Serum from Hemodialysis Patients Inhibits Basal and Cytokine-Stimulated Tissue Factor Expression in Vitro

PAOLA MADERNA, PATRICK COLEMAN, CATHERINE GODSON, YVONNE M. O’MEARA, and HUGH R. BRADY
Centre for Molecular Inflammation and Vascular Research, Department of Medicine and Therapeutics, Mater Misericordiae Hospital, University College Dublin, Ireland.

Abstract. Hemorrhagic complications are common among hemodialysis (HD) patients. The mechanisms by which HD perturbs the coagulation cascade are still being defined. This study evaluated the influence of HD serum on cellular expression of tissue factor (TF), a procoagulant membrane-associated protein that is a pivotal regulator of blood coagulation. Serum was collected immediately before dialysis and 15, 30, and 180 min into HD using polysulfone membranes. Serum was then assessed for its ability to influence basal and cytokine-stimulated TF activity in human umbilical vein endothelial cells and ECV304 cells. Predialysis serum did not influence basal levels of TF activity. HD was associated with the appearance of a serum factor that suppressed basal TF activity (TF units/µg protein: predialysis serum 8.2 ± 0.9; 180-min dialysis serum 4.9 ± 0.6; P < 0.05) and TF activity induced by the cytokine tumor necrosis factor-α (TNFα) (TF units/µg protein: TNFα alone 15.9 ± 0.7; TNFα + 180-min dialysis serum 5.9 ± 0.9; P < 0.01). This response was not mimicked by heparin, suggesting production of an endogenous inhibitor of TF activity during HD. Dialysis was associated with a striking increase in circulating levels of tissue factor pathway inhibitor (TFPI), a physiologic inhibitor of the TF/VIIa complex. The lack of temporal correlation between TFPI levels and suppression of TF activity, however, suggested the presence of additional TFPI independent pathway(s) for modulation of TF activity. Dialysis-related suppression of TF expression may contribute to hemorrhagic complications in HD patients.

Patients with end-stage renal disease (ESRD) are prone to a variety of coagulation disorders. Hemodialysis (HD) patients carry an increased risk of systemic hemorrhage that remains a significant cause of morbidity in this population (1,2). Previously defined factors that may contribute to hemorrhagic complications in HD patients include platelet dysfunction of uremia; reduced activity of several coagulation factors, including factors II, IX, X, and XII; and heparin anticoagulation during HD (1,3). On the other hand, ESRD patients are also prone to develop thrombotic complications even in high flow conduits such as arteriovenous fistulas (4). We examined the influence of HD on endothelial cell tissue factor (TF) activity, TF being an important modulator of extrinsic pathway activation, thrombin generation, and fibrin deposition (5). TF is not expressed under basal conditions by endothelial cells, thus avoiding spontaneous thrombosis in health. In contrast, basal TF expression is observed with most cells that are not in direct contact with circulating blood. When the endothelial barrier is breached, exposure of blood to TF initiates coagulation, a key homeostatic defense against hemorrhage (5). Cytokines induce de novo TF expression by endothelial cells and augment TF expression by many extravascular cells, responses that are postulated to confer additional protection against hemorrhage during infection and inflammation and that may also promote leukocyte recruitment and angiogenesis at sites of vascular injury (5).

Materials and Methods
Patients and Dialysis Procedure
Eight ESRD patients were studied (five men, three women; ages 20 to 70; mean age 50 ± 16), each undergoing HD three times a week for 3 to 4 h with polysulfone conventional low-flux hollow fiber dialyzers (F6 or F7 dialyzers; Fresenius, Germany) with standard heparin anticoagulation (continuous infusion of 1000 U/h). Blood was collected from the afferent “arterial” line immediately before dialysis before heparinization and from the efferent “venous” line 15, 30, and 180 min after initiation of HD. Blood was collected at the described times and divided into two tubes: one containing 3.8% sodium citrate (9:1 vol/vol) and one without anticoagulant. The serum used for the experiments of TF activity was obtained by incubating the non-anticoagulated sample for 2 h at 37°C to allow clot formation and centrifugation at 600 × g for 15 min at room temperature. For plasma preparation for assay of tissue factor pathway inhibitor (TFPI) levels, anticoagulated blood was centrifuged at 600 × g for 15 min. Serum and plasma samples from patients and control subjects were stored at −20°C for approximately the same duration. The study protocol was approved by the Mater Misericordiae Hospital Ethics Committee, and informed consent was obtained from all patients.

