Fibrin Is a Determinant of Neutrophil Sequestration in the Lung

Jeffrey A. Cooper, Sui K. Lo, and Asrar B. Malik

We investigated the role of fibrin in the sequestration of neutrophils in lungs challenged with microembolism. Lungs of chronically prepared awake sheep were imaged after infusion of $^{111}$In-neutrophils and $^{131}$I-fibrinogen. Thrombin (80 units/kg) was administered to induce fibrin sequestration in the lung. One group received tranexamic acid (4 mg/kg i.v.) to inhibit fibrinolysis, and the control group received a saline infusion. Thrombin infusion increased both $^{131}$I-fibrinogen and $^{111}$In-neutrophils in the lung, but the increases were greater and more prolonged in the fibrinolysis-inhibited group. We examined the adherence of isolated neutrophils to fibrin matrix to investigate how neutrophil-fibrin interactions could mediate neutrophil sequestration. Unstimulated neutrophils showed a 37.5 ± 3.1% adherence to fibrin in contrast to a 3.9 ± 0.4% neutrophil adherence to endothelium and a 6.5 ± 0.5% adherence to agarose. Neutrophil adherence to fibrin increased to 81.7 ± 6.2% with activation by phorbol myristate acetate (10$^{-8}$ M) and was inhibited by a monoclonal antibody directed against the β-chain of the CD18 leukocyte adhesion glycoprotein. The findings indicate that retention of pulmonary fibrin microthrombi is associated with increased lung uptake of neutrophils. Fibrin serves as a substrate for neutrophil adherence, and this adherence is mediated by expression of the CD18 glycoprotein complex on neutrophils. Fibrin sequestration in the lung may contribute to lung vascular injury by inducing pulmonary neutrophil uptake. (Circulation Research 1988;63:735-741)

A    ept pulmonary edema secondary to increased permeability of the lung vasculature to fluid and protein is an essential feature of the adult respiratory distress syndrome (ARDS).1,2 Histological studies of ARDS have demonstrated that microthrombi containing fibrin and neutrophils are present in pulmonary microvessels.3 Thrombin-induced pulmonary microembolism reproduces some of the pathological features of ARDS,4,5 such as increased lung vascular permeability.5,6 The increase in vascular permeability requires fibrin clot formation.5,7 Neutrophils are also required for the expression of the increased permeability because their depletion with hydroxyurea or antileukocyte serum attenuates the permeability increase.7-9

Neutrophils accumulate in the pulmonary microvasculature after thrombin-induced microembolism in the acutely prepared anesthetized sheep.10 Neutrophil uptake appears to depend on fibrin clot formation because γ-thrombin, a form of thrombin that does not cleave fibrinogen but retains other properties of thrombin,11 did not cause pulmonary neutrophil localization.10 The chronically prepared unanesthetized sheep demonstrated transient increases in pulmonary vascular resistance and transvascular protein clearance after challenge with thrombin.2 However, after fibrinolysis inhibition, the thrombin-challenged unanesthetized sheep had greater and more sustained increases in pulmonary vascular resistance and lung transvascular protein clearance than did control sheep.2 These studies suggest that fibrinolysis inhibition enhances neutrophil-mediated vascular injury by prolonging the residence of pulmonary microemboli.

In the present study, we investigated neutrophil and fibrinogen kinetics in the lungs of awake, chronically prepared sheep challenged with thrombin. The role of fibrin entrapment in neutrophil sequestration was assessed by treating animals with a fibrinolysis inhibitor before thrombin challenge, thereby prolonging fibrin sequestration in the pulmonary vascular bed. Studies were also carried out to determine whether fibrin serves as a specific substrate for neutrophil adherence.

Materials and Methods

Neutrophil Isolation

Sheep neutrophils were isolated from peripheral blood by a modification of previously described

From the Departments of Radiology and Physiology, The Albany Medical College, Albany, New York.
Supported by HL-32418 (Program Project) from the National Institutes of Health.
Address for correspondence: Jeffrey A. Cooper, MD, Department of Radiology, The Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208.
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techniques. Sheep blood anticoagulated with EDTA (pH 7.40) was centrifuged, and the plasma and theuffy coat were discarded. The remaining red cells, which contained the neutrophil fraction, were mixed at a ratio of 1:1 with ice-cold buffer containing (M) NaCl 0.117, glucose 0.011, citrate 0.022, NaH$_2$PO$_4$ 0.086, K$_2$HPO$_4$ 0.0016, pH 7.40. The red cells were lysed with a 2× volume of ice-cold distilled water andisotonicity was reconstituted with 4× buffer. The lysate was pelleted, resuspended in buffer, layered over preformed continuous Percoll density gradient, and centrifuged. The neutrophil band was aspirated and washed. The isolate contained at least or more than 99% neutrophils and was free of platelets. Neutrophil chemotaxis to zymosan-treated serum, superoxide production at baseline and in response to phorbol myristate acetate (PMA; 10$^{-8}$ M) and adherence to endothelium at baseline and in response to PMA were similar to previously reported values. Sheep Experiments

