Nuclear factor κ B mediates a procoagulant response in monocytes during extracorporeal circulation

Elizabeth N. Morgan, MD
Timothy H. Pohlman, MD
Craig Vocolka, BS
Angela Farr, BA
Gina Lindley, BS
Wayne Chandler, MD
Jeanette M. Griscavage-Ennis, PhD
Edward D. Verrier, MD

Objective: The objective of this study was to examine the mechanism of procoagulant activity and inhibition in whole blood during extracorporeal circulation.

Methods: In this study we examine the development of procoagulant activity and monocyte activation in heparinized whole blood passing through a closed circuit consisting of a pump and silicone envelope membrane oxygenator for 6 hours.

Results: Anaphylatoxins, C3a and C5a, determined by means of enzyme-linked immunosorbent assay, appeared in the blood within 30 minutes of circulation. Circulated blood developed a marked potential for coagulation demonstrated in a 1-step clotting assay that reached maximal activity by 4 hours of circulation. This procoagulant activity was neutralized by anti-tissue factor antibody, suggesting a prominent role for the extrinsic pathway in pump-induced intravascular coagulation. Isolation of monocytes from circulated blood revealed that tissue factor expression is upregulated on the cell surface. Furthermore, we observed nuclear factor κ B nuclear translocation in monocytes from blood passing through the circuit, suggesting that tissue factor expression was due to monocyte stimulation and transcriptional activation of the tissue factor gene. Tissue factor expression resulted in an approximately 30-fold increase in thrombin generation. Monocyte nuclear factor κ B activation, monocyte tissue factor expression, thrombin generation, and the procoagulant activity of blood in extracorporeal circulation were all blocked by the proteasome inhibitor MG132.

Conclusions: We conclude that intravascular tissue factor expression during extracorporeal circulation of blood is due to nuclear factor κ B-mediated activation of monocytes (possibly by complement), which can be controlled pharmacologically.

From the Department of Surgery, Division of Cardiothoracic Surgery, University of Washington School of Medicine, Seattle, Wash.

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Address for reprints: Timothy H. Pohlman, MD, Harborview Medical Center, 325 9th Ave, Box 359796, Seattle, WA 98104 (E-mail: tpohlman@u.washington.edu).

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Despite advances in cardiothoracic surgery, there remains considerable potential for morbidity and mortality resulting from the whole-body inflammatory and coagulopathic response caused by cardiopulmonary bypass (CPB). Multiple events contribute to the coagulopathic response to CPB, including hypothermia, hemodilution of clotting factors, and platelet dysfunction. Also, recent data suggest a prominent role for activation of the extrinsic, or tissue factor (TF)-dependent, pathway of coagulation in hemostatic abnormalities in patients subjected to CPB.¹⁻³

The extrinsic pathway of coagulation is initiated when TF contacts and activates clotting factor VII (VIIa).⁴ This dual-enzyme complex, TF/VIIa, then activates both factors IX and X, ultimately resulting in the generation of thrombin and fibrin.

Normally, TF is constitutively expressed only in the extravascular space. However, under conditions of oxidative and inflammatory stress, TF expression can be induced on intravascular cells, such as monocytes and endothelial cells. The resulting intravascular activation of the extrinsic pathway of coagulation contributes to a systemic inflammatory response syndrome and organ failure.^{5,6}

The inducible expression of TF is mediated, in part, by activation of the transcription factor nuclear factor (NF) κ B. NF- κ B is held inactive in the cytoplasm by I κ B α . During stress, I κ B α is ubiquitinated and degraded by the proteasome complex, freeing NF- κ B to translocate to the nucleus, where it initiates the transcription of *TF*, as well as other proinflammatory and procoagulant genes.⁷ NF- κ B also induces transcription of I κ B α to initiate a negative feedback loop, regulating NF- κ B-mediated cellular responses. TF expression also requires the coordinated action of other transcription factors, including AP-1 and Egr-1.⁸ Moreover, NF- κ B might have other roles in cardiac surgery that include mediating the cellular responses to oxidative stress (eg, ischemia-reperfusion injury), microbial invasion, and mechanical stress, and NF- κ B mediates activation of genetic programs that control tissue repair. NF- κ B is now recognized to have a potential role in programmed cell death (apoptosis) and the adaptive response to hypoxia. NF- κ B might also mediate other cytoprotective functions, such as preconditioning. Delineating these pathways might allow the selective modulation of homeostasis to the benefit of the patient during cardiac surgery and other clinical settings that require the extracorporeal circulation of blood.

