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Mukul S. Goel and Scott L. Diamond

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# Neutrophil Enhancement of Fibrin Deposition Under Flow Through Platelet-Dependent and -Independent Mechanisms

Mukul S. Goel, Scott L. Diamond

**Abstract**—We examined the effect of adherent neutrophils on fibrin deposition under laminar flow conditions. Perfusion of recalcified citrated platelet-free plasma (PFP) over neutrophils adherent to fibrinogen-coated glass at a venous wall shear rate of  $62.5 \text{ s}^{-1}$  for 15 minutes resulted in dense deposition of fibrin around each neutrophil, whereas fibrin deposition on glass alone was sparse. Fibrin deposition on neutrophils was markedly reduced by anti-CD18 or anti-CD11b or a higher shear rate ( $250 \text{ s}^{-1}$ ). Significantly less fibrin was deposited around adherent fibrinogen-coated beads, indicating that nonspecific “cross-sectional capture” effects were not responsible for the massive fibrin deposition on neutrophils. Direct visualization of fibrin capture by neutrophils and elimination of fibrin deposition at 15 minutes by a factor XIIa inhibitor ( $50 \text{ }\mu\text{g/mL}$  corn trypsin inhibitor [CTI]) or elastase/cathepsin G inhibitors (Methoxysuccinyl-Ala-Ala-Pro-Ala-Chloromethyl-Ketone/Z-Gly-Leu-Phe-CMK,  $100 \text{ }\mu\text{mol/L}$ ) indicated that neutrophils can capture short fibrin strands flowing in recalcified PFP lacking CTI and can also promote thrombin generation through pathways attenuated by inhibitors of factor XIIa, elastase, and cathepsin G. When neutrophils were allowed to interact with platelets on a fibrinogen surface before perfusion of recalcified CTI-treated PFP, the fibrin deposition was observed to be dramatic compared with that over surfaces coated with platelets alone or neutrophils alone and compared with that formed on platelets adherent to collagen. This neutrophil promotion of platelet-mediated fibrin formation was attenuated by inhibitors of elastase or cathepsin G but not anti-tissue factor antibody. Neutrophils can interact with platelets via released proteases to increase platelet procoagulant activity and fibrin formation in CTI-treated plasma under the low-flow conditions expected in venous thrombosis or inflammation. (*Arterioscler Thromb Vasc Biol.* 2001;21:2093-2098.)

**Key Words:** P-selectin ■ CD11b/CD18 ■ thrombin ■ deep vein thrombosis

**B**lood coagulation on the inner wall of blood vessels is the major cause of cardiovascular diseases, such as deep vein thrombosis, myocardial infarction, and stroke. In a Dacron graft model, Palabrica et al<sup>1</sup> showed that neutrophils enhance fibrin deposition through P-selectin-dependent mechanisms. Adherent neutrophils may promote thrombosis via pathways such as deencryption of tissue factor,<sup>2</sup> release of proteases that activate platelets,<sup>3,4</sup> Mac-1 (CD11b/CD18) binding of factor X,<sup>5</sup> and improved capture of flowing platelets via protrusion into the flow stream.<sup>6</sup>

The capture of neutrophils by the vessel wall involves neutrophil P-selectin glycoprotein ligand-1 (PSGL-1)-mediated rolling<sup>7</sup> and membrane tethering<sup>8</sup> on P-selectin presented by activated endothelium or spread platelets,<sup>9</sup> followed by  $\beta_2$ -integrin-mediated firm arrest.<sup>10</sup> The neutrophil  $\beta_2$ -integrin Mac-1, when upregulated, can bind endothelial intercellular adhesion molecule-1 and an unknown platelet ligand,<sup>11</sup> which may be glycoprotein Ib $\alpha$ .<sup>12</sup> Adherent neutrophils can also mediate the capture of flowing neutrophils near the vessel wall via an L-selectin-PSGL-1 mechanism.<sup>13</sup> Additionally, Mac-1 can bind  $\gamma$ 190-202 dodecapeptide on the D domain of fibrin(ogen).<sup>14</sup>

The relationships among flow, neutrophil and platelet adhesion, and blood coagulation are intricately coupled. Collagen-adherent platelets are comparatively more procoagulant<sup>15,16</sup> than are fibrinogen-adherent platelets in promoting fibrin formation. Interactions of neutrophils with platelets in aggregometer-mixed cell suspensions<sup>17</sup> have been found to cause neutrophil oxidative burst and arachidonic acid exchange.<sup>18</sup> However, the effects of neutrophil-platelet interactions, which primarily occur during adhesion and heterotypic aggregation, on subsequent fibrin formation have not been previously studied in a system that decouples flow-regulated adhesion events from flow-regulated coagulation biochemistry.

The objective of the present study was to examine the role of adherent neutrophils in fibrin formation and accumulation under laminar flow conditions. We hypothesized that neutrophils enhance fibrin formation through distinct platelet-independent and platelet-dependent mechanisms. We designed experiments to eliminate the role of flow effects in cell adhesion to allow a focus on subsequent events of plasma function under venous flow. Fibrinogen-coated surfaces were

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used as an analogue of the Dacron shunt model of Palabrica et al<sup>1</sup> to understand platelet-neutrophil cross talk during coagulation.

