Complement anaphylatoxin C5a contributes to hemodialysis-associated thrombosis

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Abstract

Thrombosis is a common complication of end-stage renal disease (ESRD), particularly in patients on hemodialysis. Although substantial progress has been made in preventing thrombotic complications in various other groups of patients, the mechanisms of thrombosis during hemodialysis require clarification. In this report, we demonstrate that complement activation triggered by hemodialysis biomaterials, and the subsequent generation of the complement anaphylatoxin C5a, results in the expression of functionally active tissue factor (TF) in peripheral blood neutrophils. Since TF is a key initiator of coagulation in vivo, we postulate that the recurring complement activation that occurs during long-term hemodialysis contributes to thrombosis in dialyzed ESRD patients. Furthermore, we found that complement contributed to the induction of granulocyte colony stimulating factor (G-CSF), which has been implicated in the pathogenesis of thrombosis in patients treated with the recombinant form of this molecule. Importantly, the inhibition of complement activation attenuated the TF expression and G-CSF induction in blood passing through a hemodialysis circuit, suggesting that the complement system could become a new therapeutic target for preventing thrombosis in patients with chronic renal failure who are maintained on hemodialysis.

Key words: hemodialysis, thrombosis, complement, C5a, neutrophils, tissue factor, compstatin
Introduction

Several primary kidney and systemic disorders lead to end-stage renal disease (ESRD), which is manifested as renal failure. The treatment of choice in ESRD is kidney transplantation. However, the shortage of available kidney donors, combined with contraindications to transplantation in some patients, result in the necessity for sustaining these individuals on renal dialysis for various periods of time. Although renal dialysis is a life-saving procedure, patients undergoing this treatment are at risk of various complications, including thrombosis. Thrombotic complications are thought to be a net outcome of both underlying kidney disease, complicated by renal failure, and its management through dialysis.

The most common thrombotic complication in hemodialyzed ESRD patients is thrombosis of vascular access, which is a major cause of hemodialysis-associated morbidity. In addition, recent studies have found that age-adjusted rates of hospitalization due to pulmonary embolism are higher in patients on dialysis than in the general population. Importantly, thrombotic events fatally complicate the course of various cardiovascular diseases that are the leading cause of death among patients suffering from chronic kidney diseases. For example, coronary artery thrombosis increases the risk of myocardial ischemia and subsequent myocardial infarct. Cardiovascular diseases contribute to more than half of deaths in patients with ESRD. Furthermore, mortality due to cardiovascular diseases is notably higher in patients on dialysis than in the general population.

These epidemiological data have triggered research efforts to elucidate the pathogenesis of cardiovascular disorders and accompanying thrombotic complications in patients with chronic kidney diseases who are undergoing dialysis, and to provide plausible therapeutic strategies to reduce mortality associated with these complications. However, although remarkable progress has been made in reducing the risk of cardiovascular death and
thrombotic complications in the general population, these pathologies remain a major clinical challenge in patients with ESRD, particularly those on dialysis regimens.  

The mechanisms contributing to an increased risk of thrombosis during hemodialysis are complex and still not well understood. However, several studies have pointed to inflammation as an important contributor to thrombotic complications in hemodialyzed patients. In fact, ESRD is currently perceived as a state of chronic or recurrent inflammation. Furthermore, the inflammatory response is exacerbated in hemodialyzed patients as a result of bioincompatibility. Elevated levels of inflammatory mediators and acute-phase reactants such as TNF, IL-6, C-reactive protein, and fibrinogen have been repeatedly demonstrated in patients on dialysis. Another contributor to dialysis-associated inflammation is complement. Its activation during the initial phase of dialysis was demonstrated more than three decades ago. Although considerable progress has been made in reducing this activation through modifications of the surfaces of the biomaterials used to manufacture hemodialysis filters and elements of extracorporeal circuits, activation of complement as a result of bioincompatibility still induces adverse reactions in patients maintained on hemodialysis.

Since we have found that activation of complement and subsequent generation of the complement anaphylatoxin C5a contributes to upregulation of tissue factor (TF), a major trigger of coagulation in vivo, in patients with antiphospholipid syndrome (APS) and acute respiratory distress syndrome (ARDS), and since TF levels are elevated in ESRD patients, we hypothesized that complement activation might contribute to the pathogenesis of hemodialysis-associated thrombosis by upregulating TF. Endothelial cell damage in ESRD patients also contributes to the upregulation of TF in these cells. However, considering that a low-grade inflammatory response is constantly present in these patients and that hemodialysis exacerbates this inflammation, it is likely that inflammatory mediators, including complement effectors, also activate peripheral blood leukocytes to produce functionally active TF. Since
monocytes and neutrophils have been shown to produce TF when activated by inflammatory mediators and complement effectors, we have postulated that these cells are an important source of TF in the hemodialysis setting.