In the present study, using an extracorporeal CPB system, we demonstrate NF- κ B activation in monocytes during oxygenated pump circulation, which is associated with an increase in TF expression on monocytes and thrombin generation in circulated blood. Furthermore, a selective proteasome inhibitor, MG132, blocked NF- κ B activation, TF expression, and thrombin generation, suggesting that NF- κ B plays an essential role in the coagulopathic response to CPB.

Material and Methods

Simulated Extracorporeal Circuit

Extracorporeal circulation was carried out in a closed system with surface area/blood volume ratios equivalent to those of a clinical circuit used for CPB. Each perfusion circuit is assembled from silicone rubber tubing, polycarbonate connectors, a polyvinyl chloride venous reservoir bag, and a 0.4-m² spiral-coil membrane oxygenator. All components of the circuit were sterilized, and blood samples were exposed to sterile laboratory equipment. Human blood (440 mL) was drawn from healthy donors through 16- or 18-gauge needles and polyvinyl tubing directly into a venous reservoir bag containing beef lung heparin (5 U/mL) and dextrose (2.25 mg/mL). The venous reservoir bag for the treatment groups additionally contained 3 μ mol/L MG132 (Calbiochem Novabio-

chem Corporation, San Diego, Calif) or proline dithiocarbamate (ProDTC) at 0.07, 0.21, and 0.42 mg/mL (provided by Norbert Frank, PhD, German Cancer Research Center). Informed written consent was obtained from all donors, and the protocols for the study were approved by the Institutional Review Board of the University of Washington. Eighteen simulated extracorporeal circuits were run at 37°C: 6 control, 6 MG132-treated, and 6 ProDTC-treated circuits. All donors were taking no medications 1 week before donating blood.

Blood and gas compartments of the circuits were flushed with 100% carbon dioxide for 15 minutes before priming and then were evacuated by applying suction to the sample port to debubble. The system was primed with Plasmalyte electrolyte solution, which is used clinically. The perfusion circuit was then filled with blood, avoiding bubble formation. After blood was added, as much priming volume as possible was removed; this can be compared with retrograde autologous priming used clinically in CPB. Blood was circulated for 6 hours at 300 mL/min and 37°C with a calibrated, barely occlusive roller pump. The oxygenator was ventilated with a mixture of 95% oxygen and 5% carbon dioxide (0.71 L/min). Blood was obtained from the venous reservoir before connection to the circuit and incubated in polypropylene tubes at 37°C for 6 hours before processing. Experimental samples for TF analysis were taken at 2, 4, and 6 hours of circulation. Experimental samples for NF- κ B analysis were taken directly from the venipuncture site (baseline) and at 5, 30, 60, and 120 minutes after initiation of the circuit.

Thrombin Measurement

Citrated plasma was prepared by centrifuging 9 parts whole blood to 1 part sodium citrate (0.105 mol/L) at 1500g for 10 minutes. Plasma samples were frozen at -70°C and assayed within 1 month. An F1.2 enzyme-linked immunosorbent assay (Dade Behring, Inc, Deerfield, Ill) based on the sandwich principle was used for the immunochemical determination of human prothrombin fragment F 1+2 in nanomoles per liter.

Monocyte Separation

Ficoll-Paque solution (Pharmacia Corporation, Peapack, NJ) was used for separation of mononuclear cells, as previously described.¹ Two milliliters of blood was withdrawn from the closed circuit system in ethylenediamine tetraacetic acid-containing tubes and diluted with 2 mL of Tris-buffered saline solution, pH 7.4. The diluted blood was carefully overlaid on 3 mL of Ficoll-Paque solution (Pharmacia) and centrifuged for 35 minutes at 18°C and 400g. The buffy coat, containing monocytes, was isolated and washed twice with 1 $\times$ Tris-buffered saline solution at 4°C for 30 seconds. Cells were counted with a Cell Dyn 3500 counter (Abbott Laboratories, North Chicago, Ill).

