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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 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 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 μg/mL corn trypsin inhibitor [CTI]) or elastase/cathepsin G inhibitors (Methoxysuccinyl-Ala-Ala-Pro-Ala-Chloromethyl-Ketone/Z-Gly-Leu-Phe-CMK, 100 μmol/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

Blood 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. showed that neutrophils enhance fibrin deposition through P-selectin–dependent mechanisms. Adherent neutrophils may promote thrombosis via pathways such as deencryption of tissue factor, release of proteases that activate platelets, Mac-1 (CD11b/CD18) binding of factor X, and improved capture of flowing platelets via protrusion into the flow stream.

The capture of neutrophils by the vessel wall involves neutrophil P-selectin glycoprotein ligand-1 (PSGL-1)–mediated rolling and membrane tethering on P-selectin presented by activated endothelium or spread platelets, followed by β₂-integrin–mediated firm arrest. The neutrophil β₂-integrin Mac-1, when upregulated, can bind endothelial intercellular adhesion molecule-1 and an unknown platelet ligand, which may be glycoprotein Ibα. Adherent neutrophils can also mediate the capture of flowing neutrophils near the vessel wall via an L-selectin–PSGL-1 mechanism. Additionally, Mac-1 can bind γ190-202 dodecapeptide on the D domain of fibrinogen. The relationships among flow, neutrophil and platelet adhesion, and blood coagulation are intricately coupled. Collagen-adherent platelets are comparatively more procoagulant than fibrinogen-adherent platelets in promoting fibrin formation. Interactions of neutrophils with platelets in aggregometer-mixed cell suspensions have been found to cause neutrophil oxidative burst and arachidonic acid exchange. 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...
used as an analogue of the Dacron shunt model of Palabrica et al.1 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. Methoxyxycinnyl-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 Z-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. 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 × 10^7 cells per milliliter, respectively. (Please see online data supplement at http://www.ahajournals.org for details.)

Microcapillary Flow Chambers

Rectangular glass capillaries (Vitromed) with a cross section of 0.2 × 2.0 mm, a length of 7 cm, and a wall thickness of 0.15 mm were used as previously described8 to construct the flow chambers. To enable cell adhesion, microcapillary flow chambers were incubated with human fibrinogen solution (100 μg/mL) for 120 minutes at room temperature or with calf skin collagen (30 μg/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 μg/mL) or anti-CD11b (20 μg/mL) to block the β2-integrin, Mac-1. In other experiments, surface-adherent neutrophils were incubated with either pAB-TF (50 μg/mL), or Z-Gly-Leu-Phe-CMK (100 μmol/L), MeOSuc-Ala-Pro-Ala-CMK (100 μmol/L), or Z-Gly-Leu-Phe-CMK (100 μmol/L) to inhibit possible transfer of tissue factor from neutrophils to platelets19 or to suppress any coagulation that can be initiated when blood comes in contact with an artificial surface. In selected experiments, P-AB-TF (20 μg/mL), anti-CD62P (P-selectin), anti-CD11b (20 μg/mL), MeOSuc-Ala-Pro-Ala-CMK (100 μmol/L), or Z-Gly-Leu-Phe-CMK (100 μmol/L) was added to citrated PFP before perfusion. Factor VII–depleted plasma was prepared by treating PFP with anti-human factor VII/VIIa (10 μg/mL). In other experiments, PPACK (50 μmol/L) or hirudin (25 U/mL) was used to inhibit thrombin.

During flow experiments, the microcapillary flow chambers were mounted on a Zeiss Axiostar 135 microscope with ×4.6/1.40 oil immersion objective lens for contrast-enhanced differential interference contrast microscopy.8

Fluorescence Microscopy and Quantification of Fibrin Deposition

In some experiments, neutrophils were incubated with SYTO 17 fluorescent red nucleic acid stain (25 μmol/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 μg/mL) at 25 μL/min for 20 minutes. To avoid side-wall influences, we also measured fluorescent fibrinogen incorporation by using circular microcapillary chambers (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 mg/mL), was perfused into the chamber at 93 μL/min, corresponding to a wall shear stress of 0.625 dyne/cm² (by τw = 4Qμ/πR²) and a wall shear rate of 62.5 s⁻¹ (by γw = 4Q/πR³). After 20 minutes, the flow chamber was rinsed with HBSS and perfused with 1 mL plasmin (2 μmol/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 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...
Shear rates of 62.5 s⁻¹ and 250 s⁻¹ for 25 minutes. The density of fibrin deposited around each neutrophil at a wall shear rate of 250 s⁻¹ (Figure 2B) was significantly lower than that at a wall shear rate of 62.5 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 µg/mL) was perfused over adherent neutrophils at a wall shear rate of 62.5 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 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 1 (please see http://www.ahajournals.org) shows an acquired capture event in which a neutrophil captures an entire fibrin fiber within 830 ms (γₛ = 50 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 U/mL thrombin to 200 nmol/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 β₃-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 (γₛ = 62.5 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 µmol/L) or Z-Gly-Leu-Phe-CMK (100 µmol/L) in separate chambers to inhibit elastase or cathepsin G, respectively. Recalciﬁed 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...
Neutrophil Enhancement of Fibrin Formation via Platelet-Dependent Mechanisms

Recalcified citrated PFP (containing CTI) was perfused concurrently (wall shear rate 62.5 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 XIa 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 μmol/L) or Z-Gly-Leu-Phe-CMK (100 μmol/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 μg/mL) containing recalcified citrated PFP (CTI present) over neutrophils and platelets that had also been incubated with pAB-TF (50 μg/mL) did not block fibrin formation (Figure 5D). The same was true when PFP (CTI present) containing 2096 Arterioscler Thromb Vasc Biol. December 2001

To determine fibrin density (mass per unit area) that was due to the presence of neutrophils with platelets, circular 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 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...
fibrin deposition at 62.5 s

Adherent neutrophil-platelet mixtures cause the same levels of fibrin deposition as collagen-adherent platelets and fibrinogen-adherent 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 platelet surfaces (C) to the levels supported by collagen-adherent platelets.

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 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 platelet surfaces (C) to the levels supported by collagen-adherent platelets.

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 or cathepsin G enhancement of activation of spread platelets or (2) elastase/cathepsin G cleavage of plasma zymogens such as factor V and/or factor X. 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, 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 (XIIa generation) and the antithrombotic effects of P-selectin antibodies.

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, turning them into fully procoagulant structures.
agulant structures via released elastase/cathepsin G, even when factor XIIa is inhibited.

Acknowledgments
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References
**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 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 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, 1 mM MgCl$_2$, 3.8 mM NaH$_2$PO$_4$, 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 µl 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 µm polystyrene beads, which were prepared by incubating polystyrene beads (Coulter) with fibrinogen (200 µg/ml) for 4 hours at 4°C, were mixed with isolated neutrophils ($10^6$ cells/ml; 2 x $10^5$ beads/ml) prior to their perfusion into the flow chamber. Nonadherent cells and beads
were rinsed away by perfusing Ca$^{2+}$/Mg$^{2+}$-free HBSS for 5 min at 62.5 s$^{-1}$. Flow rates of 40, 50 and 200 µl/min corresponded to wall shear stresses of 0.5, 0.625 and 2.5 dyne/cm$^2$, and wall shear rates of 50, 62.5 and 250 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 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.