Sheep blood anticoagulated with EDTA (pH 7.40) was centrifuged, and the plasma and theuffy coat were discarded. The remaining red cells, which contained the neutrophil fraction, were mixed at a ratio of 1:1 with ice-cold buffer containing (M) NaCl 0.117, glucose 0.011, citrate 0.022, NaH$_2$PO$_4$ 0.086, K$_2$HPO$_4$ 0.0016, pH 7.40. The red cells were lysed with a 2× volume of ice-cold distilled water and isotonicity was reconstituted with 4× buffer. The lysate was pelleted, resuspended in buffer, layered over preformed continuous Percoll density gradient, and centrifuged. The neutrophil band was aspirated and washed. The isolate contained at least or more than 99% neutrophils and was free of platelets. Neutrophil chemotaxis to zymosan-treated serum, superoxide production at baseline and in response to phorbol myristate acetate (PMA; 10$^{-8}$ M) and adherence to endothelium at baseline and in response to PMA were similar to previously reported values.

Neutrophil Labeling

The neutrophils were suspended in 5 ml buffer and 0.5 mCi $^{111}$In-oxine (Amersham, Arlington Heights, Illinois) was slowly added with agitation. After incubation at room temperature for 30 minutes, the labeled neutrophils were washed with buffer to remove unbound $^{111}$In. Labeling efficiency was equal to or more than 80%. The labeled neutrophils routinely showed at least or more than 98% Trypan blue dye exclusion.

Fibrinogen Isolation

Sheep fibrinogen was isolated according to the method of Kazal et al. Plasma anticoagulated with 20 mM citrate and 3 mM benzamidine was precipitated twice with BaSO$_4$ and MgSO$_4$, and the precipitate was discarded. Fibrinogen was precipitated twice from the supernatant with glycine. Plasminogen and fibrinogen were removed by passage over lysine and collagen affinity columns. The isolated fibrinogen was 97% coagulable and free of contaminants by polyacrylamide gel electrophoresis.

Fibrinogen Labeling

Fibrinogen labeling was performed by the lactoperoxidase method. The fibrinogen (250 μg in 100 μl buffer) and 5 μg lactoperoxidase were added to Na$^{131}$I (pH 7; New England Nuclear, Boston, Massachusetts), and the reaction was started with 10 mM H$_2$O$_2$. The mixture was agitated for 30 minutes and then quenched with 5 mM cysteine. Carrier KI (0.1 M) was added and free iodine removed by passage over a G10 Sephadex column. Isotopic clotability was more than 85%. Gel chromatography indicated that degradation of the fibrinogen did not take place. Free iodine determined by trichloroacetic acid precipitation was less than 1%.

Gamma Camera Imaging

Pulmonary radioactivity was determined with a large field of view gamma camera (ElScint, Haifa, Israel). For studies with $^{111}$In-neutrophils alone, the camera was fitted with a medium energy (300 KeV), medium resolution (12 mm) collimator. For studies with $^{131}$I-fibrinogen either alone or in combination with $^{111}$In-neutrophils, a high-energy (400 KeV), medium-resolution (14 mm) collimator was used. Windows were set at 173±26 KeV for $^{111}$In and 361±54 KeV for $^{131}$I. A digital computer (MV/6000, Data General, Westborough, Massachusetts) acquired the spatial distribution of the pulmonary radioactivity every 5 minutes. The data were recorded in matrices having 128×128 picture elements, with separate matrices for each isotope. Each picture element corresponded to a 3×3-mm area.

