Application of Ex Vivo Flow Chamber System for Assessment of Stent Thrombosis

Mamoru Sakakibara,* Shinya Goto,* Koji Eto, Noriko Tamura, Takaaki Isshiki, Shunnosuke Handa

Objective—Factors influencing platelet accumulation around stents were to be investigated by an ex vivo flow chamber system.

Methods and Results—Platelet accumulations on collagen surfaces under flow conditions were augmented in the presence of stents, especially at sites downstream from coil stents. Densitometric analysis revealed that 4.9±0.8 times more platelets accumulated downstream from coil stents than were formed downstream from tube stents ($P<0.01$), suggesting that stent morphology is an important determinant factor of its thrombogenicity. Platelet accumulations around stents were significantly inhibited by a combination of ticlopidine and aspirin, whereas aspirin alone produced only modest inhibition. Anti–glycoprotein IIb/IIIa (abciximab) inhibited platelet accumulation around stents in a dose-dependent manner, whereas the antibody blocking von Willebrand factor binding to glycoprotein Ibα, which had been shown to inhibit platelet thrombus formation under high shear rates, did not inhibit the accumulation downstream from the coil stents. Our results suggest that the important characteristics of in vivo stent thrombosis, ie, augmented platelet accumulation with coil stents and the strong antithrombotic effect of the combination antplatelet agents and an anti–glycoprotein IIb/IIIa, can be reproduced in ex vivo perfusion model.

Conclusions—We conclude that an ex vivo perfusion system is useful in the assessment of the thrombogenicity of various stents and in the screening of effective antplatelet agents. (Arterioscler Thromb Vasc Biol. 2002;22:DOI:10.1161/01.ATV.0000027102.53875.47)

Key Words: stent thrombosis • platelets • glycoproteins • flow chamber • von Willebrand factor

Coronary stent implantation is now an established procedure for the prevention of abrupt occlusion after unsuccessful coronary interventions.1 However, thrombotic occlusion of the coronary arteries2–4 (even though the incidence has been greatly reduced by high-pressure inflation and appropriate antithrombotic therapy)5 and restenosis occurring later in ≈15% of patients6 are unresolved issues regarding the use of stents. The results of clinical trials have clearly demonstrated that therapy with antplatelet drugs, a combination of aspirin and ticlopidine, was more effective than persistent powerful anticoagulation with an oral anticoagulant,7–5,7 suggesting an important role of platelets in the onset of stent thrombosis. Platelet accumulation around the stent is also believed to play a role in the later event of restenosis via local release of bioactive materials, such as platelet-derived growth factor8,9 or recruitment of leukocytes,10 although there are, as yet, no convincing clinical data showing the effects of antplatelet agents on restenosis.11 Nevertheless, it is reasonable to speculate that inhibition of the platelet accumulation around stents would facilitate reducing not only acute vascular events but also the subsequent event, restenosis. Indeed, clinical experiences and animal experiments have suggested that patient factors,12 vascular factors (such as small vessel diameter),13 and stent factors (such as the shape of the stent)14,15 may affect platelet accumulation, thus influencing the rate of restenosis.

In the present study, we constructed a flow chamber equipped with an epifluorescence videomicroscope. This device enabled us to visualize platelet thrombus formation on collagen surfaces under flow conditions. We attempted to clarify the effects of stent morphology, stent thickness, and the type of metal used on platelet accumulation around the stent by comparing the thrombogenicity of several stents having different characteristics. To test the validity of our assay system, we tested in an ex vivo flow chamber system the effects of antplatelet therapy known to be effective for preventing platelet thrombosis, such as anti–glycoprotein (GP) IIb/IIIa agents16,17 and a combination of aspirin and ticlopidine,18 or less effective therapy, such as aspirin alone.7 We also tested the effects of the agent known to block von Willebrand factor (VWF) binding to platelet GP Ibα, which was reported to play crucial roles in platelet thrombus formation at sites exposed to high shear stress,19–22 on platelet accumulation around stents.