## Methods

### Materials

Anti-CD18 monoclonal antibody (mAb) IB4, anti-CD11b mAb ICRF44, and anti-CD62P mAb G1/G1-4 (Ancell), corn trypsin inhibitor (CTI) and human fibrinogen (Enzyme Research Labs), FITC-conjugated rabbit anti-human fibrin/fibrinogen (Accurate Antibodies), murine anti-human factor VII/VIIa mAb IgG (American Diagnostica), human serum albumin (HSA, Golden West Biologicals), hirudin (Sigma Chemical Co), and human fibrinogen–Alexa Fluor 488 conjugate (Molecular Probes) were stored according to the manufacturers' recommendations. Methoxysuccinyl-Ala-Ala-Pro-Ala-Chloromethyl Ketone (CMK) (human neutrophil elastase inhibitor) and Z-Gly-Leu-Phe-CMK (cathepsin G inhibitor) were from Enzyme Systems Products. Collagen (calf skin), human thrombin, and D-Phe-Pro-Arg-CMK HCl (PPACK) were obtained from Calbiochem. Rabbit anti-human tissue factor polyclonal antibody (pAB-TF) was a kind gift from Dr Yale Nemerson (Mount Sinai School of Medicine, New York, NY).

### Isolation of Neutrophils and Platelets

Human blood was collected from healthy donors by venipuncture and anticoagulated with sodium citrate (9 parts blood to 1 part sodium citrate). Neutrophils were isolated by centrifugation over neutrophil isolation medium (Cardinal Associates) as previously described.<sup>8</sup> A gel filtration column was used to isolate platelets. After isolation, neutrophils and platelets were diluted to final concentrations of  $10^6$  and  $2.5 \times 10^7$  cells per milliliter, respectively. (Please see online data supplement at <http://www.ahajournals.org> for details.)

### Microcapillary Flow Chambers

Rectangular glass capillaries (Vitrocom) with a cross section of  $0.2 \times 2.0$  mm, a length of 7 cm, and a wall thickness of 0.15 mm were used as previously described<sup>8</sup> to construct the flow chambers. To enable cell adhesion, microcapillary flow chambers were incubated with human fibrinogen solution (100  $\mu\text{g}/\text{mL}$ ) for 120 minutes at room temperature or with calf skin collagen (30  $\mu\text{g}/\text{mL}$ ) for 4 hours at 4°C. A rinse step was followed by adhesion of isolated cells/beads onto the surface. (For detailed adhesion protocol, please see online data supplement at <http://www.ahajournals.org>.) In some experiments, adhesion of neutrophils was preceded by their treatment with monoclonal anti-CD18 (20  $\mu\text{g}/\text{mL}$ ) or anti-CD11b (20  $\mu\text{g}/\text{mL}$ ) to block the  $\beta_2$ -integrin, Mac-1. In other experiments, surface-adherent neutrophils and platelets were incubated with either pAB-TF (50  $\mu\text{g}/\text{mL}$ ) or anti-CD62P (P-selectin) to inhibit possible transfer of tissue factor from neutrophils to platelets<sup>19</sup> or to suppress any possible role of P-selectin in neutrophil-platelet coactivation. To inhibit pathways due to neutrophil elastase or cathepsin G, neutrophils and/or platelets were incubated with specific inhibitors,<sup>20,21</sup> MeOSuc-Ala-Ala-Pro-Ala-CMK (100  $\mu\text{mol}/\text{L}$ ) or Z-Gly-Leu-Phe-CMK (100  $\mu\text{mol}/\text{L}$ ), to inhibit elastase or cathepsin G, respectively. All the above incubations were concurrent with the adhesion protocol and lasted for 60 minutes.

### Perfusion of PFP and Digital Imaging

Citrated platelet-free plasma (PFP; please see online data supplement at <http://www.ahajournals.org> for preparation) was recalcified to a final concentration of 20  $\text{mmol}/\text{L}$   $\text{CaCl}_2$ . This recalcified citrated PFP was immediately perfused (at 25°C) into the flow chamber containing surface-adherent cells (or beads) at a controlled flow rate of 40, 50, or 200  $\mu\text{L}/\text{min}$  for 15 to 20 minutes or until an observable amount of fibrin was deposited. The flow rate during perfusion was controlled by a syringe pump (Harvard Apparatus). The wall shear stress ( $\tau_w$ ) imposed on the surface was calculated from the solution of the Navier-Stokes equation for laminar flow of a Newtonian fluid:  $\tau_w = 6Q\mu/B^2W$ , where Q represents the flow rate ( $\text{cm}^3/\text{s}$ ),  $\mu$  represents the viscosity (0.01  $\text{poise}$ ), B represents the channel separation