Sheep Experiments

Carotid arterial catheters and external jugular vein introducer sheaths were placed in sheep (24–26 kg) at least 2 days before study. At the time of the study, the sheep were secured in a radiolucent restraining sling. A 7.5F Swan-Ganz catheter was guided into the pulmonary artery. Systemic, pulmonary artery, and pulmonary arterial wedge pressures were continuously monitored. Cardiac output was measured with the thermodilution technique. Blood was withdrawn for the isolation and labeling of autologous neutrophils. The gamma camera was positioned adjacent to the right lateral chest wall, and serial 5-minute dynamic images were started. $^{131}$I-fibrinogen (200 μCi) was injected intravenously as imaging was begun, followed in 5 minutes by 500 μCi $^{111}$In-neutrophils. At 90 minutes into the 2-hour baseline period, the fibrinolysis-inhibited group received tranexamic acid (4 mg/kg i.v.) while the control group received a saline infusion. Thrombin (supplied by Dr. John Fenton of the New York State Department of Health, Albany, New York) was administered at a dose of 80 units/kg i.v. over 10 minutes at the end of the 2-hour baseline (30 minutes after the infusion of tranexamic acid or saline). Imaging was continued for a 4-hour period. Blood was withdrawn at intervals for total white cell count, absolute granulocyte count, $^{111}$In-neutrophil radioactivity, and $^{131}$I-fibrinogen radioactivity. Plasma samples were counted to detect free $^{111}$In-radioactivity and were precipitated with trichloroacetic acid to correct for in vivo deiodination of the $^{131}$I-fibrinogen.

Endothelial Cell Monolayer Preparation

The CCL-209 bovine pulmonary artery endothelial cell line was obtained at the 16th passage from the American Type Culture Collection (Rockville, Maryland) and was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 20% fetal bovine serum, gentamicin (50 μg/ml), and Modified Eagle Medium with nonessential amino acids (10 mM). The cells were removed from the tissue culture flask by brief and gentle trypsinization, pelleted, resuspended at 8×10$^5$ cells/ml, and seeded onto plastic wells. The
wells were incubated at 37°C in a 95% air-5% CO₂ atmosphere and used 72 hours after seeding.

Neutrophil Adherence Study

Aliquots of sheep fibrinogen (0.25 mg/0.5 ml) were placed into plastic wells or plastic wells with a confluent endothelial monolayer. The fibrinogen was clotted with 1 unit/ml thrombin. Control wells contained either 0.5 ml of 7 mg/ml agarose or an endothelial monolayer alone. DMEM (0.9 ml) was added, and the wells were incubated for 3 hours at 37°C in 95% air-5% CO₂. Sheep neutrophils labeled with 31Cr-sodium chromate (2 × 10⁷ cells in 0.1 ml) were added to the wells, and either PMA (final concentration 1 μM) or a diluent control was added as a neutrophil-activating agent. The wells were incubated in 5% CO₂ in air at 37°C for 60 minutes. Nonadherent neutrophils were removed by washing in a standardized manner twice with 0.5 ml PBS and once with 1 ml PBS. NaOH (1N) was added to the wells, and the lysate of each well was counted in a well counter. The neutrophil adherence was the 31Cr radioactivity as a percentage of the total cellular radioactivity added.

We used a murine monoclonal antibody (MoAb 60.3) against the β-chain of the CD18 glycoprotein complex on the neutrophil (supplied by Dr. John Harlan, University of Washington, Seattle, Washington) to examine the role of this adherence protein in mediating neutrophil attachment to fibrin. Neutrophils incubated in buffer containing 160 μg/ml MoAb 60.3 were added to the adherence assay without washing. Fluorescence microscopy with a fluorescein-labeled antibody to mouse immunoglobulin demonstrated binding to MoAb 60.3 to the sheep neutrophil. This binding was specific and not to Fc-receptor sites because control murine antibody (antineutrophil antigen free) failed to show staining.

Data Analysis

Counts in the lungs from each of the sequential 5-minute image matrices were summed to determine the time course change in total lung neutrophils and fibrinogen. The neutrophil activities were corrected for 131I downscatter. Results are expressed as the mean ± SEM. The significance of differences was determined by analysis of variance and Student's t test.16

Results

Neutrophil Kinetics

After reinfusion of 111In-neutrophils, lung radioactivity peaked at 10 minutes, washing out over the remainder of the baseline period (Figure 1). Thrombin administration increased lung neutrophil radioactivity from 70.9 ± 3.0% to 79.7 ± 4.1% in the control group (p<0.05) and from 72.1 ± 5.0% to 88.3 ± 7.3% in the fibrinolysis-inhibited group (p<0.05). In the fibrinolysis-inhibited group, lung neutrophil radioactivity between 150 and 360 minutes was more than control (p<0.05).

Peripheral blood neutrophil radioactivity decreased to similar degrees after thrombin in the fibrinolysis-inhibited group (0.38 ± 0.04 of baseline, p<0.05) and control group (0.46 ± 0.11 of baseline, p<0.05), and remained decreased for the rest of the study (Figure 2). Absolute neutrophil counts in the control group decreased after thrombin to 2.900 ± 778 cells/μl (p<0.05) but returned toward baseline over the remainder of the study. The absolute neutrophil counts in the fibrinolysis-inhibited and control groups were parallel.