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From the Division of Cardiology, Department of Medicine, Tokai University School of Medicine, Kanagawa, Japan, and the Department of Medicine (T.I.), Teikyo University School of Medicine, Tokyo, Japan.
*These authors contributed equally to the present study.
Correspondence to Shinya Goto, MD, Division of Cardiology, Department of Medicine, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1153, Japan. E-mail shinichi@is.icc.u-tokai.ac.jp
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Methods

Preparation of Blood Samples

Blood samples were collected from healthy adult donors after obtaining written informed consent. All the donors were requested to abstain from using drugs known to interfere with platelet function, such as aspirin, for at least 1 month before the blood collection. Blood samples were drawn from the antecubital vein with the use of 19-gauge needles and immediately transferred to plastic tubes containing 1/10 of their volume of the specific thrombin inhibitor, Argatroban (Mitsubishi Kagaku). To test the effects of aspirin and the combination of aspirin plus ticlopidine, additional blood samples were collected 10 hours after oral intake of aspirin (500 mg/d) and 10 hours after oral intake of aspirin subsequent to the 1-week consecutive oral intake of ticlopidine (200 mg/d). Argatroban, instead of the common anticoagulant citrate, at a final concentration of 100 μmol/L was used for anticoagulation of the blood. In performing experiments in the presence of the physiological concentrations of divalent cations because the biological function of GP IIb/IIIa was known to be influenced by divalent cation concentrations. We did not choose heparin as an anticoagulant, even though it is commonly used in patients undergoing interventional treatment, because that anticoagulant, having various pleiotropic effects on platelet functions, is not a pure material.

Platelets were rendered fluorescent by the addition of mepacrine at a final concentration of 10 μmol/L (Sigma Chemical Co). Although mepacrine is known to affect platelet function through the inhibition of phospholipid hydrolysis, that effect can be negligible at the dose that we used.

Preparation of Flow Chamber System and Platelet Thrombus Visualization by Epifluorescence Videomicroscopy

A Hele-Shaw type of flow chamber with immobilized type I collagen was prepared as described previously. The blood samples were aspirated through the chamber with a syringe pump (Holliston, MA 01746, Harvard Apparatus) at a constant flow rate to achieve a wall shear rate on the collagen surface of 1500 s⁻¹ in the absence of the stent. To generate pulsatile flow conditions, blood flow was stopped for 1 minute after every 1 minute of perfusion. Note that the wall shear rate cannot even be roughly estimated in the presence of a stent because the local flow environment is randomly disturbed by the presence of stents.

The effects of these stents with different characteristics, as described below, on platelet thrombus formation were evaluated. The Palmaz-Schatz stent, a typical slotted tube stent with different metal thicknesses (PS14, thickness 0.635 mm; PS15, thickness 1.01 mm; Johnson & Johnson), and GFX and Wiktor stents (Medtronic), typical coil stents, were inflated with a balloon to a pressure of 7 atm. Parts of the stents were then cut and placed on the collagen-coated glass coverslips and tightly pushed to be fixed on the collagen surface as demonstrated in Figure 1. Because the flow route was exactly 2.2 mm in width, only pairs of struts could be placed. Larger parts of the PS15 and GFX stents were cut and placed in the proximal portion in the Hele-Shaw chamber, which has wider flow routes. Note that the blood flow rate should be increased by a factor of 3 to assess the levels of thrombogenicity of the larger parts of stents in the same wall shear rate condition as in the absence of stents and that only a shorter period of blood perfusion (2 minutes) was available with the limited amount of fresh blood obtainable from blood donors. Platelet thrombi forming on the collagen surface in the presence and absence of various stents were visualized by using an epifluorescence videomicroscope system (DM IRB, IRB-FLUO, Leica). The microscopic images were digitized online with a photosensitive color CCD camera (LE-500, Leica) and stored as digital images in a personal computer (Power Macintosh G3, Apple, Co, Ltd). For quantitative analysis, digital color images were converted to 256 scales of gray-scale images by using NIH image version 1.62, public domain software by Dr Wayne Rasband, National Institutes of Health. Then, the regions of interest (ROIs) were set to calculate the mean gray-scale number, which corresponded to the number of platelets deposited, in the areas described in the Figure 1 legend.