Procoagulant Activity Assay

Mononuclear cells (1×10^7) were diluted 1:1 in type-specific normal human plasma and incubated for 60 seconds at 37°C. CaCl₂ (8 mmol/L) was added, and clotting time was recorded with a STA Compact coagulation analyzer (Roche Diagnostics, Basel, Switzerland) in a 1-stage clotting assay, as previously described.⁹ Isolated monocytes were incubated with 90 μ g/mL anti-TF antibody (provided by N. Mackman, Scripps Institute) and subjected

Figure 1. Complement is activated in blood during simulated extracorporeal circulation. Whole blood from healthy donors was circulated through a membrane oxygenator, as described in the “Material and Methods” section. At time points indicated on the x-axis, blood was withdrawn, and the serum fraction of each sample was assayed for C3a (A), C5a (B), or soluble C5b9 (C) by means of enzyme-linked immunosorbent assay. Each sample was measured in duplicate. Values on the y-axis represent the means $\pm$ SEM for 4 separate experiments.

to procoagulant assay, as described above, to ensure that changes in clotting time are attributable to TF expression. The amount of TF expressed per 10^5 monocytes was determined by using a standard curve described by the following equation:

$$\text{TF (ng/mL)} = e^{(\ln[\text{PCA}]) - (4.74/-0.41)}$$

Nuclear Protein Isolation

The monocyte pellet from 2 mL of whole blood was resuspended in 400 μL of buffer (10 mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [pH 7.9], 1.5 mmol/L MgCl_2 , 10 mmol/L KCl, 0.1 mol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L dithiothreitol [DTT]) and allowed to swell on ice for 15 minutes. NP-40 (0.6%) was added, and the sample was vortexed for 10 seconds. The sample was centrifuged for 30 seconds, the supernatant was aspirated, and the pellet was resuspended in 20 μL of buffer (0.5 $\mu\text{mol/L}$ dithiothreitol, 0.1 mol/L phenylmethylsulfonyl fluoride, and 0.01 mg/mL leupeptin). The sample was incubated at 4°C for 15 minutes and centrifuged for 5 minutes at 16,000g. The supernatant containing nuclear protein was removed and quantified by means of the standard Bradford assay.

Electromobility Shift Assay

Nuclear protein (10 μg) was incubated in a binding reaction with a double-stranded, ^{32}P end-labeled oligonucleotide containing the human consensus NF- κB binding motif 5'-AGTTGAGGG-GACTTTCACAGGC-3' (Promega Corporation, Madison, Wis). Binding reactions occurred at room temperature for 20 minutes. Proteins were resolved on a 6% nondenaturing polyacrylamide gel at 100 V for 1 to 2 hours in a 0.5% Tris-borate electrophoresis buffered solution. The gels were dried and autoradiographed.

Statistical Analyses

Results are expressed as means $\pm$ SEM. Comparisons of values between groups at each time point were made by 1-way analysis of variance.

Results

Complement Activation During Extracorporeal Circulation

During CPB, blood is in contact with both the polymer surface and a gas-fluid phase interface. Both these interfaces activate complement by means of the alternate pathway generating anaphylatoxins C3a¹⁰ and C5a.¹¹ Within 30 minutes of the initiation of circulation of blood through the circuit and membrane oxygenator, levels of C3a increased sharply and continued to increase gradually for at least 2 hours after initiating circulation (Figure 1). There was a corresponding increase in levels of C5a (eg, 15-fold at 30 minutes). Soluble C5b-9 terminal complex, also a sensitive indicator of complement activation, was increased after 30 and 60 minutes of circulation. These values agree with previous studies using closed-circuit extracorporeal circulation.

