Tissue Factor Is Expressed on Monocytes During Simulated Extracorporeal Circulation

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Certain forms of extracorporeal circulation exemplified by cardiopulmonary bypass require continuous high-dose anticoagulation to prevent thromboembolic complications. We hypothesized that monocytes may be stimulated to express tissue factor (TF) during prolonged simulated extracorporeal circulation. TF was identified both by flow cytometry using three TF-specific monoclonal antibodies and functional assay of procoagulant activity (PCA). TF significantly increased between 2 and 6 hours of simulated extracorporeal circulation by both analyses. Relative fluorescence on monocytes increased from a control value of 100 to 313 ± 79 on cells from the simulated extracorporeal circuit (p < 0.05). PCA increased from 21 ± 8 to 775 ± 326 pg TF/10^4 monocytes (p < 0.05) and was blocked 99.6% by preincubation of cells with a mixture of monoclonal antibodies to TF. By 6 hours, the number of leukocytes in the circuit was decreased by 43%. The cells were recovered from the oxygenator membrane by washing with EDTA. Compared with initial values, by 6 hours, both TF antigen at 378 ± 90 (p < 0.05) and PCA at 1,357 ± 280 pg TF/10^4 monocytes (p < 0.01) were highest in the recovered cells. Cells incubated for 6 hours and not subjected to simulated extracorporeal circulation did not increase TF. Examination of monocytes for the adhesive receptor CD11b/18 (Mac-1) paralleled TF expression, providing an additional putative receptor for the coagulant proteins, factor X and fibrinogen or fibrin. Our data support the hypothesis that TF expression by monocytes is induced during prolonged simulated extracorporeal circulation and lead to the suggestion that this may significantly contribute to the increased risk of thrombotic events during prolonged extracorporeal circulation. (Circulation Research 1993;72:1075–1081)

KEY WORDS • tissue factor • cardiopulmonary bypass

Tissue factor (TF) is a cell surface transmembrane protein with an extracellular domain that is the functional receptor and catalytic cofactor for coagulation factor VII/IIa.1 Factor VIIa, assembled with TF in the functional TF–VIIa complex, proteolytically activates factors X and IX, resulting in thrombin generation by the prothrombinase complex, and ultimately leads to fibrin (thrombus) formation2,3 and platelet activation via the thrombin receptor.4,5 TF is the most potent trigger of blood coagulation and is not expressed on unactivated cells normally in contact with blood. However, in certain physiological and pathological responses, endothelial cells6 and leukocytes7 are stimulated to express TF. Bacterial lipopolysaccharide (LPS) elicits functional TF expression by monocytes8 as do antigen-derived T helper cells,9,10 lymphokines,11 antigen–antibody complexes,12 and complement-derived anaphylatoxin C5a.13 In addition, adhesion of monocytes to endothelium enhances the expression of TF14 once an initial transcriptional signal has been delivered.15 Complement activation with anaphylatoxin generation occurs during cardiopulmonary bypass,16 and cardiopulmonary bypass requires large amounts of heparin to prevent thrombotic events. Simulated extracorporeal circulation is associated with increased neutrophil superoxide generation and slight shortening of leukocyte-generated clotting times by 2 hours.17 In addition, use of extracorporeal membrane oxygenation has been associated with an increased incidence of thromboembolic complications. Erythrocytes and leukocytes are entrapped within the fibrin meshwork in the oxygenator bypass segment, and it has been proposed that the membrane oxygenator may be a source of thrombogenesis.18 In this study, we adopted an in vitro model of an extracorporeal cardiopulmonary bypass circuit that was developed19 and evaluated20–22 in previous studies. We now examine the hypothesis that elicited expression of TF by monocytes is responsible for the increased activation of the coagulation cascade during prolonged extracorporeal circulation.