In this report, we provide evidence that C5a, generated through the activation of complement, stimulates the production of TF by peripheral blood neutrophils in patients with chronic kidney diseases who are on hemodialysis; this mechanism may contribute to hemodialysis-associated thrombosis. We have also found that complement regulates the levels of granulocyte colony stimulating factor (G-CSF) which, when administrated as a recombinant protein to patients with neutropenia, has been suggested to increase risk of thrombosis. Furthermore, we have demonstrated in clinically relevant models of hemodialysis that inhibiting the complement system at the C3 level with compstatin analogues can efficiently reduce the amount of TF produced by neutrophils and attenuate G-CSF induction. These findings suggest that inhibiting the complement system represents a potentially novel treatment strategy for decreasing the risk of thrombosis in hemodialysis patients with chronic kidney disease.
Methods

More detailed methodology is provided in supplemental materials.

Patients

Blood samples were collected from three female (mean age, 65.8 ± 5.18 years) and three male (mean age, 62.5 ± 9.16 years) ESRD patients maintained on hemodialysis in the Department of Nephrology, the University Hospital of Alexandroupolis, Greece. ESRD in these patients was a consequence of chronic glomerulonephritis (two patients), chronic pyelonephritis (two patients), diabetes mellitus type II (one patient), and essential arterial hypertension (one patient). Three patients have a history of thrombotic cardiovascular complications, and two patients have had vascular access thrombosis. Anti-platelet therapy was administered to three individuals, while three other patients were treated with low molecular weight heparin. All patients were maintained on low-flux hemodialysis using polysulfone filters. Blood samples from nine healthy donors were also used. Informed consent was granted by all involved individuals. The study was performed in compliance with institutional guidelines approved by the Institutional Review Board of the University Hospital of Alexandroupolis and in accordance with the Helsinki Declaration.

Complement inhibition

A potent compstatin analogue (Ac-I[CVW(Me)QDWGAHRC]T-NH$_2$)\textsuperscript{16} or a newly designed 2.2 times more active analogue of this inhibitor (Ac-I[CVW(Me)QDWSarAHRC]I-NH$_2$), termed compstatin-10, (unpublished observation) were used to block complement activation. A linear analogue of compstatin (H-IAVVQDWGHHHRAT-NH$_2$) served as a control.\textsuperscript{17} SB-290157 was used to block C3a receptor (C3aR).\textsuperscript{18} The C5a receptor (C5aR) was blocked by a cyclic hexapeptide (AcF-[OPdChaWR]).\textsuperscript{19} These inhibitors and the appropriate controls were
synthesized as described.\textsuperscript{16-19} For the complement inhibition studies, lepirudin-anticoagulated blood (50 μg/ml) or isolated serum was pre-treated for 10 min with either compstatin analogue or its linear analogue (25 μM), and the shredded filter hollow fibers (15 mg/ml) were then added. The blood was incubated for 60 min at 37˚C. For the experiments that used serum, 50 μl of the serum, treated as described above, was added to the cell suspension and incubated for 90-120 min at 37˚C. C3aR or C5aR inhibitor or the appropriate control (10μM) was added for 10 min, and the serum from patients on hemodialysis or filter fiber-activated serum was then added to the cell suspension. Approximately 0.8–1×10^6 cells from healthy donors were used for these experiments. Preliminary experiments showed that the effects of complement inhibition were dose-dependent, peaking at the doses showed in Results.

\textit{Ex vivo hemodialysis model}

To mimic the hemodialysis conditions \textit{ex vivo}, we designed a blood recirculation system consisting of a peristaltic pump (Easyload Masterflex, Cat No, XX80ELO04, Millipore, Billerica, MA), a mini-dialyzer (HPH Junior polysulfone dialyzers, Minntech Corporation, Minneapolis, MN, USA) and standard hemodialysis bloodline components that served as a tubing system (Set a/v for Fresenius 2008/4008, HMC Premedical S.p.A., Mirandola, Italy). To prevent any ultrafiltration, the dialysate compartment of the filter was filled with saline solution (0.9% NaCl) and clamped. The circuits were pre-rinsed with saline for approximately 30 min before being filled with 30 ml of blood. The blood sample from one donor, collected with lepirudin, was divided, with half preincubated with either compstatin analogue (40 μM, three experiments) or compstatin-10 (20 μM, one experiment), and half preincubated with an equal concentration of control peptide, for 10 min at 37˚C. “Predialysis” samples were collected before compstatin analogues or control peptide-treated blood was placed into the circuit system. For each experiment (donor), two identical circuit systems were used in parallel: one for blood treated
with compstatin, and the other for blood with the control peptide. Blood circulated in these systems at 37°C with a flow rate of 150 ml/min for 120 min. Samples from these two systems were collected at 2, 15, 30, 60, 90, and 120 min after the circulation began.