(0.02 cm), and W represents the width (0.2 cm). The wall shear rate,  $\dot{\gamma}_w$  ( $\text{sv}^{-1}$ ), is given as  $\dot{\gamma}_w = 6Q/B^2W$ . After perfusion of recalcified PFP, Hanks' balanced salt solution (HBSS) containing 1% HSA was perfused through the chamber at 125  $\text{s}^{-1}$  without effect on deposited fibrin. In some experiments, the contact pathway of coagulation was blocked by the addition of CTI (50  $\mu\text{g}/\text{mL}$ ) to citrated PFP before its recalcification and perfusion. CTI is a specific inhibitor of coagulation factor XIIa<sup>22</sup> and is used to inhibit the contact pathway of coagulation that can be initiated when blood comes in contact with an artificial surface. In selected experiments, pAB-TF (20  $\mu\text{g}/\text{mL}$ ), anti-CD62P (P-selectin), anti-CD11b (20  $\mu\text{g}/\text{mL}$ ), MeOSuc-Ala-Ala-Pro-Ala-CMK (100  $\mu\text{mol}/\text{L}$ ), or Z-Gly-Leu-Phe-CMK (100  $\mu\text{mol}/\text{L}$ ) was added to citrated PFP before perfusion. Factor VII-depleted plasma was prepared by treating PFP with anti-human factor VII/VIIa (10  $\mu\text{g}/\text{mL}$ ). In other experiments, PPACK (50  $\mu\text{mol}/\text{L}$ ) or hirudin (25 U/mL) was used to inhibit thrombin. During flow experiments, the microcapillary flow chambers were mounted on a Zeiss Axiovert 135 microscope with a  $\times 63$  (numerical aperture 1.40) oil immersion objective lens for contrast-enhanced differential interference contrast microscopy.<sup>8</sup>

### Fluorescence Microscopy and Quantification of Fibrin Deposition

In some experiments, neutrophils were incubated with SYTO 17 fluorescent red nucleic acid stain (25  $\mu\text{mol}/\text{L}$ , Molecular Probes) for 15 minutes before use in the flow chamber. After perfusion, the flow chamber was washed with HBSS (1% HSA) and perfused with FITC-conjugated anti-human fibrin/fibrinogen (10  $\mu\text{g}/\text{mL}$ ) at 25  $\mu\text{L}/\text{min}$  for 20 minutes. To avoid side-wall influences, we also measured fluorescent fibrinogen incorporation by using circular microcapillaries (radius 0.316 mm, length 6.4 cm). After cell adhesion, CTI-treated recalcified citrated PFP, doped with Alexa Fluor 488–fibrinogen conjugate (0.2  $\text{mg}/\text{mL}$ ), was perfused into the chamber at 93  $\mu\text{L}/\text{min}$ , corresponding to a wall shear stress of 0.625  $\text{dyne}/\text{cm}^2$  (by  $\tau_w = 4Q\mu/\pi R^3$ ) and a wall shear rate of 62.5  $\text{s}^{-1}$  (by  $\dot{\gamma}_w = 4Q/\pi R^3$ ). After 20 minutes, the flow chamber was rinsed with HBSS and perfused with 1 mL plasmin (2  $\mu\text{mol}/\text{L}$ ) to elute the degraded fibrin. The fluorescence intensity of the effluent was measured (Perkin-Elmer LS50) to calculate the surface density of fibrin by using a nominal ratio of 1.5 mg native fibrinogen per milliliter supplemented with 0.2 mg fluorescent fibrinogen per milliliter for plasma diluted 1:2 before the perfusion.

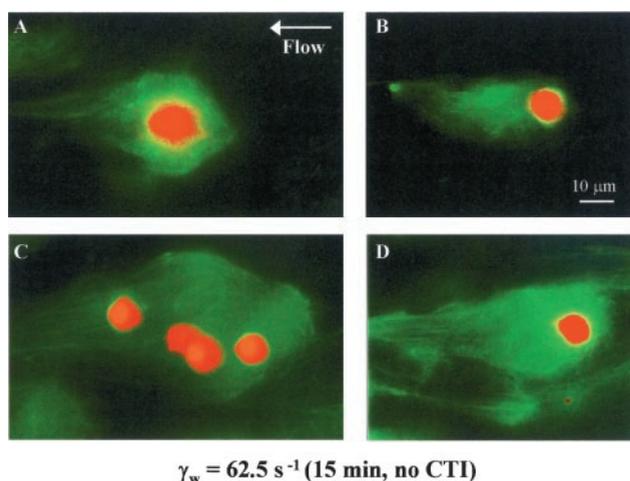
## Results

### Formation of Dense Fibrin Around Neutrophils

Recalcified citrated PFP (no CTI) was perfused over SYTO-17–treated surface-adherent neutrophils at a venous wall shear rate of 62.5  $\text{s}^{-1}$  for 15 minutes. In all instances, fibrin fibers formed dense deposits in proximity to the neutrophils, especially downstream from each cell (Figure 1). The density of the fibrin formed decreased with distance from each neutrophil. Little fibrin was deposited on the surface at sites without neutrophils or in regions only a few microns lateral or upstream from an adherent cell. To quantify the relative density of fibrin deposited around the neutrophils with respect to the background, a density profile plot (Scion Image) for each image in Figure 1 was obtained. At 1 neutrophil length downstream, the fibrin formed around neutrophils was 14- to 55-fold greater ( $n > 8$  cells) than was the background fluorescence. Identical results were obtained with neutrophils not treated with SYTO-17. Of 200 cells observed per chamber, >90% showed dense fibrin deposition on them.