Fibrinogen Kinetics

After infusion of the 131I-fibrinogen, lung radioactivity rapidly reached a steady state with 86.1 ± 2.8% of the initial radioactivity present after the 2-hour baseline period (Figure 3). Administration of thrombin increased lung 131I-radioactivity in both groups (control, from 86.5 ± 3.7% to 95.5 ± 1.0%, p<0.05; fibrinolysis-inhibited, from 85.6 ± 3.7% to 110.5 ± 2.0%, p<0.05). In the control group, lung 131I-radioactivity returned toward baseline over the next 90 minutes, whereas the fibrinolysis-inhibited group 131I-radioactivity remained above the control group (p<0.05) for the 150–240-minute period.

In the peripheral blood, protein-associated 131I-radioactivity decreased after thrombin in both groups (p<0.05) and remained decreased (Figure 4). Differences between the two groups were not detectable.

Hemodynamic Data

Pulmonary hemodynamic data are shown in Figure 5. Pulmonary blood flow decreased with thrombin administration in the control group (from 5.15 ± 0.49 to 2.56 ± 0.49 l/min, p<0.05). Thrombin had a similar effect in the fibrinolysis-inhibited group (from 4.28 ± 0.40 to 2.34 ± 0.27 l/min, p<0.05). In both groups, pulmonary blood flow returned toward baseline values.
Thrombin administration caused similar increases in pulmonary artery pressure in both groups (control, from $16.0 \pm 1.4$ to $49.0 \pm 5.9$ mm Hg, $p<0.05$; fibrinolysis-inhibited, from $16.0 \pm 1.0$ to $45.0 \pm 1.9$ mm Hg, $p<0.05$). After 255 minutes, pulmonary artery pressure returned to baseline values in the control group but remained above baseline and above the control group in the fibrinolysis-inhibited group ($p<0.05$). Changes in the pulmonary arterial wedge pressure were not different. The initial increases in pulmonary vascular resistance were similar (control, from $2.15 \pm 0.40$ to $16.1 \pm 5.9$ l/min/mm Hg, $p<0.05$; 

**FIGURE 2.** Peripheral blood $^{111}$In-neutrophil (PMN) radioactivity (fraction of maximum radioactivity) and absolute neutrophil counts ($\times 10^9$ cells/µL) versus time. Thrombin infusion at 120 minutes decreased $^{111}$In-neutrophil radioactivity and absolute neutrophil counts in the peripheral blood. $^{111}$In-neutrophil radioactivity remained depressed for the remainder of the study while peripheral blood absolute granulocyte counts returned toward baseline. The patterns were similar between the two groups.

**FIGURE 3.** Lung radioactivity of $^{131}$I-fibrinogen and its derivatives [fibrin(ogen)] expressed as a percentage of initial maximal lung $^{131}$I-radioactivity versus time. Thrombin infusion at 120 minutes increased lung $^{131}$I-fibrin(ogen) radioactivity in both groups. The increase was greater in the fibrinolysis-inhibited group ($p<0.05$ compared with control).

**FIGURE 4.** Peripheral blood radioactivity of protein-associated $^{131}$I-fibrinogen and its derivatives [fibrin(ogen)] expressed as the fraction of the maximum $^{131}$I-radioactivity versus time. Thrombin infusion at 120 minutes decreased $^{131}$I-fibrin(ogen) radioactivity and absolute neutrophil counts in the peripheral blood. The changes were similar between the two groups.
FIGURE 5. The effect of thrombin on pulmonary hemodynamics. Pulmonary blood flow ($Q_L$) in liters per minute, mean pulmonary artery pressure ($P_{PA}$), mean pulmonary arterial wedge pressure ($P_{PAW}$), and pulmonary vascular resistance (PVR) versus time. Thrombin caused decreases in $Q_L$ and increases in $P_{PA}$, $P_{PAW}$, and PVR that were similar between the two groups. However, the increases $P_{PA}$ and PVR were more sustained in the fibrinolysis-inhibited group (*p<0.05 compared with control).

