Quantification of Fibrin Deposition in Flowing Blood With Peroxidase-Labeled Fibrinogen

High Shear Rates Induce Decreased Fibrin Deposition and Appearance of Fibrin Monomers

Pim N.M. Tijburg, Martin J.W. IJsseldijk, Jan J. Sixma, and Philip G. de Groot

To study fibrin incorporation into thrombi at different wall shear rates, a new method to study fibrin deposition on extracellular matrixes underlying stimulated endothelial cells under flow conditions was developed. For this method, we used fibrinogen labeled with peroxidase (Fg-PO). Fg-PO was fully exchangeable for Fg in the clotting assays tested, and PO activity was bound to fibrin-specific fragments. Fg-PO containing fibrin could be stained for microscopic studies with 3,3′-diaminobenzidine and could be quantified by oxidation of phenylenediamine. The absorbance values at 492 nm were converted to fibrin quantities via a standard curve. To study fibrin deposition, Fg-PO was added in trace amounts to whole blood anticoagulated with low-molecular-weight heparin, and perfusion studies were performed over endothelial cell matrixes containing tissue factor. In parallel perfusion studies, 125I-labeled Fg was added in trace amounts to whole blood instead of Fg-PO. Both quantitative methods demonstrated decreased fibrin deposition after perfusions at 1,300 sec⁻¹ compared with fibrin deposition after perfusions at 300 sec⁻¹, while fibrinopeptide A generation was independent of the wall shear rate. The decrease in fibrin deposition at 1,300 sec⁻¹ was accompanied by the appearance of fibrin monomers in the perfusate. This suggested that the decrease in fibrin incorporation at 1,300 sec⁻¹ was due to the impaired polymerization of fibrin monomers. This impairment was probably due to a decrease in local fibrin monomer concentration as a result of the increased removal of monomers from the surface at 1,300 sec⁻¹. (Arteriosclerosis and Thrombosis 1991;11:211–220)
lets and fibrinogen (Fg) in the formation of a thrombus, both in the normal situation and in different groups of patients.

Methods

Materials

Human Fg and H-d-Phe-t-Pip-Arg-p-nitroanilide (S 2238) were purchased from KabiVitrum, Stockholm, Sweden. Horseradish peroxidase (PO), human thrombin (3,000 units/ml), 3,3'-diaminobenzidine 4 HCl, and 4-β-phorbol-12-myristate-13-acetate (PMA) were from Sigma Chemical Co., St. Louis, Mo. 1,2-Phenylenediamine was purchased from Merck, Darmstadt, F.R.G. All culture plastics were obtained from Nunc, Roskilde, Denmark. The other tissue culture supplies (media, antibiotics, and tryptophan) were from Gibco Biocult, Paisley, Scotland. Low-molecular-weight heparin (LMWH; Fragmin) was a generous gift of KabiVitrum. All other chemicals obtained from commercial sources were of the highest purity grade available.

Cell Cultures

Human umbilical vein endothelial cells (ECs) were isolated from umbilical veins and cultured according to Jaffe et al21 with some minor modifications.22 The cells were identified by their typical characteristics such as the presence of von Willebrand factor. ECs of the second passage were subcultured on gelatin-coated Thermaxan coverslips (Flow Laboratories Inc., Rockville, Md.). Before seeding the cells, the gelatin on the Thermaxan coverslip was fixed with 0.5% glutardialdehyde. Cell monolayers, grown to confluence in 5-7 days, were used (approximately 60,000 cells/cm²). At confluence, the ECs were stimulated for 4 hours with tumor necrosis factor (TNF) or for 16 hours with PMA. Human recombinant TNF was generously provided by D. Stern, Columbia University, New York, N.Y. TNF was dissolved in distilled water containing 0.1% bovine serum albumin (BSA) and diluted 1:1,000 (vol/vol) in the culture medium, resulting in 600 pM TNF in the culture medium. PMA was dissolved in dimethyl sulfoxide (DMSO) and diluted 1:1,000 (vol/vol) in the cell culture medium. PMA was added to a final concentration of 0.1 μg/ml in the culture medium.

