIALS was added to 500 μ l of citrated blood (blood:3.2% citrate=9:1) and incubated for 2 or 15 min at 37°C with stirring (1,000 rpm). To the mixture, 500 μ l of Tyrode solution containing 2 mM CaCl_2 was added. The mixture was incubated for 1 min and platelet aggregation was induced with 5 or 20 μ l collagen (1 mg/ml, Collagen reagent Horm). In this experiment, 1/100 volume of antibody was added to blood to correspond to the concentration expected in the laser experiment (0.77 ml antibody solution/kg).

Measurement of blood velocity. Erythrocyte velocity was measured by the fiber-optic laser-Doppler method [36]. Two He-Ne laser beams were crossed on the lumen of the blood vessel through the objectives of the microscope. Light scatter, reflected by the erythrocytes, was recovered by a collecting fiber optic and the signal, transformed by a photomultiplier, was analysed on a personal computer. Mean *in vivo* red cell velocities were converted to mean wall shear rates using the formula;

Mean wall shear rate = $8 \times (\text{mean flow velocity} / \text{blood vessel inner diameter})$.

Statistical analysis. The differences in the mean number of irradiations necessary to induce occlusive thrombosis were analysed by the Mann Whitney *U*-test, correlation coefficient by the Pearson test, regression line by the least squares test, and platelet aggregation and the content of adhesive proteins in plasma by paired Student's *t*-test. Values are expressed as means $\pm$ SEM.

RESULTS

Electron microscopic observation of thrombi in microvessels

The thrombi formed in mesenteric arterioles and venules were examined with transmission electron microscopy. They were composed of packed platelets, and degranulation and pseudopod formation were observed in many platelets (Fig. 1). However, although the endothelial membrane was slightly damaged in some preparations and electron-dense intracellular structures were occasionally observed, the monolayer remained and gap formation between endothelial cells was not evident. Similar morphological features of the

endothelium were seen in both arterioles and venules, although platelets appeared to be more loosely packed in venules than in arterioles. Leucocytes were often incorporated into the thrombi in venules.

Velocities and shear rates in pial and mesenteric microvessels

The erythrocyte velocities and calculated wall shear rates in the mesenteric microvessels are shown in Fig. 2. Erythrocyte velocity increased with diameter in both arterioles and venules. Calculated wall shear rates remained constant, however, over a wide range of diameters in arterioles, and decreased slightly in larger venules. The mean shear rates ($\pm$ SEM) observed in the arterioles (ID: 20–25 μ m diameter) and venules (ID: 30–35 μ m) were 641 ± 40 and $280 \pm 20 \text{ s}^{-1}$, respectively.

In contrast to the mesenteric microvessels, a considerable difference in shear rates was observed in the pial arterioles and venules. The calculated mean shear rate in pial arterioles (ID: 30–40 μ m) was $1,935 \pm 102$ and $179 \pm 18 \text{ s}^{-1}$ in pial venules (ID: 30–40 μ m). The shear rate in the pial arterioles was statistically related to vessel diameter (Fig. 3) [37].

Changes in shear rates during thrombus formation

In this study thrombi were formed in mesenteric and pial microvessels with diameters described above and where the calculated overall mean shear rates were 641 and 280 s^{-1} , in mesenteric arterioles and venules, and 1,935 and 179 s^{-1} in pial arterioles and venules, respectively. In order to examine shear rates more closely during thrombosis, erythrocyte velocities were measured in mesenteric arterioles (ID: approximately 40 μ m) and venules (ID: approximately 50 μ m) during the formation of occlusive thrombi. The mean wall shear rates were calculated assuming that the stenosed lumen was circular and red-cell velocity pattern was parabolic. The changes in mean wall shear rates are shown in Fig. 4. The mean wall shear rates in arterioles and venules were 448 ± 59 ($n=5$) and $208 \pm 24 \text{ s}^{-1}$ ($n=6$), respectively, prior to thrombosis. The shear rates increased, however, as the diameter of the blood vessels were occluded by the developing thrombus, and reached similar levels at the peak shear rates; $2,266 \pm 71 \text{ s}^{-1}$ in arterioles and $2,150 \pm 195 \text{ s}^{-1}$ in venules. Shear rates decreased as stenosis progressed.