**Modified prothrombin time (mPT) assay**

Previous studies have shown that the modified prothrombin time assay (mPT) can be used to evaluate the TF-dependent procoagulant properties of bronchoalveolar lavage fluid and cell culture supernatants.14,20 TF in these fluids is probably an alternatively spliced TF isoform20 or originates from TF microparticles.21 Therefore, we used this method to estimate the procoagulant activity (TF/FVIIa binding activity) of supernatants from blood leukocytes as previously described.13,14 The supernatants of leukocytes incubated with sera from ESRD patients, hemodialysis filter fiber – treated sera and/or various complement inhibitors were isolated by centrifugation at 1000 x g for 10 min; supernatants were additionally checked to confirm the absence of cells. After performing the classical PT test (100 µl of platelet poor plasma plus 200 µl of thromboplastin; Instrumentation Laboratory, Milan, Italy), the modified PT assay was conducted. Briefly, 125 µl of leukocyte supernatant and 75 µl of thromboplastin were added to 100 µl of platelet poor plasma PT was measured. 125 µl of PBS was used instead of cell supernatant as negative control. To verify that the thromboplastic activity was due to TF, supernatants were incubated for 30 min at room temperature with a specific mouse anti-human TF monoclonal antibody (Mab; No 4509, American Diagnostica, Greenwich, CT., USA) and isotype controls13 at 10 µg/ml before the assay.
Complement activation assays

The magnitude of the complement activation was evaluated by ELISAs measuring the amounts of the terminal complement complex (TCC) or C3 cleavage products. TCC formation was quantified using Mab aE11 recognizing a neoeptope of C9 exposed when it is incorporated into the fluid-phase sC5b-9 complex, as previously described.\textsuperscript{22} C3 products were quantified using Mab C3-9 as previously described.\textsuperscript{23} This antibody binds to a neoantigen exposed in C3(H$_2$O), C3b, and C3c, but not to non-activated C3.

Statistical analysis

Data are presented as means±SD or means±SEM. The statistical significances of observed differences were tested using paired and unpaired t-test, Wilcoxon matched pairs, Mann-Whitney tests, and Two-way analysis of variance (ANOVA). The Bonferroni post-test correction was applied to control for the occurrence of false-positives. All statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA). \textit{P} values of ≤ .05 were considered significant.
Results

Sera of hemodialyzed ESRD patients stimulate neutrophils and monocytes to produce functionally active TF

The increased thrombogenicity of the blood in patients with ESRD, particularly those on hemodialysis, is likely the result of upregulation of TF, since TF activity is a major trigger of coagulation in vivo, and TF levels are increased in these patients. However, the sources of TF in hemodialyzed patients remain elusive. Peripheral blood monocytes produce TF in various inflammatory conditions. Also, recent studies have shown that complement-activated neutrophils constitute an important source of functionally active TF in patients with APS and ARDS. Therefore, we hypothesized that these cells are stimulated in the circulation of hemodialyzed ESRD patients to produce TF. To test this hypothesis, we measured the procoagulant activity of supernatants from PMNs and PBMCs obtained from healthy donors (n=4) and incubated with ESRD patients’ sera (n=6), which were collected before and at various times after the beginning of hemodialysis.

Supernatants from PMNs incubated with sera of ESRD patients before hemodialysis exhibited a moderate procoagulant activity, as illustrated by the shortening of the mPT when compared to supernatants from unstimulated cells or PMNs incubated with serum from healthy individuals (Figure 1A). A more pronounced shortening of the mPT was observed when supernatants from PMNs incubated with sera from hemodialyzed ESRD patients were assayed (Figure 1A), indicating that hemodialysis increases the procoagulant properties of the blood in these individuals. The increase in these procoagulant properties was observed immediately after hemodialysis was begun, reaching a maximum at 15 min and returning to levels observed before hemodialysis after 240 min. This transient and rapid increase in procoagulant activity indicates that an increased thrombogenicity of blood during hemodialysis is an acute and transient phenomenon—the opposite of the steady and more pronounced increase in the
prothrombotic properties of the blood that was observed in APS patients. Given these properties of APS patients’ blood, their sera were chosen for use as positive controls (Figure 1A).

Addition of neutralizing TF antibody to supernatants obtained from PMNs that had been incubated with sera collected from ESRD patients 15-30 min after the beginning of hemodialysis completely abolished their procoagulant activity, as illustrated by the return of mPT values to the levels seen in healthy individuals’ sera (Figure 1A). This effect of TF antibody indicates that the procoagulant activity of PMN supernatants is dependent on TF production by these cells. Furthermore, control antibodies added to PMN supernatants at various concentrations did not affect the mPT values in a similar manner, confirming the specificity of these findings. Thus, we concluded that sera from ESRD patients contain factors that stimulate neutrophils to produce functionally active TF. Given that these sera are likely to contain small amounts of TF, we needed to confirm that the shortening of the mPT was exclusively dependent on TF released from the neutrophils in vitro and was not affected by serum-derived TF. To achieve this goal, we tested the effect of ESRD sera alone on mPT values. The lack of shortening of the mPT that we observed (data not shown) excluded the contribution of serum-derived TF to the procoagulant activities of PMN supernatants. The same experiments described for PMNs were also conducted using PBMCs to validate the results for PMNs, since the monocytes included in the PBMC population are a well-known source of TF in various inflammatory conditions. We found that factors present in ESRD sera stimulated these cells to produce functionally active TF in a manner similar to that observed for PMNs (Figure 1B).