The incorporation of Fg-PO in the fibrin clot was demonstrated as follows. Fibrin clots were formed by incubation of normal plasma, with or without addition of Fg-PO (Fg/Fg-PO=200:1), on matrices of PMA-stimulated ECs (see "Cell Cultures"). Fibrin/Peroxidase Enzyme-Linked Immunosorbent Assay

The exchange of Fg-PO with native Fg in the formation of a surface-bound fibrin network was demonstrated with an enzyme-linked coagulation assay previously described by Doellgast et al24 with some minor modifications. Briefly, microtiter plates were coated with 100 μl/well of 10 μg/ml Fg in 50 mM sodium carbonate (pH 9.6) overnight at 4°C. Variable Fg/Fg-PO molar ratios were added to the wells, and incubations were performed for 10 minutes at room temperature in the presence of 1 unit/ml thrombin. The wells were extensively washed and developed for PO activity using phenylenediamine (0.4 mg/ml, 50 mM citric acid, 100 mM NaH2PO4, 0.0015% [vol/vol] H2O2, pH 5.0). The absorbance at 492 nm (A492) was read against a blank with a Titertek Multiscan, Flow Laboratories Inc.

Synthesis of Peroxidase-Labeled Fibrinogen

Peroxidase-labeled fibrinogen (Fg-PO) was prepared according to the method originally described by Nakane and Kawai.23 Briefly, free amino groups on the PO molecules were blocked with dinitrofluorobenzene (DNFB). The carbohydrate portion of the molecule was oxidized to form aldehyde groups with sodium periodate. After dialysis to remove excess DNFB, the aldehyde groups were coupled to free amino groups of the Fg molecule. Labeled Fg was separated from free PO with a Sephadex G-100 column and stored at -80°C. The molar ratio of PO to Fg was 1:1.

Enzyme-Linked Coagulation Assay

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Fibrin/Peroxidase Enzyme-Linked Immunosorbent Assay

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Clotting Assays

Thrombin times were assessed by addition of 0.1 ml thrombin (3 units/ml) to 0.2 ml normal plasma (containing various amounts of Fg-PO) or to 0.2 ml Fg (2.0 mg/ml) preincubated at 37°C for 2 minutes. The clotting time was recorded with an automated coagulometer (model KC 10, Amelung, GmbH, Lemn, F.R.G.).
Perfusion Studies and Fibrin Quantification

Cephalin–kaolin times were determined in the following way. One tenth milliliter cephalin-kaolin (Boehringer-Mannheim, Mannheim, F.R.G.) was preincubated with 0.1 ml normal plasma (containing various amounts of Fg-PO) at 37°C for 3 minutes. Clotting times were recorded with the automated coagulometer after addition of 0.1 ml CaCl₂ (0.33 M).

Fibrin Monomer Assays

The presence of fibrin monomers in plasma samples before and after perfusions was determined with a fibrin monomer agglutination test (Boehringer). To do so, 0.1 ml plasma was incubated with 0.1 ml fibrin monomer reagent provided in the kit for 10 minutes at 37°C. The reaction mixture was subsequently transferred to a slide and spun for 6 minutes. Aggregation was compared with the aggregation of the negative and positive fibrin monomer controls provided in the kit.

The influence of the incorporation of PO molecules on the reaggregation of fibrin monomers was determined as previously described. Briefly, Fg/Fg-PO mixtures in molar ratios of 200:1, 100:1, and 50:1 were clotted by addition of thrombin. The fibrin clot was dispersed in 1 M NaBr, 0.05 M sodium acetate, pH 5.3. Reaggregation of the fibrin was effected by adding 25 μl fibrin monomer preparation to 475 μl 0.08 M phosphate, pH 6.3. Reaggregation was monitored by measuring the scattered light in a UVIKON Model 810 spectrophotometer, Kontron, AG, Zurich, Switzerland.