Materials and Methods

Antibodies

Murine monoclonal antibodies TF8-5G9, TF9-6B4, and TF9-9C3 to human TF23,24 and LM2 directed against the leukocyte integrin Mac-125 were prepared by
ascites growth of the hybridomas and isolation of the immunoglobulin (Ig) G1 antibody using immobilized protein A as previously described. Unrelated mouse IgG and fluorescein isothiocyanate–labeled sheep anti-mouse IgG were purchased from Sigma Chemical Co., St. Louis, Mo.

**Simulated Extracorporeal Circuit**

This model system provides defined and appropriate flow geometry and hemodynamic stresses, proper surface area/blood volume ratios, and ready access to serial blood samples during a single trial. The system has recently been modified to decrease the volume, surface area, and flow. Each perfusion circuit has a surface area of 0.46 m² and is assembled from silastic tubing (Avocor, Inc., Plymouth, Minn.), polycarbonate connectors, a polyvinyl chloride venous reservoir bag (Gish Biomedical, Santa Ana, Calif.), and a 0.4-m² spiral/coil membrane oxygenator (model 0400-2A, Sci-Med Life Systems, Inc., Minneapolis, Minn.). All components of the circuit were sterilized, and blood samples were only exposed to sterile labware throughout cell separation. Human blood (300 ml) was drawn from healthy male donors (aged 24–32 years) through a 16-gauge 1.5-in. needle and polyvinyl tubing directly into a venous reservoir bag containing beef lung heparin (5 units/ml blood, The Upjohn Co., Kalamazoo, Mich.) and dextrose (2.25 mg/ml blood). Informed written consent was obtained from all donors according to the provisions of the Declaration of Helsinki, and the protocols for the study were approved by the Institutional Review Board of the University of Pennsylvania. Six simulated extracorporeal circuits were run at 37°C. All donors abstained from all medications for at least 2 weeks before giving 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. The perfusion circuit was then filled with blood, avoiding bubble formation. Blood was recirculated for 6 hours at 0.3 l/min at 37°C by a calibrated, barely occlusive roller pump (Sarns, Ann Arbor, Mich.). The oxygenator was ventilated with a mixture of 95% oxygen and 5% carbon dioxide flowing at 0.7 l/min. The 0-minute time samples and standing (uncirculated) control samples were obtained from the venous reservoir before connection to the circuit. The standing control sample from the reservoir that contained heparin (5 units/ml) and dextrose (2.25 mg/ml) was incubated in the water bath for 6 hours at 37°C before it was processed.

Experimental samples were taken at 2, 4, and 6 hours of recirculation. The blood was drained from the system and replaced with 150 ml wash solution containing 0.01 M EDTA. The wash solution was recirculated for 15 minutes. The wash samples contained all of the adherent leukocytes and were processed and analyzed identically to the whole blood samples; these are described as recovered cells. All blood (including the 0-minute and standing control samples) and wash samples were collected into citric acid–sodium citrate–dextrose (9:1 [vol/vol]).

**Leukocyte Separation**

At each time point, samples were removed for leukocyte count and differential staining. Whole blood was layered onto Ficoll Hypaque (Sigma) and centrifuged at 400g for 30 minutes at room temperature. For Mac-1 studies, to avoid upregulation by temperature, cell separation was carried out at 4°C. Mononuclear cells were harvested from the interface and washed twice in Hanks’ balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) without Ca²⁺ and Mg²⁺ (HBSS). Cell count was adjusted to 0.5 × 10⁷/ml for flow cytometry analysis and at 1 × 10⁷/ml for assay of procoagulant activity (PCA) of intact viable cells. Viability was typically greater than 95% as checked by trypan blue exclusion test.