Cell Culture
Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics Corp. (San Diego, CA). In agreement with most
assessments of TF expression by endothelial cells in vitro and in vivo, HUVEC do not express TF under basal conditions, but robust TF expression is observed after cytokine activation. HUVEC were used between the second and the fourth passage and propagated in medium 199 (Sigma, St. Louis, MO) supplemented with 20% fetal calf serum (FCS) (Life Technologies, Grand Island, NY), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) in the presence of 50 μg/ml heparin and 50 μg/ml endothelial cell growth factor (Sigma). ECV304 is a spontaneously immortalized cell line that expresses TF under basal conditions and displays increased TF expression in response to cytokine stimulation (6). This cell line provides a convenient model for assessment of both constitutive and inducible TF expression displayed by many other cell types and was used for the majority of experiments. ECV304 cells were maintained in medium 199 supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For each experiment, cells were cultured in 12-well tissue culture plates and incubated for 4 h with FCS-free medium 199 or with FCS-free medium 199 supplemented with 25% serum obtained from HD patients or healthy control subjects in the presence or absence of 10 ng/ml recombinant human tumor necrosis factor-α (TNFα) (R&D Systems, Minneapolis, MN). The integrity of ECV304 cells was not affected during incubation with dialysis serum, as determined morphologically by light microscopy and by assay of LDH release (LDH in medium: vehicle 70.0 ± 7.1; serum 76.7 ± 16.7; “typical” normal range, 100 to 350 U/ml) (Sigma). At the end of the incubations, the medium was discarded and cells were washed twice with serum-free medium, and the plates were stored at ~80°C until assayed. Endotoxin contamination of reagents was routinely excluded with the chromogenic Limulus amebocyte lysate assay (Sigma).

Determination of TF Activity and Plasma Levels of TFPI

For determination of TF activity, the cells were lysed with 15 mM octyl-β-D-glycopyranoside, and TF activity was determined in cell lysates at 37°C by a one-stage clotting assay. The assay mixture contained 0.1 ml of cell lysates, 0.1 ml of citrated, pooled normal plasma, and 0.1 ml of 25 mM CaCl₂. Values were converted in arbitrary units of procoagulant activity by comparison with a standard curve of clotting times obtained by serial dilutions of rabbit brain thromboplastin (Sigma). Total protein concentration was determined using the method of Bradford (7). TFPI antigen levels were determined in citrated plasma, using a commercially available enzyme-linked immunoassay that detects both intact and truncated forms of TFPI (IMUBIND® TFPI kit; America Diagnostica, Inc., Greenwich, CT).

Statistical Analyses

Values are expressed as means ± SEM. Statistical significance of the differences was tested by paired t test.

Results

Influence of HD Serum on Cellular TF Activity

HUVEC do not express TF activity under basal conditions, and this state was not affected by incubation with dialysis serum (n = 3). Constitutive expression of TF was not altered by treatment of ECV304 cells with serum collected immediately before dialysis (Figure 1), whereas exposure to serum collected after 15, 30, and 180 min of HD inhibited TF activity, the magnitude of inhibition increasing with the duration of HD

Figure 1. Effect of hemodialysis (HD) serum on tissue factor (TF) activity. ECV304 cells were incubated for 4 h with serum-free 199 medium (baseline) or with 199 medium supplemented with 25% patient serum obtained from the afferent “arterial” line immediately before dialysis (predialysis) and from the effluent “venous” line 15, 30, 180 min after initiation of HD. Values are means ± SEM. n = 4. *P < 0.05 versus baseline.

Figure 2. Effect of HD serum on tumor necrosis factor-α (TNFα)-induced TF activity. ECV304 cells were incubated for 4 h with TNFα in the presence or absence of 25% serum obtained from healthy subjects or from dialysis patients. Blood was drawn from the afferent “arterial” line immediately before dialysis (predialysis) and from the effluent “venous” line 15, 30, 180 min after initiation of HD. Values are means ± SEM. n = 4. *P < 0.05 versus TNFα.
dialysis also suppressed TNFα-induced TF expression (ECV304 TF activity: U/μg protein: TNFα 17.4 ± 1.1; TNFα + 180-min serum from “arterial” blood line 12.9 ± 1.6; n = 4; P < 0.05). Furthermore, serum drawn from venous blood suppressed TNFα-induced TF expression 1 h after termination of dialysis (TF units/μg protein: TNFα 17.4 ± 1.1; TNFα + postdialysis serum 11.7 ± 1.6; n = 4; P < 0.05).