Blood Collection

LMWH was diluted in saline to a concentration of 200 units/ml. Blood was collected by clean venipuncture in 1:10 (vol/vol) of this heparin/saline. Anticoagulation with citrate was obtained by collecting blood in 1:10 (vol/vol) 110 mM citrate. EDTA-anticoagulated blood was obtained by collecting blood in 1:10 (vol/vol) 110 mM EDTA. Blood from patients on oral anticoagulation therapy was obtained from the local Thrombosis Service of the Dutch Red Cross.

Factor II–deficient plasma was prepared by incubating normal human plasma (6 ml) with a polyclonal antibody to factor II, coupled to CNBr-activated Sepharose. The polyclonal antibody was immune-purified goat anti-human factor II. Factor II–deficient plasma contained less than 0.01 unit/ml factor II as determined by a one-stage clotting assay. No decrease in other clotting factors was found. Reconstitution was performed by adding purified factor II (54 units/ml, 10.9 mg/ml) to a plasma concentration of 2 units/ml. Factor II and the polyclonal antibody to factor II were generous gifts from J. Meijers and B.N. Bouma from our laboratory.

Perfusion Studies and Fibrin Quantification

Perfusions with steady flow were performed with an annular and a rectangular perfusion chamber.
Fg-PO-containing fibrin was stained for microscopic evaluation by incubation of the Thermanox coverslip with 3,3'-diaminobenzidine 4 HCl (0.5 mg/ml, 0.1 M sodium acetate, 0.1% H$_2$O$_2$, pH 6.0) for 5 minutes.

**Standard Curve**

A standard curve was prepared by spraying 1 mg/ml Fg/Fg-PO (molar ratio, 200:1) in 0.05 M ammonium acetate, pH 7.4, onto glass coverslips with a retouching airbrush (Badger model No. 100, Badger Air-Brush Co., Franklin Park, Ill.). The airbrush was connected to a nitrogen cylinder operating at a pressure of 1 atmosphere. Just before spraying, 1 unit/ml human thrombin was added to the solution and mixed thoroughly. The applied amount of fibrin deposited on the surface was determined by weighing the coverslips with a microbalance before and after spraying and correlated with the PO activity found on the same coverslip.

In parallel experiments, the amount of fibrin on the Thermanox coverslip was determined by addition of trace amounts of $^{125}$I-Fg to the Fg/Fg-PO mixtures (Fg to $^{125}$I-Fg=1,000:1). The Thermanox coverslips were assayed for PO activity and subsequently counted in a gamma counter. The amount of fibrin was calculated from the measured radioactivity on the Thermanox coverslip. The $A_{492}$ values from the standard curve ranged from 0.0 to 3.0 for fibrin quantities of 0.0-32.5 $\mu$g/cm$^2$. The SEM for each point was within 10%.

**Fibrinopeptide A Assays**

A radioimmunoassay kit (Mallinckrodt, St. Louis, Mo.) was used for FPA measurements. Samples of 900 $\mu$l were collected before and after the perfusions and added to the 100-$\mu$l anticoagulant mixture provided in the kit. Instructions of the manufacturer were followed. FPA values were expressed in nanograms per milliliter plasma. FPA generation was calculated from the increase in FPA level compared with the initial value just before perfusion. All samples were assayed in duplicate. Baseline values were less than 3 ng/ml in all experiments. The results presented are the mean of at least three experiments.