Neutrophil Adherence In Vitro

Unstimulated neutrophils had a baseline adherence to endothelial monolayers of 3.9 ± 0.4% and an adherence to agarose of 6.5 ± 0.5%. Fibrin increased the adherence to 37.5 ± 3.1% (p<0.01 compared with baseline and agarose) (Figure 6). The adherence of neutrophils to fibrin was not enhanced by forming the fibrin over endothelial cells. Neutrophils activated with PMA showed an increased degree of adherence to fibrin (91.6 ± 1.6%, p<0.01 compared with unstimulated neutrophils) that was comparable to PMA-stimulated neutrophil adherence to endothelium (81.9 ± 6.2%). MoAb 60.3, a murine monoclonal antibody to the CD18 glycoprotein on neutrophils, reduced the baseline adherence of unstimulated neutrophils to fibrin (from 21.7 ± 3.6% to 9.3 ± 1.2%, p<0.01) (Figure 7).

Discussion

We examined the role of fibrin in the pulmonary neutrophil sequestration associated with pulmonary microembolism. The pulmonary neutrophil uptake induced by microembolism could be prolonged by increasing the duration of fibrin retention in the microcirculation. In vitro studies indicated that neutrophils adhere to fibrin by expression of the CD18 leukocyte adhesion glycoprotein complex on the neutrophil cell membrane.

Sheep neutrophils isolated with the present method demonstrated chemotaxis, phagocytosis, endothelial adherence, aggregation, and superoxide production. At 1 hour after infusion, 30% of the neutrophils were circulating and 35% were in the lung. Intra-arterial infusion also resulted in lung retention, indicating that the observed uptake was specific for the pulmonary vascular bed and was not
The amount of fibrin in the lung is related to the rate of clot formation and lysis. If the rate of clot lysis is similar to or faster than the rate of clot formation, the quantity of lung fibrin at any time would be less than the total amount formed. The similar decreases in blood radioactivities (Figure 4) suggest that comparable amounts of fibrin were formed. Therefore, the observation that the increase in lung \(^{131}\text{I}\) radioactivity in the tranexamic acid-treated group was more than the control group reflects fibrinolysis inhibition.

The studies indicating that unstimulated neutrophils adhered to fibrin in vitro indicate a mechanism by which fibrin can contribute to pulmonary leukostasis. Adherence to fibrin was specific because neutrophils did not adhere to agarose. Moreover, adherence to fibrin was dependent on expression of the CD18 leukocyte glycoprotein adhesion complex.\(^{15}\) The expression of neutrophil adhesive glycoprotein complex may mediate neutrophil sequestration after intravascular fibrin entrapment.

The finding that phorbol myristate acetate increased neutrophil adherence to fibrin suggests that neutrophil-activating substances, such as those generated after thrombin challenge,\(^{17-19}\) contribute to pulmonary neutrophil uptake by enhancing adherence to fibrin. The role of fibrin in mediating pulmonary neutrophil uptake after microembolism is supported by the finding that fibrinogen depletion prevents the associated neutropenia\(^5,6\) and that fibrinolysis inhibition enhances neutrophil-mediated lung vascular injury.\(^5\) These results further support the concept that fibrin is an important mediator of neutrophil sequestration.

The in vitro data indicate a specific mechanism of neutrophil-fibrin interaction. Another explanation of the data is that fibrin microembolization mediates pulmonary neutrophil uptake by reducing regional pulmonary blood flow.\(^{27,28}\) However, we observed similar pulmonary hemodynamics in the two groups.
and reduced pulmonary blood flow did not alter neutrophil kinetics in the sheep lung. Another mechanism of uptake of neutrophils and fibrin may be the stationary intravascular mononuclear phagocytes. We have shown that uptake of particles (99m-Tc-sulfur colloid) by pulmonary intravascular macrophages in sheep is sustained up to 24 hours in contrast to the present observations. Phagocytosis cannot explain the 90-minute washout of neutrophils and fibrin in the control group.

Nonspecific effects of tranexamic acid may influence neutrophil kinetics and neutrophil-fibrin interactions. Although tranexamic acid is known to inhibit C1 esterase, this occurs at concentrations of more than 0.03 M, far more than that achieved in this study. Therefore, C1 esterase inhibition by tranexamic acid may not be a factor in the observed neutrophil kinetics. Furthermore, we have shown that tranexamic acid had no independent effect on neutrophil adherence.

In summary, the results indicate that thrombin-induced pulmonary microembolism in sheep induces the pulmonary localization of fibrin and neutrophils. Inhibition of fibrinolysis, which is required for the mediation of pulmonary vascular injury after thrombin microembolism, increases the degree and duration of neutrophil and fibrin uptake in the lungs. Neutrophil adherence to fibrin occurs by the expression of neutrophil CD18 glycoprotein adhesion complex. The results indicate that neutrophil-fibrin interaction is an important determinant of pulmonary neutrophil sequestration.

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