These conclusions were further strengthened by an increase in the TF gene expression in PMNs (Figure 1C) and PBMCs (data not shown) obtained and stimulated in the same way as described above. Sera from ESRD patients before hemodialysis induced a moderate increase in TF gene expression in PMNs when compared to its expression in cells incubated with sera from healthy individuals, whereas stimulation of these cells with sera obtained during hemodialysis
resulted in more pronounced increase, peaking at 30 min after the beginning of this procedure. This increase in TF gene expression corresponded to the increased procoagulant properties of supernatants from PMNs and PBMCs, as demonstrated by a shortening of the mPT values (Figure 1A,B).

**TF is expressed in blood leukocytes obtained from ESRD patients, and hemodialysis transiently enhances its expression**

Since our experiments have demonstrated that sera from ESRD patients contain mediator/s that stimulate PMNs and PBMCs from healthy individuals to produce TF *in vitro*, we next asked whether similar mechanisms are responsible for an increased tendency for thrombosis to occur *in vivo*. For this purpose, we examined the expression of TF isoforms in leukocytes obtained from ESRD patients before and during hemodialysis. TF and asTF mRNA levels were slightly increased in their PMNs and PBMCs even before hemodialysis, when compared to leukocytes from healthy individuals (Figure 2A, i and ii). Hemodialysis further transiently upregulated TF isoform expression. The maximum level of expression was observed at 30 min, with asTF being the dominant isoform for PMNs.

The changes in TF protein expression, as measured by flow cytometry (Figure 2B) and western blotting (Figure 2C), followed the pattern observed for TF mRNA.

**Upregulation of TF in neutrophils of hemodialyzed ESRD patients is complement-mediated**

Previous studies have suggested that C5a, which is generated as a result of complement activation, induces production of TF by neutrophils circulating in the blood or accumulating in the pulmonary alveoli of APS\textsuperscript{13} and ARDS\textsuperscript{14} patients, respectively. In addition, C5a contributes to fetal loss in an animal model of APS by upregulating TF in neutrophils.\textsuperscript{26}
Furthermore, hemodialysis activates complement. Therefore, we hypothesized that complement activation and subsequent generation of anaphylatoxins play a role in the upregulation of TF in the neutrophils of ESRD patients during hemodialysis. To confirm this hypothesis, we initially analyzed complement activation in these patients by monitoring the levels of TCC, which is generated as a result of final steps in the complement cascade activation, in the plasma of ESRD patients before and during hemodialysis. Hemodialysis induced significant complement activation, which reached a maximum 60 min after the beginning of this procedure (Figure 3A). In agreement with previous reports, we found that this activation was associated with a decrease in C5aR surface expression on PMNs in three out of four analyzed ESRD patients (Figure 3B). The moderate attenuation of C5aR expression, which correlated with low grade complement activation (Figure 3A), was observed in PMNs from ESRD patients even before hemodialysis; however, a clearer decrease in C5aR expression was observed only after hemodialysis began. In contrast, C5aR expression evaluated in permeabilized leukocytes did not decrease but showed a moderate, although not statistically significant, increase during hemodialysis when compared to predialysis status (Figure 3C). These data suggest that C5aR was internalized due to C5a binding and that hemodialysis induced a moderate upregulation of this receptor when compared to its levels in PMNs from patients before hemodialysis. The decreased C5aR expression observed in both experimental settings in ESRD patients when compared to healthy donors (Figure 3B, C) also suggests that inflammation associated with chronic kidney disease contributed to moderate downregulation of this receptor.

Next, we examined the effect of the inhibition of anaphylatoxin signaling in PMNs obtained from healthy volunteers and stimulated with sera from hemodialyzed ESRD patients, as described above, on the procoagulant properties of supernatants from these cells. We used ESRD sera obtained 15-30 min after the beginning of hemodialysis, since we found that these sera induced the most pronounced shortening of the mPT by PMN supernatants (Figure 1A) and
corresponding upregulation of TF (Figure 1C). Incubation of PMNs with the C5aR inhibitor prior to stimulation with ERSD sera completely abrogated the procoagulant activity of the supernatants, as demonstrated by a return of the mPT values to those for supernatants from cells incubated with sera from healthy donors (Figure 3D). Furthermore, blocking C5aR inhibited the upregulation of TF expression in these PMNs (Figure 3E). Conversely, blocking C3aR did not affect the mPT of the supernatants from PMNs stimulated with ESRD serum (Figure 3D). These results indicate that the TF-mediated procoagulant properties of neutrophils are C5a-dependent.