**Results**

**Characteristics of Peroxidase/Fibrinogen**

No degradation of Fg was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis after the synthesis of Fg-PO (not shown). Purified Fg-PO alone was incapable of forming stable fibrin networks after incubation with thrombin. However, incubation of Fg-PO with thrombin induced FPA generation. Thrombin incubation also induced the formation of fibrin monomers (not shown). When Fg-PO was added to plasma up to molar ratios of Fg/Fg-PO=1:50 (50 $\mu$g/ml Fg-PO), no detectable

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Photomicrograph of fibrin deposition on extracellular matrix of stimulated endothelial cells. Endothelial cells were stimulated with 4-B-phorbol-12-myristate-13-acetate (20 ng/ml) for 16 hours. Perfusion was performed for 5 minutes at 300 sec$^{-1}$. The molar ratio of fibrinogen (Fg) to peroxidase-labeled Fg (Fg-PO) in the perfusate was 200:1. Fibrin/PO was stained with 3,3'-diaminobenzidine as described in "Methods." Preparations were photographed with a Zeiss photomicroscope III. x575

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Bar graph showing incorporation of Fg-PO in fibrin. Fibrin was formed by plasma incubations on matrices of PMA-stimulated endothelial cells. After digestion of fibrin by plasmin, the mixture was incubated in DD3b6f22-coated enzyme-linked immunosorbent assay wells in the presence of 4% BSA. Bound peroxidase activity was developed with phenylenediamine for 10 minutes, and the absorbance values at 492 nm ($A_{492}$) were measured against a blank. Fg-PO, peroxidase-labeled fibrinogen; PMA, 4-B-phorbol-12-myristate-13-acetate; BSA, bovine serum albumin; Fib, fibrin formed by native fibrinogen; Fib-PO, fibrin formed in the presence of Fg-PO. Values are expressed as mean±SEM (n=6).
Quantification of Fibrin Deposition With Peroxidase/Fibrinogen After Perfusion Studies

Fg-PO was then used to study fibrin deposition in a thrombosis model. In this model, matrixes of cultured ECs were exposed to flowing blood anticoagulated with LMWH. Stimulation of ECs with PMA induces the synthesis of tissue factor by the ECs and deposition of tissue factor on the subendothelial matrix. Tissue factor, present in the matrix, is able to induce thrombin formation and consequently platelet aggregation and fibrin deposition. LMWH as an anticoagulant completely inhibits the formation of disturbance of the fibrin network was detected by microscopic evaluation. Fg-PO–containing fibrin was stained by incubation with 3,3′-diaminobenzidine. All fibrils were stained brown, indicating the incorporation of Fg-PO in all different sizes of fibrin fibrils (Figure 1). Addition of 100 μg/ml Fg-PO to normal plasma did not influence the thrombin time or the cephalin-kaolin clotting time (not shown).

The incorporation of Fg-PO into fibrin was further shown by plasmin-digested fibrin formed in the presence of Fg-PO. The digest was incubated in ELISA wells coated with the monoclonal antibody DD3b6/22. This monoclonal antibody recognizes a fibrin-specific epitope that is not present in fibrinogen. The specific increase in A492, only when the mixture of Fg/Fg-PO was allowed to polymerize, therefore demonstrated the incorporation of Fg-PO into fibrin (Figure 2).

Aggregation of fibrin monomers was studied as previously described.27 Fg-PO incorporation in fibrin (molar ratio of Fg/Fg-PO=200:1, 100:1, and 50:1) did not disturb the aggregation of fibrin monomers at all ratios tested (not shown).

The exchange of Fg-PO with Fg in the formation of a surface-bound fibrin network was demonstrated with an enzyme-linked coagulation assay. To do so, Fg-PO was mixed with native Fg at various molar ratios in wells coated with Fg. Fibrin polymerization was started by addition of thrombin. A linear correlation was found between the A492 values and the percentage of Fg-PO present in the Fg/Fg-PO mixture (Figure 3).

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thrombin in the fluid but has hardly any effect on thrombin generated at and bound to the surface.