Inhibition of thrombus formation by anti-vWF, anti-Fg, and anti-FN

The injection of specific antibody to rat vWF inhib-

Fig. 1. Transmission electron microscopic observation of a thrombus formed in mesenteric arteriole and venule. (A) Arteriole without laser irradiation (control), magnification, $\times 4,400$; (B) laser-induced thrombus in an arteriole, $\times 3,600$; (C) thrombus in an arteriole, the arrow shows an electron-

dense endothelial cell induced by irradiation, $\times 6,400$; (D) Venule without laser irradiation (control), $\times 2,000$; (E) laser-induced thrombus in a venule, $\times 2,200$; (F) thrombus in a venule, $\times 4,500$, the arrow shows an injured part of an endothelial cell. Bars = $1\ \mu\text{m}$.

Fig. 2. Relationship between mean erythrocyte velocities (A) or wall shear rates (γ) (B) and vessel diameters in mesenteric microvessels. (A) Arterioles: velocity (V) = $9.8 \times 10^{-2} \times \text{diameter } (D) - 0.41$, $r = 0.596$, $p = 0.0001$, $n = 182$; venules: $v = 2.4 \times 10^{-2} \times D + 0.92$, $r = 0.405$, $p = 0.0001$, $n = 218$. (B) Arterioles: $\gamma = -1.9 \times 10^{-5} \times D + 0.80$, $r = 0.001$, $p = 0.995$; venules: $\gamma = -2.0 \times 10^{-3} \times D + 0.46$, $r = -0.218$, $p = 0.0012$.

Fig. 3. Relationship between mean wall shear rates and diameters in pial microvessels. Arterioles: $\gamma = 27 \times D + 970$, $r = 0.497$, $p = 0.0001$, $n = 33$ rats; venules: $\gamma = -0.24 \times D + 200$, $r = -0.194$, $p = 0.2019$, $n = 4$ rats. Wall shear rates were reproduced from fig. 4 of Seki *et al.* [37].

Fig. 4. The change in shear rates during thrombosis. Five typical data for arterioles and six for venules are shown.

Fig. 5. Inhibition of thrombosis by anti-vWF antiserum in pial (A) and mesenteric (B) microvessels. O, control group; ●, anti-vWF rabbit antiserum group; $n=7-14$ and $6-8$ in each stage of pial and mesenteric microvessels, respectively. * $p < 0.05$, ** $p < 0.01$.

ited the formation of occlusive thrombi in arterioles and venules (Fig. 5). Significant differences between the controls and treated animals were observed from approximately 20 min after the initial injection and generally persisted for 50 min.

The effects of specific antibody to Fg on the occlusion of pial and mesenteric blood vessels are illustrated in Fig. 6. Significant inhibition of thrombus for-

mation was demonstrated in venules. There was a tendency for the antibody to delay thrombosis in arterioles, but the difference did not achieve statistical significance.

The injection of specific antibody to FN caused a significant delay in thrombus formation in pial venules but not in pial arterioles (Fig. 7A). A similar tendency was noted for mesenteric blood vessels (Fig. 7B).

Fig. 6. Inhibition of thrombosis by anti-Fg antibody in pial (A) and mesenteric (B) microvessels. $\circ$, control group; $\bullet$, anti-Fg antibody group; $n=6-8$ in each stage. * $p < 0.05$, ** $p < 0.01$.

Fig. 7. Inhibition of thrombosis by anti-FN antiserum in pial (A) and mesenteric (B) microvessels. $\circ$, control group; $\bullet$, anti-FN antiserum group; $n=6-15$ and $6-8$ in each stage of pial and mesenteric microvessels, respectively. * $p < 0.05$, ** $p < 0.01$.