### Shear and Factor XIIa Dependence of Fibrin Deposition on Neutrophils

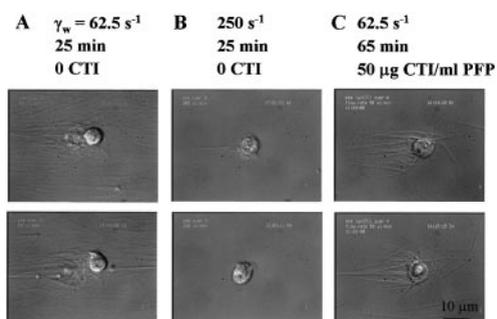
Recalcified citrated PFP (no CTI) was perfused over surface-adherent neutrophils in microcapillary flow chambers at wall



**Figure 1.** Deposition of fibrin around neutrophils. As recalcified citrated PFP was perfused over surface-adherent neutrophils at a shear rate of  $62.5 \text{ s}^{-1}$  for 15 minutes, dense fibrin deposition was observed around each neutrophil, especially downstream from each cell. Neutrophils were dyed with the use of SYTO 17 fluorescent red nucleic acid stain; fibrin (green) was labeled with the use of FITC-conjugated anti-human fibrin/fibrinogen. Flow is from right to left.

shear rates of  $62.5 \text{ s}^{-1}$  and  $250 \text{ s}^{-1}$  for 25 minutes. The density of fibrin deposited around each neutrophil at a wall shear rate of  $250 \text{ s}^{-1}$  (Figure 2B) was significantly lower than that at a wall shear rate of  $62.5 \text{ s}^{-1}$  (Figure 2A), demonstrating a strong inverse correlation between fibrin deposition on neutrophils and wall shear rate via mechanisms completely independent of platelet deposition or red blood cell motions.

Recalcified citrated PFP treated with CTI ( $50 \mu\text{g}/\text{mL}$ ) was perfused over adherent neutrophils at a wall shear rate of  $62.5 \text{ s}^{-1}$ . Compared with the control perfusions (no CTI), in which fibrin accumulation could be observed even after 15 minutes and was prominent by 25 minutes (Figure 2A), fibrin deposition was completely undetected at 25 minutes (as will be discussed in Figure 4) and detectable only after 65 minutes in the presence of CTI (Figure 2C). This 40- to 50-minute increment in the time required for fibrin deposition by CTI treatment of plasma further supported the conclusion that



**Figure 2.** Fibrin deposition around neutrophils at wall shear rate of  $62.5 \text{ s}^{-1}$  after 25 minutes in the absence of CTI (A), wall shear rate of  $250 \text{ s}^{-1}$  after 25 minutes in the absence of CTI (B), and wall shear rate of  $62.5 \text{ s}^{-1}$  after 65 minutes in the presence of CTI (C). Comparison of panels A with B indicates an inverse correlation between shear and fibrin accumulation; a comparison between panels A and C demonstrates a reduction in the rate of fibrin deposition in the presence of CTI. Identical results were obtained in 3 separate experiments; each was conducted with individual donors. Downloaded from [ahajournals.org](http://ahajournals.org) at University of Pennsylvania on August 6, 2009

during the prior perfusion experiments shown in Figure 1 and 2A, neutrophils were promoting factor XIIa-dependent fibrin formation or were capturing short fibrin protofibril bundles that were flowing in recalcified plasma. Direct observation of fibrin capture by neutrophils under the microscope demonstrated that capture played a role in the local fibrin accumulation. Online Figure I (please see <http://www.ahajournals.org>) shows an acquired capture event in which a neutrophil captures an entire fibrin fiber within 830 ms ( $\gamma_w = 50 \text{ s}^{-1}$ ). However, this type of event may not be highly efficient. In place of recalcified PFP, when fibrin monomer solution (prepared by addition of  $0.01 \text{ U}/\text{mL}$  thrombin to  $200 \text{ nmol}/\text{L}$  fibrinogen in HBSS) was perfused over neutrophils for 30 minutes, no neutrophil-bound fibrin was observed. The complete blockade of fibrin accumulation on neutrophils at 25 minutes by CTI indicated that either (1) neutrophils were capturing flowing fibrin species, and/or (2) factor XIIa and the intrinsic pathway are critical for thrombin production by adherent neutrophils in the absence of platelets.