Increasing amounts of Fg-PO were added to whole blood and perfused for 5 minutes at a wall shear rate of 300 sec\(^{-1}\). The increase in \(A_{492}\) values was linear with the increase in the percentage of Fg-PO added (Figure 4). In all further experiments, Fg-PO was added in a molar ratio of 1:200 of Fg-PO to Fg, resulting in an Fg-PO concentration of approximately 10 \(\mu\)g/ml. Disturbance of fibrin polymerization on the matrix was only observed after addition of Fg-PO in concentrations higher than 100 \(\mu\)g/ml.

The following perfusion conditions were chosen as a standard: 1) stimulation of the ECs with PMA; 2) 5 minutes' perfusion over the matrix of these cells; 3) wall shear rate of 300 sec\(^{-1}\); and 4) 20 units/ml LMWH as anticoagulant (Table 1). Quantification of fibrin deposition under these conditions showed a fibrin deposition of 18.5±2.0 \(\mu\)g/cm\(^2\) (Table 1). All parameters were changed one by one to study the effect of the individual parameters while all other parameters remained the same (column 1 in Table 1). Fibrin deposition at 1,300 sec\(^{-1}\) was markedly decreased as was the fibrin deposition after perfusion over matrices of nonstimulated or TNF-treated ECs (Table 1). Because of its stability and powerful action, PMA was used in all further experiments with cultured cells to characterize the system. Fibrin quantification was not limited to the matrix of cultured cells but could also be assayed on inverted arteries in the annular perfusion chamber (Table 1). Perfusions with citrate-anticoagulated whole blood demonstrated that the presence of calcium was essential for fibrin formation (Table 1).

In separate experiments, perfusions with \(^{125}\text{I-Fg}\) were performed, and fibrin deposition was quantified by the amount of radioactive label deposited on the matrix (Table 1). It appeared that the fibrin deposits obtained with both methods correlated well. Only under perfusion conditions where fibrin deposition was low were the values found with the \(^{125}\text{I-Fg}\) assay somewhat higher. To measure the formation of Fg without FPA (Fg des-A) molecules (as a first step in fibrin formation), the FPA levels in the perfusate were determined (Table 1).

The possibility that the measured PO activity was due to Fg binding to the extracellular matrix was investigated with factor II–deficient perfusates or by addition of 2 units/ml hirudin to the perfusate. In both cases, thrombin activity was absent or at least strongly inhibited. Addition of purified factor II to the factor II–deficient perfusate almost completely restored fibrin deposition and FPA generation in the perfusate (Table 1).

**Effect of Shear on Fibrin Deposition**

The time dependence of fibrin deposition on the extracellular matrix was determined at low (300 sec\(^{-1}\)) and high (1,300 sec\(^{-1}\)) wall shear rates. At both wall shear rates, an increase in fibrin deposition was observed during the first 5 minutes of perfusion. This increase gradually slowed and stabilized after 5 minutes. The final fibrin deposition after 5 minutes' perfusion at 300 sec\(^{-1}\) was significantly higher than the fibrin deposition at 1,300 sec\(^{-1}\) (Figure 5, upper panel). Variation of the wall shear rate (100–1,300 sec\(^{-1}\)) demonstrated a gradual decrease in fibrin deposition at increasing shear rates (not shown).

At both wall shear rates, a fast increase in FPA concentration was observed during the first 5 minutes of perfusion, which stabilized after 5 minutes. In contrast with fibrin deposition, no difference in FPA generation was found at 300 and 1,300 sec\(^{-1}\) (Figure 5, lower panel).
Additional information about this discrepancy was obtained with the fibrin monomer agglutination test. It appeared that plasma samples after 5 minutes' perfusion at a high wall shear rate contained a higher amount of fibrin monomers than did plasma samples after perfusions at a low wall shear rate (Table 2).

Fibrin Deposition From Blood of Patients on Oral Anticoagulants

The method published here offers the possibility of studying fibrin deposition in plasma samples from patients with various types of coagulation disorders. As an example, patients on stable long-term oral anticoagulant therapy were chosen. Perfusions were performed for 5 minutes at 300 sec⁻¹ over stimulated and nonstimulated matrices. The fibrin deposition on stimulated matrices with blood samples from these patients was significantly lower than the fibrin deposition of controls (Figure 6). Moreover, blood samples from these patients generated markedly less FPA during the perfusion (24 versus 118 ng/ml).