To exclude a direct effect of heparin on TF activity, ECV304 cells were incubated with 1 to 10 U/ml heparin under basal and TNFα-stimulated conditions. As shown in Table 1, heparin did not directly affect TF activity.

**Effect of HD on TFPI Levels**

TFPI is a physiologic inhibitor of TF/factor VIIa complex released from endothelial cells (8). To determine whether the inhibitory effect of HD serum on TF activity correlated with release of TFPI, plasma levels of TFPI were measured during dialysis. TFPI levels were elevated in ESRD patients before dialysis, by comparison with healthy subjects, and increased further during HD (Figure 3). Interestingly, a significant increase in circulating TFPI was noted as early as 15 min after the initiation of dialysis, at a time when dialysis serum did not yet suppress TF activity.

**Discussion**

The formation of a complex between factor VII/VIIa and TF is a key step for activation of the coagulation cascade (5). The TF-VII/VIIa complex catalyzes the activation of factors IX and X, and ultimately promotes formation of thrombin and fibrin clot. In this study, we demonstrated that HD serum inhibits not only basal TF activity, but also TF activity induced by the cytokine TNFα. In health, most TF is expressed by cells that are not in contact with circulating blood (e.g., adventitial cells). It is being increasingly recognized, however, that other cell types, including vascular endothelial cells, express abundant TF when activated with cytokines. TF activity at these sites is postulated to regulate hemostasis during infection and inflammation, in addition to modulating other important functions such as leukocyte recruitment and activation, and angiogenesis (5).

In our study, the ability of HD serum to inhibit basal and cytokine-inducible TF activity was not due to a direct effect of heparin, because addition of exogenous heparin to our *in vitro* system did not influence TF activity, even when used at higher concentrations than are employed for anticoagulation during HD. The procoagulant activities of factor VIIa/TF complex and the resulting factor Xa are regulated by a Kunitz-type protease inhibitor, TFPI (8). TFPI acts by neutralizing the catalytic activity of factor Xa and by feedback inhibition of the factor VIIa-TF complex in the presence of factor X (8). Predialysis TFPI levels were significantly higher in our ESRD patients than control subjects and increased further during HD, consistent with previous reports (9,10). The majority of intravascular TFPI is bound to vascular endothelium. Heparin releases TFPI into circulating blood (11,12), and the progressive increase in TFPI levels during HD noted in our study may have been triggered, at least in part, by heparin anticoagulation. It is noteworthy, however, that TFPI levels were markedly elevated early in the course of HD, at a time when TF activity was not yet inhibited. In aggregate, these results suggest that circulating factors, other than TFPI, also contribute to suppression of TF activity in HD patients.

In summary, we report that HD serum inhibits the activity of basal and TNFα-triggered TF activity. This perturbation of TF activity further expands the array of coagulation abnormalities that characterize the uremic state and HD procedure, and may contribute to the pathogenesis of hemorrhagic complications in HD patients.

**Table 1. Effect of heparin on basal and cytokine-stimulated tissue factor activity in ECV304 cells**

<table>
<thead>
<tr>
<th>Category</th>
<th>TF Units/μg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−TNFα</td>
</tr>
<tr>
<td></td>
<td>(10 ng/ml for 4 h)</td>
</tr>
<tr>
<td>No heparin</td>
<td>7.9 ± 2.0</td>
</tr>
<tr>
<td>Heparin</td>
<td></td>
</tr>
<tr>
<td>1 U/ml</td>
<td>10.4 ± 2.6</td>
</tr>
<tr>
<td>10 U/ml</td>
<td>10.7 ± 3.5</td>
</tr>
</tbody>
</table>

*Values are means ± SEM of three experiments. TF, tissue factor; TNFα, tumor necrosis factor-α.*

**Acknowledgments**

This work was supported by grants from the Irish Heart Foundation (fellowship to P. Maderna), the Health Research Board of Ireland, and the Mater College. We thank Olive Doyle and her staff, and Alan O’Hare for their assistance with this study.
References