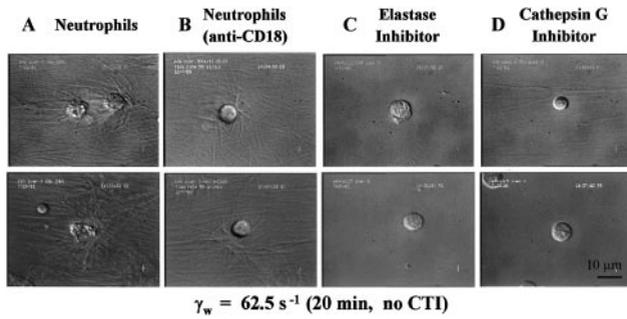
### Effect of Cross-Sectional Capture Efficiency and $\beta_2$ -Integrin Antagonism

By comparing fibrin accumulation on adherent neutrophils with that on polystyrene beads of the same size diameter (please see <http://www.ahajournals.org> for online supplemental experimental details), we demonstrated that although capture efficiency effects by mere steric protrusion into the flow stream had a small role in fibrin capture, this could only partly account for the process. Either adherent neutrophils are more efficient than are polystyrene beads at binding free-flowing fibrin species, or they are more efficient than are beads at factor XIIa-dependent thrombin generation in recalcified plasma not treated with CTI.

To determine whether the Mac-1 receptor had a role in the capture of fibrin by neutrophils, recalcified citrated PFP (no CTI) was perfused over surface-adherent neutrophils treated with anti-human CD18 or anti-human CD11b for 20 minutes ( $\gamma_w = 62.5 \text{ s}^{-1}$ ). The accumulated fibrin on neutrophils treated with either anti-CD18 (Figure 3B) or anti-CD11b (data not shown) was considerably less than that on untreated neutrophils and comparable to that observed on the polystyrene beads. This reduction in fibrin deposition indicated the role of Mac-1 (CD11b/CD18) in fibrin deposition on neutrophils under venous flow conditions by platelet-independent mechanisms.

### Role of Neutrophil Proteases in Factor XIIa-Dependent Fibrin Formation

To examine the role of neutrophil proteases in the absence of CTI, neutrophils were incubated with MeOSuc-Ala-Ala-Pro-Ala-CMK ( $100 \mu\text{mol}/\text{L}$ ) or Z-Gly-Leu-Phe-CMK ( $100 \mu\text{mol}/\text{L}$ ) in separate chambers to inhibit elastase or cathepsin G, respectively. Recalcified citrated PFP (no CTI), also containing the matching protease inhibitor, was perfused over the inhibitor-treated neutrophils. The presence of either inhibitor strongly attenuated fibrin formation (Figure 3C and 3D), demonstrating that neutrophils promote factor XIIa-dependent fibrin formation in a platelet-independent manner through pathways requiring elastase and cathepsin G. In contrast, antibodies against tissue factor or factor VII did not reduce fibrin deposition. Downloaded from [ahajournals.org](http://ahajournals.org) at University of Pennsylvania on August 6, 2009



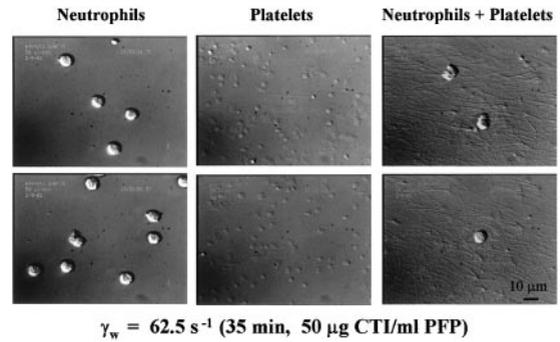
**Figure 3.** Role of Mac-1 and elastase/cathepsin-G-mediated pathways in fibrin accumulation around neutrophils. On perfusion of recalcified citrated PFP (no CTI) for 20 minutes at a wall shear rate of  $62.5 \text{ s}^{-1}$ , the amount of fibrin deposited around anti-CD18 treated neutrophils (B) was considerably less than that around untreated neutrophils (A). Fibrin accumulation around neutrophils was attenuated when either MeOSuc-Ala-Ala-Pro-Ala-CMK (C) or Z-Gly-Leu-Phe-CMK (D) was present in the PFP being perfused over neutrophils (also treated with the matching protease inhibitor). Identical results were obtained in 3 separate experiments; each was conducted with individual donors. Flow is from right to left.

$\mu\text{g/mL}$ ) was perfused over neutrophils incubated with pAB-TF, no reduction in fibrin formation was observed. The same was true when PFP treated with anti-factor VII/VIIa ( $10 \mu\text{g/mL}$ ) was perfused over neutrophils. As a control, when recalcified PFP, treated with either hirudin ( $25 \text{ U/mL}$ ) or PPACK ( $50 \mu\text{mol/L}$ ) to inhibit thrombin, was perfused over adherent neutrophils for 30 minutes, fibrin formation was completely blocked.

**Neutrophil Enhancement of Fibrin Formation via Platelet-Dependent Mechanisms**

Recalcified citrated PFP (containing CTI) was perfused concurrently (wall shear rate  $62.5 \text{ s}^{-1}$ ) over 3 different surfaces: a neutrophil-coated surface, a platelet-coated surface, and a surface coated with neutrophils and platelets (Figure 4). At 35 minutes, the neutrophil-coated surface showed no fibrin formation, and the platelet-coated surface also showed minimal deposition of fibrin. However, dramatic fibrin formation, uniformly distributed, was displayed on the surface coated with platelets and neutrophils. These observations demonstrate that the interaction of neutrophils with platelets can enhance fibrin formation under venous flow conditions in the presence of factor XIIa inhibition by CTI.