**Cell Staining for Flow Cytometry**

Isolated mononuclear cells (5 × 10⁶) were fixed overnight in 1% paraformaldehyde in HBSS and washed twice in HBSS with 0.1% azide. The cells were resuspended in 100 µl of 20% heat-inactivated normal serum in HBSS and gently agitated for 15 minutes at 4°C. After centrifugation, cells were resuspended in 100 µl of anti-human antibody at 10 µg/ml final concentration. Cells were either incubated with equal concentrations of the three TF monoclonals—TF8:5G9, TF9:6B4, and TF9:9C3—or with TF9:9C3 alone. For negative controls, the same concentration of nonimmune mouse IgG was used. Incubations were carried out for 45 minutes at 4°C. After centrifugation, supernatant was removed, and cells were washed once more in HBSS with 0.1% azide. Secondary antibody (sheep anti-mouse IgG FITC [Sigma]) was diluted in HBSS with 1% serum, and cells were incubated for 30 minutes at 4°C. Cells were washed twice and stored in the dark until analysis. Fluorescence was analyzed by an Epics Elite flow cytometer (Coulter Corp., Hialeah, Fla.). The fluorescence of lymphocytes and monocytes was analyzed by gating on each respective cell population based on their different forward and side scatter. At each point, 5,000 monocytes were analyzed. The number of lymphocytes was at least 15,000 in samples from the circuit and 5,000 in the case of recovered cells. Log fluorescence values were converted to linear units, and a 0-minute time sample was arbitrarily assigned a value of 100. Mean fluorescence of samples was compared with that of the 0-minute time sample and with the standing control sample that was not recirculated but was incubated at 37°C for 6 hours in a silastic tube.

**Procoagulant Activity Assay**

Viable cell PCA of the preparations was determined by a one-step recalification time as described previously. Mononuclear cell suspension (0.1 ml with 10⁶ cells/ml), with less than 5% neutrophil contamination, was incubated with pooled normal human plasma or factor-deficient plasma (0.1 ml) for 60 seconds. After addition of 0.1 ml of 25 mmol/l CaCl₂, the clotting times were measured by a Fibrometer (BBL Fibrosystems, Baltimore, Md.). Each sample was run in duplicate. To quantify PCA, recombinant human TF (Corvas, La Jolla, Calif.) was used, and a standard curve was obtained from serial dilutions. PCA values of cell suspensions or cell lysates were derived from the standard curve and expressed as picograms TF.

During simulated extracorporeal circulation, there is a preferential adherence of monocytes and neutrophils to the oxygenator interface. Based on the differential
counts obtained for each sample, PCA values were normalized for 10^8 monocytes. PCA analysis was also carried out on lysed cells. For this purpose, mononuclear cells were lysed by resuspending them in 15 mM n-octyl β-D-glucopyranoside (Sigma) in 25 mM HEPES-saline (pH 7.4). Cells were thoroughly vortexed and incubated for 15 minutes at 37°C. Lysates were diluted threelfold with HEPES saline, vortexed, and placed on ice. PCA was assayed within 10 minutes.

**Endotoxin Measurement**

Plasma samples were recovered at each time point and kept frozen until endotoxin was measured by a method using the Limulus amebocyte lysate chromogenic assay (Whittaker Bioproducts, Walkersville, Md.). Endotoxin concentration remained below the detection limit of 0.01 unit/ml throughout the simulated extracorporeal circulation.

**Statistical Analyses**

Results are expressed as mean±SEM. Comparisons between values within each run were made using paired t tests compared with initial values. A value of p < 0.05 was considered statistically significant.

**Results**

During the 6-hour simulated extracorporeal circuit, leukocyte counts gradually decreased, declining to 57% of the initial value by 6 hours, which correlated with the attachment of cells to the large surface area of the membrane oxygenator. Compared with lymphocytes, monocytes and neutrophils, presumably because of different adhesive receptors on their surfaces and perhaps greater density once activated, are predisposed to adhere to artificial surfaces. Based on differential counts obtained at progressive time points, the number of monocytes and neutrophils progressively decreased relative to lymphocytes in the circulating blood over the 6 hours (Figure 1). The decrease in circulating monocytes was concordant with the number of monocytes subsequently recovered. The washing procedure described in “Materials and Methods” typically eluted more than 90% of attached cells from the surfaces.