Table 1. Quantification of Fibrin Deposition on Matrix With Peroxidase-Labeled Fibrinogen and ¹²⁵I-Labeled Fibrinogen Assay and Fibrinopeptide A Generation in Perfusate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fibrin (µg/cm², Fg-PO assay)</th>
<th>Fibrin (µg/cm², ¹²⁵I-Fg assay)</th>
<th>FPA (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300 sec⁻¹</td>
<td>18.5±2.0</td>
<td>16.5±2.0</td>
<td>118±17</td>
</tr>
<tr>
<td>1,300 sec⁻¹</td>
<td>6.7±0.8</td>
<td>7.3±1.0</td>
<td>127±27</td>
</tr>
<tr>
<td>Nonstimulated ECs</td>
<td>1.1±0.4</td>
<td>3.9±0.2</td>
<td>20±6</td>
</tr>
<tr>
<td>TNF-stimulated ECs</td>
<td>11.5±1.2</td>
<td>ND</td>
<td>41±5</td>
</tr>
<tr>
<td>Inverted arteries</td>
<td>25.6±3.4</td>
<td>ND</td>
<td>105±23</td>
</tr>
<tr>
<td>Citrated blood</td>
<td>0.5±0.3</td>
<td>3.6±0.4</td>
<td>14±4</td>
</tr>
<tr>
<td>With hirudin</td>
<td>2.6±0.3</td>
<td>4.1±0.2</td>
<td>3±1</td>
</tr>
<tr>
<td>Factor II-deficient blood</td>
<td>1.0±0.4</td>
<td>ND</td>
<td>3±2</td>
</tr>
<tr>
<td>Factor II-deficient blood+2 units/ml factor II</td>
<td>18.0±2.2</td>
<td>ND</td>
<td>110±9</td>
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</table>

Perfusions were performed under the following standard conditions: 5 minutes' perfusion at a wall shear rate of 300 sec⁻¹, 16 hours' stimulation of the endothelial cells (ECs) with 4-β-phorbol-12-myristate-13-acetate, 20 units/ml low-molecular-weight heparin as anticoagulant. In column 1, one of the parameters (shear rate, cell stimulus, surface, or anticoagulant) was changed while all other parameters remained the same. Thrombin activity was inhibited by addition of 2 units/ml hirudin or by using factor II-depleted perfusates. Fibrin deposition was quantified with the peroxidase-labeled fibrinogen (Fg-PO) assay (n=12) and the ¹²⁵I-Fg assay (n=8) and expressed in µg/cm². Fibrinopeptide A generation in the perfusate (n=12) was determined by radioimmunoassay and expressed in ng/ml. ND, not determined. Values are mean±SEM.

Table 2. Fibrinogen Without Fibrinopeptide A Molecule Concentrations in Perfusate After 5 Minutes' Perfusion

<table>
<thead>
<tr>
<th>Wall shear rate (sec⁻¹)</th>
<th>Agglutination test</th>
<th>Calculated percent nonpolymerized</th>
<th>Estimated [Fg-des A] (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>Negative</td>
<td>59±4</td>
<td>12.5±1.0</td>
</tr>
<tr>
<td>1,300</td>
<td>Positive</td>
<td>85±7</td>
<td>18.5±1.5</td>
</tr>
</tbody>
</table>