To test whether release of the proteases elastase and cathepsin G had a role in platelet-dependent neutrophil promotion of fibrin formation, neutrophils and platelets were incubated with MeOSuc-Ala-Ala-Pro-Ala-CMK ( $100 \mu\text{mol/L}$ ) or Z-Gly-Leu-Phe-CMK ( $100 \mu\text{mol/L}$ ) in separate chambers to inhibit elastase or cathepsin G, respectively (Figure 5). In the presence of these inhibitors, a significant reduction in the amount of fibrin deposited at 25 minutes (Figure 5B and 5C) indicated that the interaction of neutrophils with platelets to facilitate fibrin formation is mediated through the neutrophil proteases, elastase and cathepsin G. Perfusing pAB-TF ( $20 \mu\text{g/mL}$ ) containing recalcified citrated PFP (CTI present) over neutrophils and platelets that had also been incubated with pAB-TF ( $50 \mu\text{g/mL}$ ) did not block fibrin formation (Figure 5D). The same was true when PFP (CTI present) containing  $20 \mu\text{g/mL}$  anti-CD11b (pAB-TF) was perfused over neutrophil-platelet cell mixtures that had already been incubated with the matching antibody.

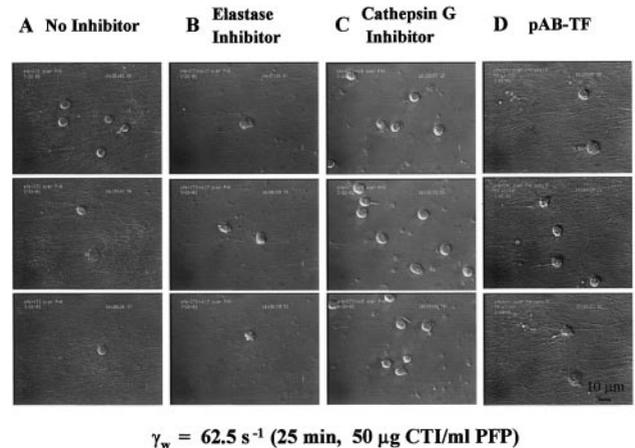


**Figure 4.** Initiation of coagulation through neutrophil-platelet interactions. Recalcified citrated PFP was perfused at a shear rate of  $62.5 \text{ s}^{-1}$  for 35 minutes over a neutrophil-coated surface, a platelet-coated surface, and a surface coated with neutrophils as well as platelets. Massive fibrin formation, uniformly distributed, was observed only on the latter surface, which had allowed neutrophil-platelet interaction before perfusion. Identical results were obtained in 3 separate experiments; each was conducted with individual donors. Flow is from right to left.

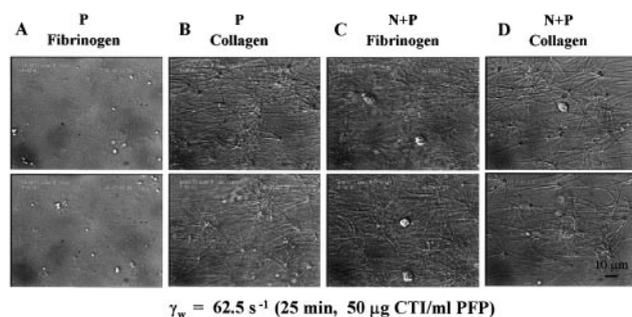
anti-CD11b was perfused over neutrophil-platelet cell mixtures that had already been incubated with the matching antibody.

To further examine the mechanism through which neutrophils interact with platelets to trigger fibrin formation, recalcified citrated PFP (containing CTI) was perfused concurrently (wall shear rate  $62.5 \text{ s}^{-1}$ ) over 4 different matrices: (1) fibrinogen-adherent platelets, (2) collagen-adherent platelets, (3) fibrinogen-adherent neutrophils and platelets, and (4) collagen-adherent neutrophils and platelets. After 25 minutes, although surfaces 2, 3, and 4 showed significant fibrin formation, surface 1 showed minimal deposition of fibrin (Figure 6). Comparable fibrin formation on surfaces 2 and 3 demonstrated that fibrinogen-adherent neutrophils enhance fibrin formation on fibrinogen-adherent platelets to the levels supported by platelets alone on a collagen surface.

To determine fibrin density (mass per unit area) that was due to the presence of neutrophils with platelets, circular



**Figure 5.** Fibrin formation was attenuated by elastase and cathepsin G inhibitors but not by pAB-TF. Fibrin deposition at a wall shear rate of  $62.5 \text{ s}^{-1}$  after 25 minutes over a surface coated with neutrophils and platelets not treated with any inhibitor or antibody (A) or treated with MeOSuc-Ala-Ala-Pro-Ala-CMK (B), Z-Gly-Leu-Phe-CMK (C), or pAB-TF (D) is shown. Identical results were obtained in 3 separate experiments; each was conducted with individual donors. Flow is from right to left.