Monoclonal Antibodies and Antiplatelet Agents Used for Functional Inhibition of Platelet Receptors

The murine monoclonal antibody against GP Iba (LJ-Ib1) was kindly provided by Dr Zaverio M. Ruggeri of the Scripps Research Institute (La Jolla, Calif) and has previously been shown to inhibit VWF–GP Ibα interaction under all experimental conditions tested. Abciximab was used as an anti–GP IIb/IIIa agent, which blocks the binding of plasma ligands such as fibrinogen and VWF to GP IIb/IIIa. Recent clinical investigations have revealed abciximab to be effective in preventing acute thrombotic occlusion of the coronary arteries after interventional treatment and stent implantation.
Statistical Analysis
The arbitrary gray-scale values in each ROI are expressed as mean ± SD unless otherwise specified. The differences between the mean gray-scale values of the different sites in the same experiments were evaluated by the Student paired t test. The mean gray scales in the absence and presence of various stents were compared by the Student unpaired t test. A value of \( P < 0.05 \) was considered statistically significant.

Results
Platelet Accumulations on Collagen Surfaces in Presence or Absence of Stents
Platelets adhered to the collagen surface and formed thrombi in the presence and absence of stents (please see Figure I, available online at http://atvb.ahajournals.org). However, more platelet deposition was demonstrated in the presence of either the typical Palmaz-Schatz tube stent (PS14 and PS15) or the typical GFX and Wiktor coil stents, the distributions of which were not similar. Indeed, significant platelet accumulation downstream from the stent, in addition to the accumulation in the space between stents and collagen, was demonstrated in the presence of coil stents but not in the presence of tube stents. No significant platelet accumulation was detected when the glass coverslip was not covered with collagen even in the presence of stents (data not shown).

Stent Characteristics Related to Thrombogenicity
As shown in online Figure I and Figure 2, there were no significant differences in the amounts of platelets accumulated around PS14 and PS15, stents with the same metallic component and similar shapes but with different thicknesses of metal, suggesting that the thickness of the metallic component of the stent is not a major determinant of their thrombogenicity. Densitometric analysis revealed that 4.9 ± 0.8 and 4.2 ± 1.2 times more platelets accumulated on the collagen surface downstream than upstream from the respective GFX and Wiktor coil stents, which had different metallic components (both \( P < 0.01 \)). These different distributions were not found with tube stents. Significant platelet accumulation was noted downstream from coil stents, but not tube stents, when the blood flow became pulsatile (data not shown) or when larger parts of multiple struts were placed (please see Figure II, available online at http://atvb.ahajournals.org).

Effects of Monoclonal Antibodies and Antiplatelet Agents
As shown in Figure III (available online at http://atvb.ahajournals.org), abciximab inhibited platelet thrombus formation around GFX stents in a dose-dependent manner. Platelet thrombus formation was completely inhibited at a dose of 2 μg/mL. Similar dose-dependent inhibition was seen with the PS15 stent (data not shown). A specific anti–GP Ibα antibody, LJ-Ib1, at a concentration enough to inhibit all available platelet surface GP Ibα, abolished platelet deposition on the collagen surface around the stents, especially at the upper reaches of flow (Figure 3). However, LJ-Ib1 did not inhibit platelet thrombus formation in the spaces between the stent and the collagen surface and platelet accumulation downstream from the coil stents. Figure 4 demonstrates the effects of commonly used antiplatelet agents on platelet accumulation around the stents. In agreement with previously published clinical experience, a combination of aspirin and ticlopidine strongly inhibited platelet accumulation around the stents (regardless of whether they were tube or coil), whereas aspirin alone showed only modest inhibition.
Discussion

Our results demonstrate that platelet thrombus formation on collagen surfaces under flow conditions is enhanced by the presence of stents, especially coil stents, the placement of which is associated with a higher risk of thrombotic occlusion and angiographically determined restenosis. Our results also show that ex vivo chamber systems demonstrate antithrombotic effects similar to those of antiplatelet agents used in vivo, ie, similar to the superior effects of the combination of aspirin and ticlopidine rather than aspirin alone. Those results (along with the finding that the inhibiting effects of abciximab on platelet accumulation around a stent in an ex vivo flow chamber system were achieved at a dose previously shown to be effective for preventing vascular complications after stent implantation in vivo) also suggest that the in vivo antithrombotic effects may be predicted by an ex vivo perfusion system.