Fluorescence reporting by flow cytometry of TF on the surface of circulating monocytes stained with monoclonal antibodies against TF increased 3.1-fold by 6 hours (p < 0.05) and 3.7-fold in cells subsequently recovered from the circuit (p < 0.05) but remained at baseline levels in the standing control cells. The adherent monocytes displayed more TF than the circulating cells at 6 hours. The TF observed at 2 and 4 hours on the circulating cells thus represents an underestimate of the TF expressed, since the more reactive adhesive cells could not be assayed at these intermediate times. Lymphocytes did not display TF by flow cytometry (data not shown).

Cell preparations incubated with nonspecific mouse IgG did not show any increase of TF by surface fluorescence, providing evidence that the observed increase after reaction with monoclonal antibodies to TF was not attributable to upregulation of the receptor for the F3 fragment of IgG. The increase in fluorescence is due to a relatively small percentage of monocytes, whereas the majority of the cells show very little change in their staining pattern. This results in a skewing or tail formation on the flow cytometry staining pattern by 6 hours compared with the 0-minute sample or the standing control sample (Figure 2). Monocytes expressing TF on their surfaces appear as soon as 2 hours (p < 0.05), and the percentage of positive cells increases up to 6 hours (p < 0.01), the highest proportion being in the cells recovered from the surface of the circuit (Figure 3).

The cells with TF antigen also had functional TF by PCA. By 4 hours, mononuclear cell suspension significantly shortened the clotting time of normal plasma (p < 0.05). Mononuclear cell PCA increased over baseline more than 15-fold (p < 0.05) by 6 hours and 25-fold in the recovered cells (p < 0.01). The activity was factor X dependent; i.e., acceleration of clotting did not occur in factor X-deficient plasma. In contrast, there was a variable factor VII dependence, since the clotting times using factor VII-deficient plasma were either prolonged, as would be the case for free TF lacking bound factor VIIa, or similar to clotting times using normal plasma, which is consistent with the presence of TF–VIIa complex on cells. However, when mononuclear cells were incubated with 10 mmol/l EDTA for 30 minutes at 37°C to dissociate factor VII/VIIa from TF and added to factor VII-deficient plasma, prolonged clotting times resulted, as compared with normal plasma. Mononuclear cells incubated with factor XI– or factor IX–deficient plasmas gave similar values to normal plasma (data not shown).

To establish whether the observed PCA is exclusively due to TF, we incubated mononuclear cells for 15 minutes on ice with 85 mg/ml TF monoclonal antibody cocktail or mouse IgG as a control. PCA of mononuclear cells was abolished by the TF antibodies, confirming that other procoagulants do not contribute to the observed PCA (Table 1).

When TF immunoreactive protein and TF functional PCA were compared with a maximal stimulation (10 μg/ml LPS for 6 hours), TF antigen on monocytes from the circuit by 6 hours reached the level of expression of TF on LPS-stimulated cells. However, TF activity of intact monocytes peaked in the recovered cells at only
49% of the LPS-stimulated ones (Figure 4). Although all TF is on the cell surface, there is a significant difference in PCA activity of intact and lysed cells. Mononuclear cell PCA from the same experiment was measured on intact and lysed cells, and data were compared to LPS-stimulated samples. After lysis, mononuclear cell PCA reached the level of LPS-stimulated cells by 6 hours, and the values for the recovered cells were similar to the flow cytometry data (Table 2).

The occupancy of the integrin Mac-1 by different ligands in the process of adherence augments the TF response. Since we have previously shown that neutrophils can upregulate Mac-1 during a 2-hour simulated extracorporeal circuit, we investigated by flow cytometry monocyte Mac-1 surface expression during the 6-hour simulated extracorporeal circuit (Figure 5). The number of Mac-1 receptors on monocytes increased up to 4 hours and then leveled off. By far, the most Mac-1 was detected in adhered cells. Monocytes from the standing control sample at 6 hours expressed Mac-1 in the same range as monocytes from the circuit by 2 hours.