Table 1. Antithrombotic effect of antibodies to adhesive proteins in rat pial and mesenteric microvessels.

	Mesenteric vessels		Pial vessels	
	Arteriole	Venule	Arteriole	Venule
Anti-Fg	+	+++	+	++
Anti-FN	+	+++	—	+++
Anti-vWF	+++	+++	+++	+++
Wall shear rate (s ⁻¹)	641	280	1,935	179
(diameter range, μ m)	(20–25)	(30–35)	(30–40)	(30–40)

—, no inhibition; +, inhibitory tendency, but not significant; ++, $p < 0.05$; +++, $p < 0.01$.

The inhibitory effects of these antibodies on thrombogenesis are summarised in Table 1 together with the observed wall shear rates. Of special notice is that anti-FN did not inhibit thrombus formation in any of the pial arteriole experiments.

Changes in vWF, Fg, and FN in the circulation after injection of antibodies

The concentrations of adhesive proteins in circulating blood were not reduced significantly after antibody injection.

Inhibition of collagen-induced platelet aggregation by antibodies to adhesive proteins

The effects of antibodies on platelet aggregation were examined by incubating whole blood with specific antibodies for 2 or 15 min at 37°C using 5 or 20 μ l collagen. Significant inhibition of platelet aggregation was not observed.

DISCUSSION

Many studies *in vitro* have previously indicated that localised shear forces play an important role in platelet binding in thrombosis [24–32]. In this study, we examined the development of microvascular thrombi *in vivo* in arterioles and venules exhibiting a broad range of vessel wall shear rates using a He-Ne laser-induced thrombosis model. This method provides an accurate and highly reproducible model for studying thrombogenesis. However, the relevance of such microvascular events to the pathology in human thrombosis remains to be clarified. In particular, the role of shear stress and extent of vascular damage in the laser-irradiated small vessels have not been fully assessed in the animal model.

In this study, the mean wall shear rates appeared to be relatively constant in a spectrum of mesenteric ar-

terioles and venules as well as pial venules with the same inner diameter, although considerable variations were observed in vessels with the same inner diameter. In contrast, the shear rate in pial arterioles was threefold higher than that in mesenteric arterioles and was similar to the rates reported by Ma *et al.* [38] and Seki *et al.* [39]. Furthermore, the shear rate in pial arterioles was dependent on vessel diameter. The constant shear stress hypothesis proposed by Mayrovitz and Roy [40] is supported, therefore, by our data for mesenteric arterioles but is not consistent with our findings for pial arterioles. Blood viscosity is considered to be low in microvessels due to the Fahraeus-Lindqvist effect and may be changed by stenosis during thrombogenesis [41–43]. Nevertheless, assuming that a blood viscosity of 0.03 s dyn/cm² was a reasonable approximation in the current experiments [44], the calculated wall shear rates corresponded to shear stresses of 6–51 (mean 19.2) dyn/cm² in mesenteric arterioles with diameters of 20–25 μ m and 3–36 (mean 8.4) dyn/cm² in venules of 30–35 μ m. These figures are similar to those reported by Seki *et al.* [45], but somewhat lower than those noted for arterioles by Sato and Ohshima [11].

Shear rates increased approximately fivefold in arterioles and tenfold in venules during the formation of occlusive thrombi (Fig. 4). These findings indicate that thrombosis occurred between approximately 10 to 100 dyn/cm² in mesenteric microvessels and between approximately 60 to 300 dyn/cm² and 6 to 60 dyn/cm² in pial arterioles and venules, respectively. Shear stress in arteries is known to be less than that in arterioles, but peak shear stresses in normal aorta and coronary arteries range from 150 to 350 dyn/cm² [46]. Shear stress can reach 3,000 dyn/cm² or more in constricted arteries [47]. It is not possible, however, for shear-forces to reach such high levels in microvessels, as stenosis in one vessel diverts flow through neighbouring branches, an option not available in larger vessels. Nevertheless, our findings indicate that shear stress during thrombosis in microvessels can reach close to the levels seen in stenosed arteries. Furthermore, He-Ne laser irradiation induces platelet-rich thrombosis in arterioles as well as venules [8, 9]. Our data confirm, therefore, that this type of study of the microcirculation could help to clarify important mechanisms of arterial thrombosis.