**Blocking complement activation abrogates the TF-dependent procoagulant activity of neutrophils**

Since hemodialysis filter fibers trigger complement activation, as demonstrated by the presence of C3 cleavage products in plasma obtained from blood after incubation with these fibers (Figure 4A), we used them as a clinically relevant *in vitro* model to explore therapeutic interventions targeting the complement system as a means of limiting the procoagulant properties of neutrophils. Initial experiments demonstrated that sera from healthy individuals incubated with shredded hollow filter fibers gained the ability to induce TF-dependent procoagulant activity in PMNs, as demonstrated by the shortened mPTs seen with supernatants from PMNs after their incubation with these sera, and the lack of this effect when TF antibody was added to the supernatants (Figure 4B). The magnitude of the mPT shortening was correlated with the duration of the incubation with the fibers. Based on these experiments, we concluded that contact between normal donor serum and the fibers induces complement activation, which results in the generation of complement effectors that subsequently upregulate TF in PMNs incubated with fiber-activated sera. Since a 60-min incubation had resulted in considerable shortening of mPT, we incubated sera collected from healthy individuals and from
ESRD patients before hemodialysis with the hemodialysis filter fibers for this same period of time. Supernatants from PMNs stimulated with fiber-activated ESRD sera induced a shortening of the mPT when compared to supernatants from untreated cells or cells treated with ESRD patient sera that were not incubated with fibers (Figure 4C). This reduction in mPT values was more pronounced than that observed for normal sera. Adding compstatin analogue to normal or ESRD patient sera before their incubation with the fibers prevented this reduction. A similar effect was observed when PMNs were pretreated with the C5aR-blocking peptide before the incubation with fiber-activated sera.

Since compstatin analogue blocks the complement cascade at C3 level, these results suggest that inhibiting complement activation could efficiently reduce the TF-dependent procoagulant activity of PMNs during hemodialysis. In addition, therapeutic targeting of C5aR had a similar beneficial effect. These conclusions are supported by the ability of compstatin analogue to reduce TF expression in PMNs stimulated with fiber-activated sera (Figure 4D,E).

**Hemodialysis-induced complement activation and subsequent TF upregulation in peripheral blood neutrophils can be efficiently reduced by inhibiting C3 cleavage**

To determine whether complement inhibition can be considered as a therapeutic approach to reduce the thrombotic complications during hemodialysis that are associated with TF expression induced by complement activation in neutrophils, we designed an experimental model mimicking hemodialysis. In this setting, a recirculation system was filled with blood from healthy donors. Passing the blood through this circuit induced complement activation, as demonstrated by an increase in C3 cleavage products in the plasma, which was proportional to the duration of the blood contact with the elements of this circuit (Figure 5A). Pre-treatment of the blood with compstatin analogues abolished this activation. These inhibitors also reduced TF expression in PMNs (Figure 5B), confirming the functional association between complement
activation and the procoagulant properties of these cells during hemodialysis. In addition, inhibition of complement in this experiment decreased the level of CD11b expression in PMNs (Figure 5C), suggesting that complement effectors are potent activators of these cells, since CD11b is an established marker of neutrophil activation.

**Complement inhibition reduces the amount of IFN-γ, IL-1RA, and G-CSF released during hemodialysis simulation**

Several cytokines and chemokines are induced in patients with ESRD as a result of hemodialysis. Some of these mediators are implicated in thrombotic complications in several diseases. Also, complement regulates the production and release of several cytokines during the inflammatory response to infections. Therefore, we hypothesized that complement, in addition to its direct regulation of TF expression in neutrophils, can indirectly contribute to thrombosis by regulating cytokine production and/or release. To test this hypothesis, we performed a high-throughput screen of cytokines released from blood cells and evaluated the effect of complement inhibition on the levels of these mediators in blood passing through a hemodialysis circuit. We found that hemodialysis simulation triggered the induction of TNF, IL-17, FGFb, PDGF-BB, GM-CSF, MIP-1α, RANTES (Supplemental Figure 1A-G), IFN-γ, IL-1RA, and G-CSF (Figure 6A-C). However, inhibition of complement significantly decreased the induction of only IFN-γ, IL-1RA, and G-CSF.
Discussion

In the present study, we have demonstrated that serum obtained from ESRD patients during hemodialysis induces the production of functionally active TF by neutrophils and monocytes from healthy donors, indicating that mediators responsible for this effect were generated in the circulation of the patients during hemodialysis. We also found that TF is transiently upregulated in neutrophils and monocytes from ESRD patients undergoing hemodialysis. These findings led us to conclude that peripheral blood leukocytes are stimulated to produce TF during hemodialysis. Since TF is a major trigger of coagulation in vivo, we postulate that this mechanism contributes to an increased blood thrombogenicity in these patients.

Activation of complement in the blood of ESRD patients and the failure of their sera to induce the expression of functionally active TF in neutrophils pre-treated with C5aR antagonist indicate that C5a, generated as a result of complement activation, contributes to thrombosis in hemodialyzed patients with ESRD. This hypothesis was further confirmed by our observation that the activation of complement was triggered by fibers used to manufacture hemodialysis filters. We also found that in the presence of the compstatin analogue, sera incubated with these fibers could no longer induce TF-dependent procoagulant activity by neutrophils. To explore the potential of complement inhibition to attenuate thrombosis in patients on hemodialysis, we designed an ex vivo model of extracorporeal circulation that mimicked this procedure. We found that inhibition of complement reduced the activation of neutrophils and TF expression in these cells that had been triggered by contact with the biomaterial surfaces. Importantly, the compstatin derivative has already been tested in patients with age-related macular degeneration. Therefore, the results of this pre-clinical research should facilitate further clinical investigation.
Although TF is a key initiator of coagulation in vivo and plays a central role in pathologic clotting-thrombosis, data concerning its role in hemodialysis-related thrombosis are surprisingly limited. Recently, increased plasma TF levels have been demonstrated in patients on dialysis, but potential mechanisms linking inflammation with coagulation were not explored. In addition, the sources of the increased TF levels in the blood of hemodialyzed patients and the stimuli responsible for this increase have not yet been determined. Thus, our findings point to novel mechanisms involving complement activation, the generation of C5a, and the subsequent induction of functional TF in blood neutrophils that can contribute to thrombosis in the hemodialysis setting.