Extracorporeal Circulation Induces Procoagulant Activity in Blood

Blood was sampled at various time points, anticoagulation was reversed, and the time required for spontaneous clot formation was observed to determine whether passage of whole blood through only a CPB circuit resulted in procoagulant activity. As shown in Figure 2, A (*open circles*), continued exposure of blood to the closed CPB circuit resulted in a 4-fold reduction in the time for clots to form. The observed reduction in clotting time began by 2 hours and shortened progressively until the end of the experiment (6 hours). This finding indicates development of significant procoagulant activity in CPB blood induced by the circuit alone without activation of coagulation by surgical trauma. Samples at each time point were treated with 90 $\mu\text{g/mL}$ TF

Figure 2. Extracorporeal circulation induces procoagulant activity (PCA) in blood. **A**, After circulation of blood for various periods of time (*x-axis*), procoagulant activity, expressed as the length of time required for blood to clot, was determined in duplicate for each time point. Clotting times were determined in the presence (*closed circles*) or absence (*open circles*) of a saturating amount of anti-TF antibody. **B**, Drawn blood from healthy donors was treated with either MG132 (final concentration, 3 μ mol/L) or an equal volume of vehicle alone. MG132-treated blood (*filled bars*) or vehicle control blood (*shaded bars*) was then circulated through the simulated extracorporeal circuit; procoagulant activity was determined at the various time points on the *x-axis*. Values (*y-axis*) represent duplicate determinations and the means $\pm$ SEM of 6 separate experiments.

monoclonal antibody cocktail (Figure 2, A, *closed circles*) to determine whether the procoagulant activity of circulated blood was due to the presence of TF on blood cells. TF antibody completely abolished the procoagulant activity, suggesting that the extrinsic pathway of coagulation is activated by passage of blood through the CPB circuit.

Intravascular TF expression requires transcriptional activation of the *TF* gene, which is mediated in part by NF- κ B. Therefore, we examined the effect of the treatment of blood with the NF- κ B inhibitor MG132 during circulation. MG132 blocks NF- κ B activation by inhibiting proteasome degradation of I κ B α .¹² The presence of MG132 during closed circulation significantly attenuated the reduction of clotting time in blood after 4 hours of circulation (Figure 2, B). After 6 hours, the reduction in clotting time was still less in the MG132-treated blood but did not approach statistical significance compared with the clotting time of circulated blood not treated with MG132. Of interest, blood treated with another NF- κ B inhibitor, ProDTC, did not inhibit the development of procoagulant activity in blood passing through a CPB circuit (data not shown).

Monocyte TF Expression

In the absence of red cell hemolysis, the most likely source of TF in whole blood is the monocyte. We isolated mononuclear phagocytes from blood passing through the closed circuit, as described in the "Material and Methods" section.

Cell-surface TF was then determined on cultured cells by means of enzyme-linked immunosorbent assay. As shown in Figure 3, by 2 hours there was a significant amount of TF present on mononuclear cells, which peaked at 4 hours. The expression of TF on monocytes could be substantially inhibited when MG132 was added to circulating blood.

NF- κ B Activation

Monocytes were isolated from samples taken directly from the venipuncture site and at 5, 30, 60, and 120 minutes after initiation of blood circulation. Isolated nuclear proteins were then probed for NF- κ B by using a DNA double-stranded probe containing the consensus binding site for the NF- κ B p65/p50 heterodimer. In Figure 4, NF- κ B is activated in nuclear proteins isolated from mononuclear phagocytes of circulated blood. In contrast, NF- κ B activity was inhibited when circulated blood was treated with MG132. Binding activity in these electrophoretic mobility shift assays was competed with a cold probe, indicating specificity for NF- κ B (data not shown).

Thrombin Generation

Because TF expression results in thrombin generation, we then assayed the thrombin byproduct F1.2 in our model. Table 1 shows that significant thrombin generation is detectable in blood at all time points as blood passes through the closed circuit, particularly at 6 hours, when there is a

Figure 3. MG132 inhibits TF expression on monocytes during extracorporeal circulation. Mononuclear phagocytes were isolated from circulated blood at various time points indicated on the y-axis. TF expression on surface membranes was determined by means of enzyme-linked immunosorbent assay, as described in the “Material and Methods” section. TF expression on monocytes from blood in the extracorporeal circuit was determined for blood treated with MG132 (3 μ mol/L) before addition to the closed circuit (*filled bars*) or after treatment with vehicle alone (*shaded bars*). Each datum point was determined in duplicate and represents the mean $\pm$ SEM of 6 separate experiments.

