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Thrombosis in the uremic *milieu*- emerging role of ‘thrombolome’

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Abstract

Chronic kidney disease (CKD) is characterized by retention of a number of toxins, which unleash cellular damage. CKD environment with these toxins and a host of metabolic abnormalities (collectively termed as uremic *milieu*) is highly thrombogenic. CKD represents a strong and independent risk factor for both spontaneous venous and arterial (post-vascular injury) thrombosis. Emerging evidence points to a previously unrecognized role of some of the pro-thrombotic uremic toxins. Here we provide an overview of thrombosis in CKD and an update on indolic uremic toxins, which robustly increase tissue factor, a potent pro-coagulant, in several vascular cell-types enhancing thrombosis. This panel of uremic toxins, which we term ‘*thrombolome*’ (thrombosis and metabolome), represents a novel risk factor for thrombosis and can be further explored as biomarkers for post-vascular interventional thrombosis in patients with CKD.

Keywords

Thrombosis; chronic kidney disease; end stage renal disease; renal failure; indoxyl sulfate; indoxyl acetate; indolic compounds; uric acid

1. Introduction

Chronic kidney disease (CKD) represents a global problem resulting in high morbidity and mortality [1,2]. According to the United States Renal Data System in 2011, more than 10% of US population of age 20 and older have CKD and this number is projected to increase by 2020 [3]. Similar figures also emerge from Europe, where almost 8% of European population has CKD [4,5]. An increasing trend of CKD incidence has been observed in Australia, New Zealand, Japan and Taiwan [6]. In essence, CKD represents a pandemic problem. CKD is a strong risk factor for various cardiovascular diseases including thrombosis[7,8], which can manifest as spontaneous venous thrombosis resulting in pulmonary embolism or unstable plaques resulting in acute coronary syndrome and stroke, etc. Another common feature of this heightened risk is the dialysis access thrombosis seen in patients with CKD and End Stage Renal Disease (ESRD) maintained on hemodialysis.

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2. Thrombosis in CKD patients

CKD increases bleeding risk on one hand and thrombosis risk on the other. Several clinical trials have shown increased risk of both spontaneous venous and arterial thrombosis along the entire spectrum of CKD, beginning from CKD stage 2 to stage 5 patients [9–13]. The relative risk for spontaneous venous thrombotic events is estimated to be 28% higher with stage 2 CKD and almost doubles with stage 3 or 4 compared to controls with normal renal function [14]. The incidence of pulmonary embolism is increased in CKD and ESRD patients (204 and 527 per 100,000, respectively) compared to the ones with normal kidney function (66 per 100,000) [14].

CKD patients undergo frequent endovascular procedures and the uremic *milieu* increases their risk for post-procedural complications such as post-angioplasty or coronary stent thrombosis or thrombosis of vascular access [15–18]. Annually 322,000 US patients on Medicare undergo coronary interventions. On an average, 1–2% of the stents get thrombosed [17–20] resulting in 50% mortality and need for urgent revascularization [21–23]. The first generation of coronary stents was bare metal stents, which suffered from the problems of thrombosis and stenosis due to neointimal hyperplasia. Though stenosis was partially averted with the second generation drug-eluting stents (coated with Mammalian target of rapamycin- mTOR inhibitors or Paclitaxel), stent thrombosis still remains a significant problem [24]. Next to drug nonadherence, CKD is the second most potent risk factor for post angioplasty or stent thrombosis increasing the risk by 6.5–10 fold [17,18,25]. Vascular access is the Achilles' heel in the management of CKD and ESRD patients. Vascular access thrombosis results in prolonged hospitalization and several complications costing Medicare \$700M annually [26]. Thus, thrombosis in CKD is a critical clinical problem warranting urgent attention.

3. Pathogenesis of thrombosis in the uremic *milieu*

The pathogenesis of venous and arterial thrombosis differs. The arterial thrombosis is triggered by the damage or denudation of endothelial cells exposing the underlying subendothelial matrix and vascular smooth muscle cell (vSMCs), which serve as a reactive bed for clot formation. On the other hand, the venous thrombosis occurs spontaneously on intact endothelial layer. In general anti-platelet agents constitute the mainstay of therapy in arterial thrombosis, while drugs targeting the coagulation cascade are used in venous thrombosis [27]. CKD is characterized by a number of metabolic abnormalities including retention of several metabolites (collectively termed here as uremic *milieu*), influences almost all the components of the thrombotic and hemostatic machineries.