**Figure 6.** Neutrophils enhance platelet-dependent fibrin formation to the levels supported by collagen-adherent platelets. Fibrin formation at a wall shear rate of  $62.5 \text{ s}^{-1}$  after 25 minutes over fibrinogen-adherent platelets (P, A), collagen-adherent platelets (P, B), fibrinogen-adherent neutrophils and platelets (N+P, C), or collagen-adherent neutrophils and platelets (N+P, D) is shown. Comparison of panels A, B, and C indicates that although collagen-adherent platelets (B) are more procoagulant than are fibrinogen-adherent platelets (A), neutrophils can augment fibrin formation on fibrinogen-coated platelets (C) to the levels supported by collagen-adherent platelets.

microcapillaries were used to avoid side-wall effects. The surface density of fibrin deposited on fibrinogen-adherent platelets was  $0.012 \text{ mg/cm}^2$  greater than that on a blank surface (fibrinogen alone, no cells). In contrast, the increase in fibrin density on a fibrinogen surface coated with neutrophils and platelets (relative to the blank surface) was  $0.057 \text{ mg/cm}^2$ . This value was equivalent to the increase in fibrin density on collagen-adherent platelets ( $0.055 \text{ mg/cm}^2$ ), demonstrating that collagen-adherent platelets and fibrinogen-adherent neutrophil-platelet mixtures cause the same levels of fibrin deposition at  $62.5 \text{ s}^{-1}$  (25 minutes of perfusion).

## Discussion

We demonstrated that under venous flow conditions, individual adherent neutrophils can significantly enhance fibrin deposition by CD18-dependent capture of fibrin protofibrils that are flowing in plasma and factor XIIIa-dependent thrombin generation through pathways attenuated by MeOSuc-Ala-Ala-Pro-Ala-CMK (elastase inhibitor) as well as Z-Gly-Leu-Phe-CMK (cathepsin G inhibitor). Also, neutrophils promote fibrin formation in a platelet-dependent manner through pathways attenuated by inhibitors of elastase or cathepsin G when platelets are adherent to fibrinogen.

Capture of fibrin protofibrils by neutrophils was also directly visualized, and the inhibition of the contact pathway of coagulation by CTI was accompanied by a 40- to 50-minute prolongation in the time required for fibrin deposition on neutrophils. The reduction in fibrin accumulation in the absence of either elastase or cathepsin G (CTI absent) demonstrated that fibrin capture was not solely responsible for fibrin deposition; neutrophils were also promoting factor XIIIa-dependent fibrin formation in a platelet-independent manner through pathways mediated by the released proteases, elastase and cathepsin G.

It is difficult to fully distinguish the role of Mac-1 on fibrin capture versus its role in thrombin formation because Mac-1 binds fibrin(ogen) and factor X to the neutrophil.<sup>5</sup> Because neutrophils can bind high molecular weight kininogen (which can bind prekallikrein and factor XI) as well as factor XII,<sup>23</sup> antibodies against CD11b/CD18 from rabbit anti-mouse CD11b/CD18

fibrin binding but may also attenuate pathways leading to Xa and thrombin formation on the neutrophil surface. Also, factor XIIIa is a known activator of neutrophils,<sup>24</sup> and CTI would be expected to attenuate kallikrein-mediated release of elastase by neutrophils exposed to recalcified plasma.<sup>25</sup> Still, the direct observation of fibrin capture by neutrophils (online Figure I) is consistent with a role for Mac-1 in capture.

The reduction in fibrin formation by either the blockade of Mac-1, inhibition of XIIIa, or inhibition of either one of two proteases, cathepsin G and elastase, may be attributed to the suppression of prothrombinase formation (factor Xa and factor Va) on the neutrophil surface. Because cathepsin G has been found to activate Mac-1-bound factor X,<sup>5</sup> decline in factor Xa levels can be expected by inhibition of cathepsin G or the blockade of Mac-1. Moreover, inhibition of elastase or cathepsin G can suppress factor V activation by these proteases,<sup>26</sup> resulting in reduced levels of factor Va. Inhibition of factor XIIIa may reduce the formation of the prothrombinase complex.

We have observed that neutrophils interact with platelets to trigger fibrin formation under flow, thus indicating that neutrophil-platelet aggregation and adhesion can have profound roles in promoting coagulation. Rather than being localized on and near neutrophils, the formed fibrin was deposited uniformly over the entire platelet surface of the flow chamber. This observation suggested that it was a consequence of some intercellular signaling event and/or catalysis of coagulation biochemistry between neutrophils and platelets that is mediated by elastase/cathepsin G. Several pathways exist by which neutrophils may trigger thrombin production and subsequent fibrin formation in a platelet dependent manner, including: (1) elastase<sup>3,27</sup> or cathepsin G<sup>4</sup> enhancement of activation of spread platelets or (2) elastase/cathepsin G cleavage of plasma zymogens such as factor V<sup>26</sup> and/or factor X.<sup>5</sup> Although mechanisms of P-selectin-mediated adhesion under flow are eliminated in the present study, P-selectin-dependent platelet-neutrophil signaling did not appear to play a role in the process, because anti-P-selectin had no effect.