**Discussion**

In addition to their well-characterized role in immunological functions, monocytes have increasingly been recognized to participate in pathophysiological changes in the cardiovascular system. Leukocytes can generate reactive oxygen metabolites that are capable of creating substantial cellular injury and are responsible for the endothelial injury secondary to ischemia and reperfusion in the coronary microvasculature. Monocytes and

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**TABLE 1. Mononuclear Cell–Generated Procoagulant Activity After Neutralization by Tissue Factor Monoclonal Antibodies**

<table>
<thead>
<tr>
<th>Clotting time (seconds)</th>
<th>PCA (pg TF/10^6 monocytes)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antibody</td>
<td>25.5</td>
<td>1,768</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>26.6</td>
<td>1,664</td>
</tr>
<tr>
<td>TF monoclonals</td>
<td>128.5</td>
<td>8</td>
</tr>
</tbody>
</table>

PCA, procoagulant activity; TF, tissue factor; IgG, immunoglobulin G. Percent inhibition is compared with no antibody.

Mononuclear cells, eluted from the oxygenator surface, were incubated with the TF monoclonal antibody cocktail (25 μg/ml each) or the same amount of nonimmune mouse IgG for 15 minutes on ice. Clotting times were determined by recalcification and PCA derived from standard curves compared with recombinant TF. After blocking with neutralizing TF monoclonal antibodies, PCA was totally inhibited.
neutrophils have both been shown to elicit endothelium-independent vasoconstrictive responses that in the case of monocytes has been shown to be augmented in atherosclerotic arteries.31 Monocytes participate in initiation and propagation of the coagulation protease cascade, not only by assembling factor VII/VIIa on TF once expressed on the surface32 but also by binding factor V/Va.33 They can also synthesize factor XIII34 and, when activated by appropriate agonists, PCA,35 now defined as TF. It has recently been recognized that Mac-1, present on activated neutrophils, monocytes, and natural killer lymphocytes, is a receptor for fibrinogen36 and coagulation factor X,37 the latter being associated with proteolytic activation. The display of enzymes and their receptors/cofactors on monocytes enables monocytes to initiate and propagate thrombotic processes.

To our knowledge, this is the first study to demonstrate that this central procoagulant3 of the extrinsic pathway is produced during prolonged extracorporeal perfusion. Previously, only the intrinsic system has been observed to be activated.28 Using simulated extracorporeal circulation as in the present study, we previously observed a significant increase in the number of Mac-1 receptors on neutrophils after 2 hours of recirculation, and it paralleled human neutrophil elastase release.28

Elastase cleaves TF pathway inhibitor, a potent inhibitor of factor Xa and the TF–VIIa complex, thereby augmenting TF-initiated activation of the coagulation pathways.38 Mac-1 can be conceived of as a procoagulant receptor, since it is capable of propagating the coagulation cascade by binding factor X and fibrinogen. However, the most potent trigger of blood coagulation is TF. The assembly of TF with its ligand, factor VII/VIIa, to form the binary complex TF–VIIa, results in efficient conversion of factors X and IX to factors Xa and IXa. The overall proteolytic efficiency is increased by approximately 107 relative to free factor VIIa.39 Since the monocyte is the only leukocyte capable of expressing TF, we examined mononuclear cells during prolonged simulated extracorporeal circulation for TF expression. Initially, no TF could be detected immunochemically, nor was significant PCA associated with monocytes. A significant increase in TF was noted by flow cytometry by 4 hours, which increased further at 6 hours. Similar results were obtained when PCA was quantified. The delay is probably attributable to the requirement for transcriptional initiation and biosynthesis.15

Mac-1 is an adhesive receptor that increases in density on both neutrophils and monocytes during 6 hours