(A). Venous thrombosis

Blood, flow and vessel wall- called the Virchow's triad are traditionally considered as three critical components of spontaneous venous thrombosis (Figure 1) [28,29].

(i). Blood component—CKD alters the balance of pro- and anti-thrombotic factors in blood. Several pro-thrombotic hemostatic mediators are elevated in CKD patients, including fibrinogen [30], soluble thrombomodulin [31], soluble tissue factor, thrombin-anti-thrombin

complex (TAT) [32], von Willebrand factor (vWF) [32], factor VIII, c-reactive protein (CRP) [32,33]. The generalized inflammatory state, endothelial dysfunction and possibly poor clearance of some of the thrombotic mediators may account for this derangement [30]. Platelet abnormalities are common in CKD patients, however, they are linked to bleeding rather than thrombotic disorders [34]. Hemodialysis is known to activate platelets [35], however, its role in thrombosis remains to be defined.

(ii) Flow component—Normal laminar flow maintains endothelial cells largely in anti-inflammatory and anti-thrombotic state. Such healthy endothelium inhibits platelets and leukocytes adhesions and platelet aggregation by upregulating several mediators such as prostacyclins, nitric oxide, activated protein C and plasminogen activator, etc [36–38]. In CKD patients, the endothelium is constantly exposed to exacerbated hemodynamic forces, such as hydrostatic pressure (due to volume overload) and fluid shear stress (due to hypertension) compromising its anti-thrombotic functions. Furthermore, extensive atherosclerotic changes introduce heterogeneity in shear forces that increase platelet adhesion, aggregations, essentially converting the endothelium to pro-thrombotic state augmenting spontaneous venous thrombosis [39].

(iii) Vessel wall component—Inflamed endothelial layer in uremia constitute the reactive vascular bed for spontaneous venous thrombosis [30,39]. Recent evidence also implicates disruption of endothelial glycocalyx as an important mediator of thrombosis [40]. The endothelial glycocalyx is a negatively charged layer present in the luminal side of all blood vessels and is composed of proteoglycans, glycosaminoglycans and associated plasma proteins. It has a vasculoprotective and anti-thrombotic effects and disruption of which activates coagulation *in vivo* [40]. CKD and ESRD patients have been shown to have a disrupted glycocalyx layer, which contributes to the increased risk of thrombosis [41,42]. The endothelial cells in uremic patients express elevated levels of tissue factor, a crucial pro-coagulant activating the extrinsic coagulation cascade [44,45]. Uremic endothelial cells also release small extracellular vesicles called microparticles loaded with TF that augments thrombosis [45–47].

(B) Arterial thrombosis is commonly precipitated by plaque rupture or endovascular procedures such as angioplasty, stent implantation or vascular procedures such as arteriovenous fistula creation or bypass, etc. Denuded endothelial layer and exposed subendothelial matrix and vSMCs characterize the reactive vascular bed for arterial thrombosis. Exposed vSMCs express the highest levels of tissue factor among all vessel wall-types and thus effectively serving as inciting cells for thrombus formation (Figure 2) [48]. This is further augmented by the fact higher circulating levels of activated factor VIIa, a co-factor for tissue factor which accelerate the coagulation cascade[49].

Vascular interventions also disturb the laminar flow pattern and predispose to oscillatory low shear forces upregulating several molecules like ICAM-1, VCAM-1 or E-selectin to increase platelet and polymorphonuclear adhesions [36–38]. The arterial stiffness in CKD patients due to arteriosclerosis and vascular calcification may exacerbate flow disturbances predisposing to thrombosis [50]. Furthermore, the vasculature of CKD patients with accelerated atherosclerosis harbors several areas of lipid droplets and lipid-laden

macrophages in subendothelial matrix that serve as highly thrombogenic nexuses by directly activating CD36 receptors on platelets to increase their adhesion and aggregation [51].

4. Role of uremic toxins/solutes in thrombosis

Co-morbidities such as diabetes, hypertension and hypercholesterolemia are some of the main causes of CKD and are also well known risk factors for thrombosis. However, their presence fails to explain the higher incidence of thrombosis in CKD patients. Several clinical trials showed CKD as a strong risk factor for thrombosis independent of these co-morbidities [13,52,53]. Furthermore, conventional anti-thrombotics and anti-platelet agents exhibit suboptimal efficacy in preventing stent or fistula thrombosis in CKD patients, indicating the presence of uremia-specific factors that could not be counteracted by conventional anti-thrombotics [54]. Recent studies now identify an independent role of CKD in thrombosis by defining uremia-specific pro-thrombotic risk factors [43,48,55].