29-fold increase in thrombin generation over baseline values (before circulation). However, treatment of blood with MG132 abolished thrombin formation during closed circulation of blood through a membrane oxygenator, which is consistent with MG132-mediated inhibition of NF- κ B activation, TF expression on monocytes, and whole-blood procoagulant activity in this closed-circuit model.

Discussion

An intense prothrombotic state during CPB is attributed to activation of the intrinsic pathway through factor XII after blood comes in contact with the artificial surfaces of the extracorporeal circuit. However, recent studies have examined thrombin generation independent of intrinsic pathway-mediated coagulation through activation of the TF/factor VII-dependent pathway during^{3,13-15} or after¹⁶ CPB. Using a closed-loop extracorporeal circuit to simulate CPB, Kappelmayr and coworkers¹ demonstrated that monocyte TF expression and activity on monocytes in whole blood increase during circulation through the circuit, although the means of initiation and mechanisms of TF expression on monocytes were not addressed in that study. Here we demonstrate that TF expression significantly increased between 2 and 6 hours of extracorporeal circulation. We observed that the onset of TF expression follows the activation of complement and the generation of C3a, C5a, and sC5b9. Furthermore, we demonstrate that the transcription factor NF- κ B is activated in leukocytes circulating in the extracorporeal circuit and that NF- κ B inhibition with the proteasome inhibitor MG132 results in a reduction in both TF expression and procoagulant activity.

Complement activation, principally through the alternate

pathway, contributes to the systemic inflammatory response and capillary leak syndrome caused by CPB.¹⁷ Complement anaphylatoxins have been shown to stimulate expression of numerous proinflammatory mediators that might initiate or propagate deleterious systemic inflammatory reactions after CPB. C5a and C3a receptors are present on human monocytes¹⁸ and nonmyeloid cells,¹⁹ such as vascular endothelial cells. In vitro, C5a induces expression of interleukin (IL) 1, IL-6, and IL-8 on human monocytes or in various monocyte cell lines.²⁰ Earlier studies with rabbit alveolar macrophages have suggested that C5a might also induce procoagulant activity on mononuclear phagocytes.²¹ C5a induces procoagulant activity on endothelial cells (in part through upregulation of TF),²² a cell type that responds to several inflammatory mediators in a fashion similar to that of monocytes. Taken together, these observations lead us to suggest that the upregulation of procoagulant activity on peripheral blood monocytes during extracorporeal circulation is due to complement activation and the interaction of C5a (and/or C3a) with monocytes.

The increased procoagulant activity we measured in extracorporeal-circulated blood might be due to increased expression of TF on circulating monocytes.¹ Other sources of TF in vivo, primarily injured vascular tissue and pericardial blood,² are eliminated in this ex vivo study. Lymphocytes and granulocytes are not sources of TF, and platelets are a target of TF-generated thrombin but do not express TF. However, activated platelets release platelet factor 4, which has been reported to induce TF expression on monocytes, particularly in synergy with lipopolysaccharide stimulation of monocytes.²³ Increases in plasma concentrations of platelet granule contents, including platelet factor 4, occur during

Figure 4. NF- κ B activation in monocytes during extracorporeal circulation is blocked by MG132. Mononuclear phagocytes were isolated from blood in the closed circuit at the time points indicated at the top of the lanes. Nuclear proteins were purified from these cells and probed by means of electrophoretic mobility shift assay for NF- κ B translocation to the nucleus. Circulation of blood was carried out after treatment with MG132 (3 μ mol/L) or MG132 vehicle. This figure is representative of 4 similar electrophoretic mobility shift assays.

CPB,²⁴ and therefore complement stimulation of TF expression on monocytes during CPB could be enhanced by CPB-induced platelet activation. Erythrocytes might induce TF-dependent extrinsic pathway activation after hemolysis and might have contributed to the procoagulant response that we observed. Although we did not specifically measure hemolysis, there was no visual evidence of red cell destruction after passage through the ex vivo circuit.