There is controversy, however, on the degree of endothelial damage induced by He-Ne laser irradiation. Evans blue is thought to convert laser energy to heat, and this has been reported to cause endothelial denudation in mesenteric small arteries [8] but not in microvessels [9]. This study confirmed that He-Ne

laser irradiation does not induce endothelial denudation in microvessels. Vasoconstriction was observed at sites upstream and/or downstream from the centre of irradiation although no such vasoconstriction was seen at the laser points where thrombi were formed. It is possible, therefore, that high-energy irradiation induced functional disturbances of endothelial cells in the absence of overt morphological changes. Thrombogenesis might be mediated by impairment of antiplatelet mechanisms such as the release of prostacyclin and nitric oxide.

Platelet adhesion and aggregation are known to be modulated by shear-dependent interactions of intercellular receptors and adhesive proteins. Fg and FN are major ligands of platelet reactions in conditions of low shear, whereas vWF is involved in high-shear-dependent mechanisms [24–32]. Using specific polyclonal antibodies to these glycoproteins, we demonstrated that thrombus formation was inhibited dependent on haemodynamic conditions. Mean shear rates (shear stress) obtained in the current study were 280 s^{-1} (8.4 dyn/cm^2) and 641 s^{-1} (19.2 dyn/cm^2) for mesenteric venules and arterioles, respectively, but the peak shear stresses were similar (approximately 90 dyn/cm^2) for both vessels during thrombosis. It appears, therefore, that the role of Fg and FN in platelet function *in vivo* is most prominent under low-shear conditions, for example less than approximately 20 dyn/cm^2 , whereas the role of vWF in these mechanisms is most evident under high shear, such as greater than 20 dyn/cm^2 . These findings are in keeping with those of others [27, 29] who have demonstrated *in vitro* that the adhesive proteins may influence platelet function in a shear-dependent manner. The shear stress threshold for vWF appeared to be lower in our *in vivo* experiments than in previously reported *in vitro* studies. The reasons for the discrepancy have not been elucidated but may be due at least in part to the co-existence of erythrocytes or leucocytes, and specific properties of the vascular wall.

The concentrations of antibodies used in the laser experiment did not affect the levels of the respective proteins in circulating plasma, and did not impair whole-blood platelet aggregation *in vitro*. Nevertheless, laser-induced thrombus formation was inhibited *in vivo* 10 or 20 min after the injection of antibody. It is reasonable to speculate that anti-vWF would not repress collagen-induced platelet reactions under low shear [29], but both anti-Fg and anti-FN could be expected to interfere with these mechanisms under similar conditions. Our findings suggest that antibody might have accumulated on the surface of cells, such as endothelial cells and platelets, and at sufficient con-

centration could have obstructed cell-cell interactions which may be mediated essentially by the localised release of specific glycoproteins from Weibel Palade bodies in endothelial cells or α -granules in platelets.

Overall, these morphological and rheological data support our hypothesis that the laser-induced thrombosis technique provides a useful model for human haemostasis and thrombosis *in vivo* without causing structurally marked vessel wall injury. The adhesive proteins, von Willebrand factor, fibrinogen and fibronectin, were shown to be involved in microvascular thrombosis although there were significant differences in the roles of the different macromolecules in the development of vascular occlusion in arterioles and venules. The results are consistent with previous *in vitro* studies which demonstrated that haemodynamic forces play an important role in platelet-rich thrombogenesis. The findings presented here have confirmed and extended the role of adhesive glycoproteins in vascular occlusion and offer additional information for strategies for thrombolytic and preventive therapy of arterial thrombosis.

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