**Table 2.** Mononuclear Cell–Generated Procoagulant Activity of Intact and Lysed Cells

<table>
<thead>
<tr>
<th>Cell condition</th>
<th>PCA (pg TF/10^6 monocytes)</th>
<th>LPS-stimulated monocytes (%)</th>
<th>PCA (pg TF/10^6 monocytes)</th>
<th>LPS-stimulated monocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simulated circulation (hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>0.2</td>
<td>11</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>157</td>
<td>2.9</td>
<td>414</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>511</td>
<td>9.6</td>
<td>7,300</td>
<td>54</td>
</tr>
<tr>
<td>6</td>
<td>800</td>
<td>15</td>
<td>10,800</td>
<td>81</td>
</tr>
<tr>
<td>6 R</td>
<td>2,200</td>
<td>42</td>
<td>12,590</td>
<td>95</td>
</tr>
<tr>
<td>LPS-stimulated</td>
<td>5,330</td>
<td>100</td>
<td>13,300</td>
<td>100</td>
</tr>
</tbody>
</table>

PCA, procoagulant activity; TF, tissue factor; LPS, lipopolysaccharide; R, recovered cells.

Mononuclear cells from the same experiment were either lysed or kept intact and analyzed simultaneously for PCA.
of extracorporeal circulation. We found that monocytes recovered from the oxygenator surface expressed the most Mac-1. The occupancy of Mac-1 by cognate and certain surrogate ligands or by surface or endothelial adhesion was shown to enhance the TF response when the cells were activated by agonists such as LPS or lymphokines. Thus, we suspected that the most TF would be present on cells attached to the oxygenator surface, which proved to be true when assayed by activity or by immunoreactivity. However, when compared with LPS stimulation, we found that by 6 hours TF antigen display on cells from the circuit approximated that of LPS, but that of PCA peaked at only 50% of the LPS positive control. In endothelial cells, it has been reported that after LPS stimulation the TF antigen peaks TF activity peak by 2 hours. TF is fully cell surface-expressed; however, there is a marked difference and variation in specific functional activity that is attributable to the availability of charged phospholipids.

Indeed, when we analyzed PCA after lysis in a nonionic detergent and compared the results with LPS-stimulated cells, TF activity by 6 hours and activity in the recovered cells reached the level of LPS-stimulated samples. Thus, in our study, the discrepancy between TF protein and functional activity is fully concordant with studies that demonstrate full exposure of TF antigen and the capacity to bind factor VII/VIIa but the enhancement of PCA by lysis or detergents that make available negatively charged phospholipids. This modulation of specific functional activity may be of biological importance.

In our studies, we did not intend to address the origin and mechanisms of induction of TF expression by monocytes. Endotoxin was below the detection level in a highly sensitive assay. Since complement activation has been detected during cardiopulmonary bypass, it seems reasonable to suggest that complement activation fragments such as C5a may be causative. Whether the mechanisms operate directly on monocyte or indirectly through lymphocyte activation is not known.

Finally, it is important to stress that these findings in a simulated extracorporeal circuit may differ from those in clinical cardiopulmonary bypass. In many respects, events in simulated extracorporeal circulation closely parallel in vivo effects. Thus, platelet dysfunction resulting from loss of fibrinogen receptors in simulated extracorporeal circulation was confirmed in clinical cardiopulmonary bypass. Neutrophil activation as manifested by elastase release occurs both in vivo and in vitro. However, fibrinolysis, which occurs in clinical cardiopulmonary bypass, does not occur in simulated extracorporeal circulation, probably because of the lack of endothelial cells, which produce and release plasminogen activators. Interleukin 1 can induce TF biosynthesis in vitro in endothelial cells. Interleukin 1 reaches peak concentrations 24 hours after clinical cardiopulmonary bypass; thus, it is likely that, in addition to monocytes, endothelial cells could also express TF postoperatively, leading to a thrombogenic activity. Indeed, it has been suggested that endothelial damage occurs after clinical cardiopulmonary bypass on the basis of analysis of changes in factor VIII proteins. In addition, the subendothelial matrix contains small amounts of TF. Other factors must be taken into consideration in vivo, such as changes in the circulating TF pathway inhibitor as well as monocyte turnover.

In summary, we have observed that TF, the major cellular initiator of the coagulation cascade, is expressed on monocytes during extracorporeal circulation and may contribute to thromboembolic complications associated with such states.

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