Given that ESRD is associated with persistent low-grade inflammation even without hemodialysis, it is likely that other inflammatory mediators also contribute to upregulation of TF expression in PMNs in ESRD patients. However, C5a, generated as a result of complement activation triggered by the recurrent contact of blood with biomaterials, appears to be a primary agent causing TF induction in these leukocytes during hemodialysis. Therefore, we postulate that C5a is an important inflammatory mediator that bridges inflammation and thrombosis in this clinical situation. This conclusion is further strengthened by our finding that complement inhibition decreases the amount of the potent chemokine G-CSF in blood passing through a hemodialysis circuit. The importance of G-CSF for hemodialyzed patients was initially suggested by data showing that this mediator is strongly induced in these individuals. Although G-CSF has not yet been linked to hemodialysis-associated thrombosis, patients treated with recombinant G-CSF for various conditions associated with neutropenia have been suggested to have an increased risk of thrombosis. Therefore, we propose that complement, in addition to enhancing TF expression in neutrophils, can enhance the risk of thrombosis indirectly by regulating G-CSF. This chemokine is a potent activator of neutrophils and mobilizes these cells from the bone marrow. Since during hemodialysis, an initial transient neutropenia is
quickly reversed to leukocytosis,\textsuperscript{33} it is likely that G-GSF induced by hemodialysis contributes to this process. In consequence, a high number of hyperactivated neutrophils, which are a source of TF further heightens the risk of thrombosis. Also, G-CSF enhances platelet aggregation,\textsuperscript{34} increasing the tendency of the blood to clot; an increase in PDGF-BB, which we observed, confirms this process. Although we found that complement inhibition also attenuated the induction of IFN-\(\gamma\) in blood passed through the hemodialysis circuit, the significance of this finding for hemodialyzed patients needs to be established, since increased amounts of this cytokine have not yet been reported in such individuals. Similarly, the role of IL-17 induction remains unclear, although recent studies have implicated this cytokine in the pathogenesis of atherosclerosis,\textsuperscript{35} which is greatly enhanced in ESRD patients. We also found that complement inhibition attenuated the release/production of IL-1RA during hemodialysis stimulation. This mediator has anti-inflammatory activity, and its levels are increased in hemodialyzed patients.\textsuperscript{36} However, the associations between IL-1RA and coagulation are unknown. High amounts of TNF and chemokines such as GM-CSF, MIP-1\(\alpha\), and RANTES can be associated with thrombosis, since TNF is a well-established enhancer of coagulation,\textsuperscript{37} and chemokines can contribute to trafficking and activation of peripheral blood leukocytes.

A thorough understanding of the interconnections between complement and coagulation that are triggered by blood-biomaterial interactions during hemodialysis should provide new insights into the adverse effects of bioincompatibility in other clinical settings that utilize artificial surfaces. Intense efforts are being directed toward eliminating these adverse effects.\textsuperscript{38} Complement activation has been described as a primary event inducing and enhancing these undesirable reactions.\textsuperscript{39} Thus, we anticipate that mechanisms similar to those reported here for hemodialysis are applicable to other conditions with bioincompatibility-associated complications. Therefore, the development of biomaterials with only limited capacity to induce complement activation or coated with complement inhibitors, the blocking of complement activation, or the
disabling of C5aR signaling may prove beneficial in preventing thrombotic complications in various groups of patients, in addition to those on hemodialysis.

The induction of TF expression by C5aR signaling during hemodialysis places ESRD in a group of acquired thrombotic disorders mediated by C5a, including APS,\textsuperscript{13} sepsis,\textsuperscript{40} and ARDS,\textsuperscript{14} and indicating that the close relationship between complement and thrombosis may be a rather common phenomenon that is present in various as-yet unexplored conditions.\textsuperscript{41} Thus, the cross-talk between complement and hemostasis may have important therapeutic implications for reducing the frequency of thrombotic complications in variety of diseases. The development of several complement inhibitors and the safe use of these drugs in humans\textsuperscript{42,43} make the inhibition of complement activation a promising strategy for preventing thrombosis in the clinic.
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Authorship

Contributions: I.K. performed the stimulation studies, real time PCR, flow cytometry and western blot experiments, analyzed the data and wrote the manuscript; M.M.M. analyzed the data and wrote the manuscript; M.D. performed the mPT assays; S.R. performed flow cytometry experiments; K.K. and I.M. carried out flow cytometry and real time PCR experiments; S.P., P.P. and V.V. selected patients for the study, provided patient samples; P.M. synthesized and characterized the complement receptor inhibiting peptides; H.Q. synthesized and characterized compstatin analogues; T.E.M. performed the cytokine assays and analyzed data; K.R. and J.D.L. conceived and supervised the project. All authors critically revised the manuscript and approved the final version.