Comparing fibrin formation on fibrinogen-adherent neutrophil-platelet cell mixtures with that on collagen-adherent platelets (no neutrophils), we found the amounts of fibrin deposited to be equivalent. Because fibrinogen-adherent platelets, unlike collagen-adherent platelets, are not fully activated procoagulant structures,<sup>15,16</sup> the above-mentioned observations demonstrated that neutrophils, in the absence of collagen, can activate platelets on their own and subsequently turn them into fully coagulating structures. This may have direct implications in studies of biomaterial thrombosis with contact pathway activation (XIIIa generation) and the antithrombotic effects of P-selectin antibodies.<sup>1</sup>

In summary, we have demonstrated that neutrophils can enhance fibrin formation in a platelet-independent and a platelet-dependent manner. Individual neutrophils can accelerate fibrin deposition by generating and capturing fibrin protofibrils in a CD18-dependent mechanism and generating thrombin and fibrin when the contact pathway is intact through elastase/cathepsin G-mediated pathways. At the same time, neutrophils can promote fibrin production by interacting with platelets and turning them into fully proco-

agulant structures via released elastase/cathepsin G, even when factor XIIIa is inhibited.

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## Figure Legends

**Figure I.** Capture of a fibrin fibril by a neutrophil. Recalcified citrated PFP was perfused over neutrophils at a wall shear rate of  $50 \text{ s}^{-1}$  in the absence of CTI. Comparison of A with B shows that the neutrophil suddenly captured a fibrin fibril (indicated by the arrow) within 830 ms. Flow is from right to left.

**Figure II.** Cross-sectional capture effects play a minor role in fibrin accumulation around neutrophils. On perfusion of recalcified citrated PFP (no CTI) for 20 min at a wall shear rate of  $62.5 \text{ s}^{-1}$ , the amount of fibrin deposited around beads (B) was considerably less than that around neutrophils (A).

## Methods

### Preparation of Cells and Platelet Free Plasma

Following isolation, neutrophils were washed and resuspended in Hanks' Balanced Salt solution (HBSS; Gibco Laboratories) containing 1% human serum albumin. Finally, cells were counted electronically by a Coulter counter (model Z2) and were diluted to a final concentration of  $10^6$  cells/ml. Platelet rich plasma (PRP) was obtained by centrifugation of anticoagulated whole blood at 130 g for 15 min. A gel filtration column was prepared using Sepharose 2B (20 mL;Sigma) and was equilibrated with Hepes-buffered tyrode's solution, pH 7.35, containing HSA (3.5 mg/ml), 137 mM NaCl, 2.7 mM KCl, 1mM  $MgCl_2$ , 3.8 mM  $NaH_2PO_4$ , 3.8 mM Hepes, and 5.5 mM glucose. PRP (2 ml) was layered onto the column and the platelets were eluted using the Hepes-buffered tyrode's solution. Platelets were collected in 500  $\mu$ l fractions with the early fractions pooled and diluted to a concentration of  $2.5 \times 10^7$  platelets/ml before use. Platelet free plasma (PFP) was obtained by centrifuging the citrated PRP at 10000 g for 10 min.

### Adhesion of Cells/Beads to Flow Chamber Surface

Isolated cells (neutrophils and/or platelets) were perfused into fibrinogen-coated or collagen-coated flow chambers, and they were allowed to settle and adhere to the surface for 60 min. To adhere both neutrophils and polystyrene beads to the fibrinogen-coated glass surface in the same flow chamber, fibrinogen-coated 10  $\mu$ m polystyrene beads, which were prepared by incubating polystyrene beads (Coulter) with fibrinogen (200  $\mu$ g/ml) for 4 hours at 4°C, were mixed with isolated neutrophils ( $10^6$  cells/ml;  $2 \times 10^5$  beads/ml) prior to their perfusion into the flow chamber. Nonadherent cells and beads

were rinsed away by perfusing  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS for 5 min at  $62.5 \text{ s}^{-1}$ . Flow rates of 40, 50 and  $200 \mu\text{l}/\text{min}$  corresponded to wall shear stresses of 0.5, 0.625 and  $2.5 \text{ dyne}/\text{cm}^2$ , and wall shear rates of 50, 62.5 and  $250 \text{ s}^{-1}$ , respectively.

## Results

### Effect of Cross-sectional Capture Efficiency

To examine the role of capture efficiency in fibrin deposition around neutrophils from flowing recalcified plasma (no CTI), the accumulation of fibrin on adherent neutrophils at a wall shear rate of  $62.5 \text{ s}^{-1}$  was compared to that on adherent polystyrene beads of the same diameter within the same flow chamber. Both neutrophils (Figure IIA) and polystyrene beads (Figure IIB) showed fibrin accumulation in their vicinity after 20 min. However, the deposition around neutrophils was massive in comparison to that around polystyrene beads.