Nevertheless, there are limitations to the methodology used in our experiments, particularly regarding the application of our ex vivo results to the understanding of events occurring in clinical situations. Obviously, we cannot perfectly reproduce in our flow chamber system the complex in vivo flow conditions prevailing around stents implanted in coronary arteries. For example, the significant platelet accumulations in the space between the stents and the collagen surface, regardless of the type of stents observed in our experiments, might not be clinically relevant, because there should be not such space when the stent expands to fit within the soft vascular tissue in the coronary arteries. As demonstrated in Figure 2 through Figure 4 (and also online Figure I through Figure III), platelets slip into the space between the stents and collagen, although we attempted to minimize the space by tightly pushing the stents onto the collagen surface before starting blood perfusion. We could not quantify the space...
generated by platelet migration, but a quantitative comparison shown by Figure 2 suggested that variations in the dimensions or effects of these spaces were not markedly different from experiment to experiment because the standard errors of platelet accumulation in the spaces between collagen and stents are similar to the values of those accumulated upstream and downstream from the stents. We speculate that the space between stents and collagen may not influence our findings regarding platelet accumulation upstream and downstream from the stents or the effects of antiplatelet agents because the variations in the spaces were minimized by our experimental setting. Moreover, in vivo pulsatile flow conditions can hardly be reproduced in our flow chamber system. As previously demonstrated, not only the extent of platelet thrombus formation but also the underlying mechanisms involved might be different under the pulsatile flow condition. Although we have experimentally concluded that the significant accumulation observed downstream from coil stents was not influenced even by the changing shear rates generated by the stop and flow conditions, our experimental conditions were indeed far different from the complex flow conditions actually prevailing in the coronary arteries.

Methodologically, there is another important limitation to our flow chamber experiments; ie, the 2 potentially important factors, thrombin and fibrin formation, could not be considered in the presence of an anticoagulant, although we attempted to minimize the influences of the anticoagulant by using the reversible thrombin inhibitor Argatroban, as mentioned in Methods. One might also claim that the effects of only parts of stents, and not entire stents, could be assessed in our system. Although we have shown that the significant accumulation of platelets downstream from the coil stents that we observed with single strut placement was not changed even in the presence of larger parts of multiple struts, our assay system has many limitations in assessing the larger parts of stents. We could conduct the experiments with only the larger parts of stents within a shorter period of blood perfusion (2 minutes), although the degrees of reproducibility of the results become poorer with shorter perfusion periods. In spite of all the above limitations; however, we would still like to emphasize that the ex vivo system, if reflecting certain important in vivo characteristics of stent thrombosis, will be useful for screening less thrombogenic stents and developing better adjuvant therapy before conducting expensive clinical trials.

Our experiments demonstrate that compared with aspirin alone, a combination of aspirin and ticlopidine is more effective in preventing platelet accumulation around stents. Because platelet attachment on the collagen surface was mediated by its binding to VWF attached on collagen and collagen itself, blocking the interaction between VWF and its corresponding receptor GP Ibα alone was not sufficient to inhibit platelet accumulation around stents. Stronganthrombotic effects of the combination of aspirin and P2Y12 inhibition might be explained by the synergistic effects of cyclooxygenase inhibition by aspirin (which has previously been reported to inhibit collagen-induced platelet activation) and VWF-induced platelet activation. An ex vivo flow chamber system may be a better tool for assessing combination drug therapy than conventional agonist-induced platelet aggregation, because multiple synergistic stimulations initiated from various receptors are involved.

In conclusion, we applied an ex vivo flow chamber system to assess factors influencing stent thrombosis, and we found it useful for screening stents less thrombogenic and for screening the antiplatelet agents effective for stent thrombosis.

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Blood Flow

Control

0.5 µg/ml

1 µg/ml

2 µg/ml