Conflict-of-interest disclosure: JDL and KR as co inventors on the following provision patent application: Inhibiting Pro-coagulant effect of biomaterials.
References


**Figure 1.** ESRD patients’ sera stimulate the production of functionally active TF in peripheral blood leukocytes from healthy donors. (A, B) The mPT values of supernatants of normal PMNs (A) and PBMCs (B) incubated with PBS (bar 1 in A and B), serum from healthy donors (HI, bar 2 in A and B), or ESRD sera isolated before (bar 3 in A and B; Pred, Predialysis) or during hemodialysis at various time points (bars 4-8 in A and B); mPT values of supernatants of PMNs (A) and PBMCs (B) either incubated with APS serum (bar 10 in A and B) or incubated with ESRD sera collected between 15-30 min after the beginning of hemodialysis and treated with TF mAb (bar 11 in A and B). Data are representative of 10 independent experiments (mean mPT values ± SD); a paired t-test was applied to examine the statistical significance (* P< .05, ** P< .001). (C) The relative expression of TF mRNA in normal PMNs cultured in the presence of PBS or ESRD patients’ sera isolated before or during hemodialysis. Data are representative of six independent experiments (mean-fold expression ± DCt SD); the Wilcoxon matched pairs test was used to assess the statistical significance (* P< .01). Horizontal lines above data bars indicate the statistical significance.

**Figure 2.** TF is expressed in the peripheral leukocytes of ESRD patients. (A) The relative expression of TF or asTF mRNA in PMNs (i) and PBMCs (ii) obtained from healthy donors (HI, bar 1) or ESRD patients before (Pred, Predialysis, bar 2) and during hemodialysis (bars 3, 4). Data are representative of six independent experiments (mean-fold expression ± DCt SD); the Wilcoxon matched pairs and Mann-Whitney tests were used to assess the statistical significance (* P< .05). (B) The induction of TF expression in PMNs (i) and PBMCs (ii) isolated from ESRD patients before (bar 2) and during hemodialysis (bars 3, 4). The results are presented as ratios of the mean fluorescence intensities (MFI) of the cells from patients to the cells from healthy donors (bar 1), stained with TF mAb, and analyzed by flow cytometry. Data are representative of one experiment (mean ± SD of four healthy donors and six ESRD
patients). The Mann-Whitney and Wilcoxon matched pairs tests were used to assess the statistical significance (* \( P < .05 \)). (C) TF expression in PMNs isolated from healthy donors (1 - HI) or ESRD patients before (2 - Pred) and during hemodialysis (3, 4). One representative western blot analysis out of four independent experiments is shown. Horizontal lines above data bars indicate the statistical significance.

**Figure 3. Procoagulant properties of ESRD sera are complement-dependent.** (A) The amounts of soluble terminal complement complex (TCC) in plasma from healthy donors (HI, bar 1) or ESRD patients before (Pred - Predialysis, bar 2) and during (bars 3 - 6) hemodialysis. Data are representative of one experiment (mean ± SD of four individuals per group). The Mann-Whitney test and paired t–test were applied to assess the statistical significance (* \( P < .05 \)). (B, C) The expression of C5a receptor on the surface of PMNs (B) or in permeabilized cells (C) from healthy donors (HI), ESRD patients before (Pred) and during hemodialysis (5, 30, 60 and 120 min). The results are presented as MFI ratios of the cells stained with C5aR mAb to the cells stained with the isotype control. Data are representative of one experiment (mean ± SD of four healthy donors and three ESRD patients). The unpaired and paired t-tests were used to assess the statistical significance (* \( P < .05 \)). (D) The mPT values of supernatants of normal PMNs treated with sera from healthy donors (HI, bar 1), ESRD patients before (Pred, bar 2), and during (bar 3) hemodialysis or pre-incubated with C5a (bar 4) or C3a (bar 5) receptor antagonists and then treated with sera obtained from ESRD patients during hemodialysis. Data are representative of six independent experiments (mean ± SD). The Wilcoxon matched pairs test was used to assess the statistical significance (* \( P < .001 \)). (E) The relative expression of TF mRNA in normal PMNs treated with sera obtained from ESRD patients during hemodialysis or pre-incubated with C5aR antagonist and then treated with sera from the same patients. Data are representative of two independent experiments (mean ± SD of three patients). The Wilcoxon
matched pairs test was used to assess the statistical significance ($P < .01$). Horizontal lines above data bars indicate the statistical significance.