The uremic *milieu* is uniquely characterized by the accumulation of a number of solutes, called uremic toxins. In 2003, the review of the European Uremic Toxins Work Group listed 90 different uremic toxins [56], and the list continues to expand [57,58]. There are a number of ways of classifying uremic toxins based on their site of origin [59]. However, classification based on their molecular size and binding affinity to albumin is well accepted, as these characteristics determine the ability of these solutes to undergo clearance with dialysis. They are most commonly divided into three groups:

- a. Small molecular weight water-soluble compounds, < 500 Dalton (Da) easily removed during dialysis (for example, urea and guanidines, etc)
- b. Protein-bound compounds that bound to serum albumin (for example indoxyl sulfate (IS), indoxyl acetate (IA), kynurenin, p-cresyl sulfate, homocysteine,). These are highly protein bound, poorly dialyzable and hence are considered major pathogenic mediators of vascular damage in ESRD [60]. The predominant chemotypes of this group are phenols and indoles [61], and these toxins originate from the colonic microbial metabolism of various amino acids [59].
- c. Middle molecules characterized by a weight >500 Da (for example, beta 2-microglobulin) and are poorly dialyzable. We direct the interested reader to comprehensive review on uremic solutes by Vanholder *et al* [56].

5. Novel pro-thrombotic role of the protein-bound uremic solutes

Recent studies have revealed previously unrecognized pro-thrombotic role of indolic uremic solutes - indoxyl sulfate (IS) and indoxyl acetate (IA). Indolic compounds induce tissue factor, a potent procoagulant in both the endothelial cells and vSMCs (Figure 2). Indolic solutes increase tissue factor mRNA levels and tissue factor in microparticles derived from endothelial cells through aryl hydrocarbon signaling, a xenobiotic pathway [43]. This mechanism is more relevant in spontaneous venous thrombosis, where the endothelium serves as reactive vascular bed.

Indolic solutes are also potent inducers of tissue factor protein in exposed vSMCs through a distinct mechanism [55]. Unlike ECs, in vSMCs these uremic solutes reduce tissue factor

degradation by inhibiting its ubiquitination [48]. Conceivably different mechanism of tissue factor regulation may offer functional and teleological advantage to vSMCs in thrombosis. Vascular injury brings blood in contact with vSMCs, which need to initiate thrombus formation rapidly. Control of tissue factor at a post-translational level, a relatively rapid mechanism compared to transcription that occurs in several hours, imparts vSMCs an ability to rapidly modulate tissue factor levels consistent with the last layer of defense against bleeding after vascular injury.

Indolic compounds are intermediate products of tryptophan metabolism [62], which undergoes fermentation by gastrointestinal colonic bacteria [63,64] through the enzyme tryptophanase to form indole moieties. These indoles absorbed by the portal circulation are further metabolized in the liver (Figure 3) by the microsomal p450 cytochrome CYP2E1 enzyme to indoxyl. This serves as a substrate for the enzyme aryl sulfonyltransferase, which conjugates sulfur moieties to synthesize indoxyl sulfate [65,66]. Both indolic metabolites (indoxyl sulfate and indoxyl acetate) circulate in body bound to albumin and are excreted by the tubular epithelial cells by specific organic anionic transporters (OAT1 and OAT3) [67,68]. This defines the gut-liver-kidney axis. As renal function deteriorates, the metabolites accumulate in the circulation and their levels increase with advancing stages of CKD (Table 1).

Dietary tryptophan is also metabolized in the liver via the kynurenine pathway, which involves the conversion of tryptophan to its metabolites ('kynurenines'), kynurenic (KYNA), anthranilic (AA) and quinolinic (QA) acids, all of which accumulate in patients with CKD [69–71]. L-kynurenin has been associated with the hypercoagulable state, the mechanism of which remains poorly defined [69]. The above body of literature underscores a critical role of the products of intermediate metabolism in thrombosis, which we term as '*thrombolome*' (a combination of thrombosis and metabolome).