Fibrinogen without fibrinopeptide A molecule concentrations [Fg-des A] in the perfusate after 5 minutes' perfusion at 300 and 1,300 sec⁻¹. The amount of fibrin monomers present in plasma was determined with a fibrin monomer agglutination test as described in "Methods." The percentages of Fg-des A molecules involved in fibrin polymerization and corresponding free Fg-des A concentrations in the perfusate were calculated with the following data: total matrix surface in perfusion system=4.4 cm², fibrin deposition of 18.5 and 6.7 µg/cm², respectively, and an Fg molecular weight (MW)=300 kd gives the amount of Fg-des A involved in polymerization. Free Fg-des A concentration was calculated with perfusate volume=15 ml, fibrinopeptide A (FPA) generation=120 ng/ml, and a MW of FPA=1.6 kd. Values are mean±SEM (n=12).
al introduced a thrombosis model in which platelet deposition and fibrin formation on the vessel wall were measured by morphometric evaluation. Recently, our laboratory introduced a new thrombosis model, which used LMWH-anticoagulated whole blood in perfusions over matrixes of cultured human cells. Also in this model, fibrin deposition was morphometrically evaluated. Although a great amount of new information and insight has been gained with these models, the morphological evaluation is a time-consuming and difficult technique, and only a relatively small part of the perfused subendothelium is evaluated.

A convenient way to study fibrin deposition in a thrombosis model is addition of trace amounts of labeled Fg to the perfusate. The use of a nonradioactive label, however, is attractive in perfusion studies where contamination is almost unavoidable. The PO labeling of Fg used two possibilities of one labeling. Fg-PO-containing fibrin could be stained with 3,3'-diaminobenzidine for microscopic evaluation or quantified by oxidation of phenylenediamine. This offers a simple and quick method for the quantification of fibrin.

The use of Fg-PO as a measure of fibrin deposition is validated because 1) treatment of purified Fg-PO with thrombin or reptilase induced FPA generation and formation of fibrin monomers. 2) The incorporation of PO groups into the fibrin network was demonstrated by microscopic evaluation and by ELISA with a fibrin-specific monoclonal antibody (Figures 1 and 2). 3) Quantification with 125I-Fg showed similar fibrin deposition on the extracellular matrix after perfusion (Table 1). Although purified Fg-PO alone did not form a stable fibrin network after incubation with thrombin, these results indicate that Fg-PO was incorporated in fibrin clots in the presence of excess native Fg. To avoid disturbance of the fibrin network as much as possible, labeled Fg was always added in trace amounts. A linear correlation between the percentage of Fg-PO and the incorporated PO activity was found (Figures 3 and 4). This indicated that the substrate activity of Fg-PO in surface-bound fibrin polymerization was normal.

The deposition of fibrin in our thrombosis model was completely dependent on the presence of thrombin. In situations in which thrombin formation was inhibited, the PO activity decreased to approximately 10% of the original value (Table 1). This indicated that the major part of the signal was due to deposited fibrin. Although the binding of Fg to ECs or their extracellular matrix plays an important role in many physiological processes linked to ECs, the contribution of matrix-bound Fg to the measured PO activity can, at most, be responsible for 10% of the signal.

A possible disturbing factor in our assay is the contribution of endogenous platelet Fg to fibrin deposition. Platelet adhesion at high wall shear rates is significantly higher than at low wall shear rates. Therefore, at high wall shear rates, the contribution of nonlabeled platelet Fg to the fibrin network may be more prominent than plasma Fg, and the percentage of labeled Fg in the boundary layer may be lower than expected. Until now, the contribution of endogenous platelet Fg to the total fibrin deposition has not become clear. A significant decrease in fibrin deposition at high shear rates was observed, while the FPA generation in the perfusate appeared to be independent of the wall shear rate (Figure 5, both panels). With the measured amount of fibrin deposited on the matrixes and the FPA generated in the perfusate, a comparison between fibrin and Fg des-A molecules was possible. It can be calculated that at low shear rates (300 sec⁻¹), 41% of the formed Fg des-A molecules were incorporated in the fibrin network, while at high shear rates (1,300 sec⁻¹), 15% were involved in the fibrin formation (Table 2). So, at low shear rate, 59%, and at high shear rate, 85% of the Fg des-A molecules escaped from the polymerization reaction. This corresponds with a calculated concentration of Fg des-A molecules in the perfusate of 12.5 and 18.5 µg/ml at low and high shear rates, respectively. An increased amount of fibrin monomers after perfusions at 1,300 sec⁻¹ could indeed be demonstrated with a qualitative assay (Table 2). This increase could be explained by the influence of shear rates on the local Fg des-A molecule concentration on the surface of the extracellular matrix. An increase of shear results in an increased removal of Fg des-A molecules, resulting in a lower monomer concentration, an impaired polymerization, and a decreased fibrin formation.