**Figure 4.** Hemodialysis filter fibers induce complement activation and enhance TF-dependent procoagulant properties in normal and ESRD sera. (A) The amounts of C3 cleavage products in plasma isolated from normal non-treated blood (bar 1), non-treated blood incubated in 37°C for 60 min (bar 2), blood treated with filter fibers (bar 3), or blood treated with filter fibers in the presence of compstatin analogue (bar 4) or control peptide (bar 5). Data are representative of four independent experiments (mean ± SD). The paired t-test was used to assess the statistical significance (* $P < .05$). (B) The mPT values of supernatants of normal PMNs incubated with sera from healthy donors (HI, bar 1), sera activated with filter fibers for various periods of time (bars 2-5) or with filter fiber-activated sera (for 60 min) and treated with TF mAb (bar 6). Data are representative of six independent experiments (mean ± SD). The Wilcoxon matched pairs test was used to assess the statistical significance (* $P < .01$). (C) The mPT values of supernatants of normal PMNs incubated with sera from healthy donors (HI, bar 1), ESRD patients before hemodialysis (Pred, Predialysis, bar 2), filter fiber-activated sera from healthy donors or patients (bars 3 and 6, respectively), sera from healthy donors or patients incubated with these fibers in the presence of compstatin analogue (bars 4 and 7, respectively) or pre-treated with C5aR antagonist and then incubated with fiber-activated sera from healthy donors or patients (bars 5 and 8, respectively). Data are representative of six independent experiments (mean ± SD). The Wilcoxon matched pairs test was used to assess the statistical significance (* $P < .01$). (D) The induction of TF expression in normal PMNs incubated with sera from healthy donors (bar 1), fiber-activated sera (bar 2), or sera treated with fibers in the presence of compstatin analogue (bar 3). The results are presented as ratios of the mean fluorescence intensities (MFI) of the cells incubated with sera treated with fibers in the presence
or absence of compstatin to the cells incubated with sera from healthy donors, stained with TF mAb, and analyzed by flow cytometry. Data are representative of six independent experiments (mean ± SD). The Wilcoxon matched pairs test was used to assess the statistical significance (* $P < .05$). (E) TF expression in normal PMNs incubated with PBS (1), APS sera (2), fiber-treated sera in the presence of compstatin analogue (3), or fiber-activated (4) or untreated sera (5). One representative western blot analysis out of four independent experiments is shown. Horizontal lines above data bars indicate the statistical significance.

Figure 5. Compstatin analogues attenuate the hemodialysis-associated activation of complement and neutrophils and upregulation of TF in these cells. Normal blood was recirculated through a hemodialysis simulation system for 2 h at 37°C. (A) The amounts of C3 cleavage products in plasma isolated from the blood before the beginning of this recirculation (Pred, Predialysis) and at various time points during hemodialysis simulation in the presence of compstatin analogues or control peptide. (B) The induction of TF and (C) CD11b expression in PMNs isolated from the blood before (Pred) and during hemodialysis simulation. The results are presented as ratios of the mean fluorescence intensities (MFI) of the cells stained with TF mAb to the cells stained with the isotype control and analyzed by flow cytometry. Data are representative of four independent experiments in A and three in B and C (mean ± SEM). The two-way ANOVA test was applied to assess the statistical significance of the difference between treatment of blood with compstatin analogues (▲) and the control peptide (■). (A) $P < .0001$, (B) $P = .0341$ and (C) $P = .0208$.

Figure 6. Complement regulates cytokine production and/or release during a hemodialysis simulation. The amounts of (A) IFN-γ, (B) IL-1RA, and (C) G-CSF during hemodialysis simulation. Data are representative of three independent experiments (mean ±
SEM). The two-way ANOVA test was used to assess the statistical significance of differences in the amounts of cytokines after treatment of the blood with compstatin analogues (▲) or the control peptide (■). (A) $P = .0177$, (B) $P = .0298$ and (C) $P = .0084$. 
Figure 1

A

PMN mPT (sec)

35
30
25
20
15

PBS  HI  Pred  1  2  3  4  5  6  7  8  9  10  11

Time (min)

B

PBMC mPT (sec)

35
30
25
20
15

PBS  HI  Pred  1  2  3  4  5  6  7  8  9  10  11

Time (min)

C

PMN TF

Relative expression (2^−DDCt)

3
2
1
0

HI  Pred  30  120

Time (min)

*  **
Figure 3

A) TCC (Arbitrary Units/ml) over time (min).

B) Surface PMN C5aR MFI ratio over time (min).

C) Permeabilized PMN C5aR MFI ratio over time (min).

D) PMN mPT (sec) over time (min).

E) PMN TF Relative expression (2^-DDCt) over time (min).
Figure 5

A. C3(H2O)/C3b/C3c (ug/ml)

- control peptide
- compstatin

B. PMN TF MFI ratio

- control peptide
- compstatin

C. PMN CD11b MFI ratio

- control peptide
- compstatin
Figure 6

A. IFN-γ

- Control peptide
- Compstatin

B. IL-1RA

- Control peptide
- Compstatin

C. G-CSF

- Control peptide
- Compstatin
Complement anaphylatoxin C5a contributes to hemodialysis-associated thrombosis

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