The nature of the procoagulant response (reduction in the time to clot) measured in extracorporeal-circulated blood was shown specifically to be TF by means of neutralization with a monoclonal antibody directed against human TF. Previous work has demonstrated that monocyte membrane vesiculation and microparticle formation is responsible for the dissemination of inducible monocyte procoagulant activity.²⁵ We did not, however, address this critical aspect of mononuclear phagocyte biology in our study.

Using gel-shift analysis on isolated mononuclear phagocytes, we have demonstrated that the transcription factor NF- κ B is activated in circulating nucleated cells of blood in our closed circuit. TF gene transcription in human monocytic cells exposed to growth factors, bacterial lipopolysaccharide (endotoxin), or cytokines (tumor necrosis factor or IL-1) is, in each case, mediated in part by activation of NF- κ B. Transcription of the TF gene also requires positive regulation by AP-1 and Egr-1.⁸ Whether activation of these 2 transcription factors occurs in blood during extracorporeal circulation has not been determined.

Under most conditions, NF- κ B activation requires phosphorylation and ubiquitination of I κ B α , the cytoplasmic inhibitor of NF- κ B. Proteolysis of ubiquitinated I κ B α occurs at the proteasome, a multicatalytic protease, freeing NF- κ B to translocate to the nucleus. Inhibition of proteasome function with the peptide aldehyde MG132 blocks

TABLE 1. MG132 inhibits thrombin generation during membrane oxygenation

Time (h)	–MG132	+MG132
2	1.2 $\pm$ 0.09	1.3 $\pm$ 0.08
4	4.5 $\pm$ 0.11	1.4 $\pm$ 0.10
6	29.4 $\pm$ 95.3	2.1 $\pm$ 0.22*

Data are expressed as fold increase of thrombin over baseline.

* $P < .05$, –MG132 versus +MG132.

I κ B α degradation and NF- κ B activation in several cell types stimulated under a variety of conditions.^{26–28} Although there is an extensive amount of information on the effects of MG132 in vitro, little is known about the efficacy of this potential pharmaceutical intervention in vivo. A related class of proteasome inhibitors, boronic acids (eg, PS-341), given orally reduces progression of polyarthritis in a rat model of bacterial cell wall–induced chronic arthritis.²⁹ Proteasome inhibition with PS-341 is currently in clinical trials for treatment of neoplasia.³⁰ However, our study is the first to investigate the use of a selective proteasome inhibitor to modify the deleterious effects of a systemic inflammatory response syndrome during CPB. Because the toxic effects of peptide aldehydes or boronic acids appear to be minimal in the in vivo studies in which they were used, the use of such compounds to improve the systemic response to CPB might be warranted. ProDTC did not block NF- κ B activation in our model of CPB, although dithiocarbamates are considered to be effective inhibitors of NF- κ B activation in vitro in a number of cell types.³¹ It is unknown why ProDTC failed to block monocyte NF- κ B activation under these conditions.

During contact activation, thrombin is generated and cleaves fibrinogen to fibrin, promoting thrombus formation. In addition, thrombin has been shown to stimulate monocyte chemotaxis; phagocytosis; IL-1, IL-6, IL-8, and tumor necrosis factor production³²; and monocyte chemoattractant protein-1 release.³³ The actions of thrombin on leukocytes occurs through interaction of thrombin with a specific cell-surface receptor, protease activated receptor-1 (PAR-1).³⁴ Exposure of mononuclear phagocytes to thrombin has been shown to induce NF- κ B activation,³⁵ and thrombin activation of monocytes might include NF- κ B–dependent TF expression. Therefore, during CPB, the generation of thrombin from TF on complement-activated monocytes could result in further monocyte NF- κ B activation through a PAR-1 receptor-mediated mechanism, leading to further upregulation of monocyte TF expression in a positive feedback loop. Of interest, heparin does not appear to interfere with thrombin/PAR-1 interactions³⁶ and thus would not block this phase of a procoagulant response during CPB.

In summary, we have demonstrated that complement activation and generation of the anaphylatoxins C3a and

C5a, a widely known consequence of CPB, possibly initiates TF expression on monocytes circulated through an extracorporeal membrane oxygenator. Moreover, we have found that TF expression on monocytes under these conditions involves activation of the transcription factor NF- κ B, which is subject to control through pharmacologic manipulation.

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