The effect of high shear rates on local monomer concentrations is in agreement with the work of Shainoff and Page on the relation between fibrin monomer concentrations and fibrin polymerization. They demonstrated the requirement of a threshold concentration of fibrin monomers necessary for initiation of their polymerization. A decrease in local fibrin monomer concentration by high shear below the threshold concentration will consequently cause an impaired polymerization and decreased fibrin deposition.

The dependence of fibrin deposition on the wall shear rate is in good agreement with previous studies of Weiss et al. However, they have observed a decrease in FPA generation with increasing shear rates. Weiss et al. have used nonanticoagulated blood. The use of nonanticoagulated blood permits thrombin activity not only locally on the surface but also in the fluid phase. While the formation and activity of thrombin bound to surfaces is not influenced by shear rates, in the fluid phase it is probably sensitive to an increased supply of inhibitors at higher shear rates. The decrease in FPA generation demonstrated by Weiss et al. can be explained by an increased inhibition of fluid-phase thrombin by protease inhibitors at higher shear rates. Investigations of the role of protease inhibitors at different shear rates are in progress.

At present, it is difficult to determine the role of factor XIII in the stabilization of the deposited fibrin matrix. The presence of PO molecules might influ-
ence the action of factor XIII on the fibrin network and thus cause a less stable fibrin network. The experiments with 125I-Fg and studies without labeled Fg, however, strongly support a mechanism in which decreased fibrin deposition at high shear rates is explained by impaired polymerization of fibrin monomers and not by removal of already deposited fibrin. Nevertheless, additional experiments are required to establish the effect of factor XIII on the fibrin network at different wall shear rates.

The high shear rate in this study (1,300 sec⁻¹) is mostly observed in vivo in arterioles that have a diameter of approximately 50 μm. In our study, this shear rate was created in a perfusion chamber with a width of 600 μm. Whereas fibrin deposition and platelet aggregate formation in vivo may significantly decrease the diameter and consequently increase the shear rate in the vessel, the percent effect will be much lower in the perfusion chamber with its greater diameter.

The method of studying fibrin deposition described here can be used for patients with various coagulation disorders. As an example, it was demonstrated that patients on oral anticoagulation therapy who had stabilized vitamin K-dependent coagulation factor levels of 15–30% had a fibrin deposition of 2.1 ±0.2 μg/cm², which is significantly lower than the 18.5 ±2.0 μg/cm² of the healthy controls (Figure 6). These studies can easily be extended to patients with other deficiencies or disorders in coagulation factors. Furthermore, quantitative studies on the importance of platelets and platelet functions for fibrin deposition are now within reach.

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References

26. Hunt FA, Rylatt DB, Hart RA, Bundesen PG: Serum crosslinked fibrin (XDP) and fibrinogen/fibrin degradation

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products (FDP) in disorders associated with activation of the coagulation or fibrinolytic systems. *Br J Haematol* 1985;60:715–722

27. Laudano AP, Doolittle RF: Studies on synthetic peptides that bind to fibrinogen and prevent fibrin polymerization, structural requirements, number of sites and species difference. *Biochemistry* 1980;59:1013–1019


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Quantification of fibrin deposition in flowing blood with peroxidase-labeled fibrinogen. High shear rates induce decreased fibrin deposition and appearance of fibrin monomers.

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