P-cresyl sulfate, a protein-bound uremic toxin, is a product of the tyrosine metabolism and accumulates in renal failure. It has been implicated in cardiovascular and peripheral vascular diseases and vascular access dysfunction [72]. The exact mechanism of pro-thrombotic property of p-cresyl sulfate remains poorly understood, however, it seems to increase the shedding of endothelial microparticles and induce endothelial dysfunction enhancing thrombosis [73].

6. Other uremic toxins influencing thrombosis

Uric acid is a product of protein metabolism and is commonly elevated in CKD patients. It has been associated with various forms of cardiovascular diseases with a poorly defined mechanism. Uric acid robustly induces tissue factor in vSMCs and enhances arterial thrombus formation after interventional procedures [55]. Homocysteine is a homologue of cysteine and is elevated in CKD and ESRD patients [74]. Several studies have suggested elevated total plasma homocysteine as an independent risk factor for both arterial and venous thromboses in patients with CKD [74,75]. Homocysteine may enhance thrombosis through multiple mechanisms including increases in tissue factor activity [76] and platelet

reactivity, and inhibition of the fibrinolysis process. However, clinical studies have yet to demonstrate a conclusive association between homocysteinemia and thrombosis [77,78].

7. Poor effectiveness of conventional anti-thrombotics in uremic *milieu*

It is note worthy that both the anti-platelet and the anti-thrombotics agents exhibit suboptimal efficiency to prevent coronary stent or vascular access thrombosis in patients with CKD and ESRD. For example, platelets from CKD patients are less responsive to Clopidogrel (an anti-platelet agent) compared to controls with normal renal function [79]. Similarly, Clopidogrel and Coumadin showed minimal effect in improving suitability or patency of arteriovenous fistula and polytetrafluoroethylene arteriovenous graft in CKD patients, respectively [54,80,81]. Thus, uremic *milieu* fosters resistance to these agents, which can be multi-factorial in etiology. Two aspects of uremia in particular are likely to be significant contributors. First, highly-protein bound nature of the pro-thrombotic uremic solutes precludes their effective removal with dialysis perpetuating the thrombotic state. Second, more importantly, none of the conventional anti-thrombotic or anti-platelet agents target these prothrombotic uremic solutes, underscoring a need for agents particularly targeting uremia-specific risk factors.

8. Future directions and conclusions

Several aspects of thrombosis in CKD warrant further investigations, including better characterization of thrombogenic microparticles, different components of gut-liver-kidney-vascular thrombosis axis and the effects of these pro-thrombotic uremic toxins on other cell-types critical for thrombosis. Like tryptophan metabolites, there are number of other products of metabolism that may constitute ‘*Thrombolome*’, which deserves further characterization. That several uremic toxins are generated in the gastrointestinal tract makes it a potential site for therapeutic intervention. The oral gastrointestinal absorbent Kermzin® (AST-120) has been shown to decrease the serum levels of protein bound uremic toxins, and cardiovascular risk markers [82,83] and may decrease the complications, including thrombosis mediated by these protein-bound uremic toxins. Several proteins undergo post-translational modifications in the uremic environment and their collective impact on thrombosis remains to be determined.

In conclusion, better definition of ‘*thrombolome*’ will provide deeper understanding of thrombosis in the uremic *milieu*, which can serve as biomarkers for thrombotic risk in CKD patients. Development of anti-thrombotics specifically targeting these pro-thrombotic uremic toxins is critical to prevent thrombosis in CKD population, which is already plagued by high cardiovascular morbidity and mortality.

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Figure 1.

Alteration of Virchow's triad in uremic *milieu*. Uremia perturbs all the components of Virchow's triad predisposing to venous thrombosis.

Figure 2.

Protein-bound uremic solutes enhance post-vascular interventional arterial thrombosis. Vessel wall injury after procedures such as angioplasty, stent placement or arteriovenous fistula results in denudation of endothelial cells (ECs) and exposure of subendothelial matrix and vascular smooth muscle cells (vSMCs). Uremic toxins such as indoxyl sulfate (IS) and indoxyl acetate (IA) increase tissue factor by increasing tissue factor mRNA in ECs or inhibiting proteasomal degradation of TF in vSMCs. Thrombus formation further augmented by higher levels of circulating endothelial-derived microparticles loaded with tissue factor (black circle) and soluble factor VIIa in CKD patients.

Figure 3.

Synthesis and excretion of indoxyl sulfate and indoxyl acetate. Intestinal microbes degrade proteins and form indole compounds from tryptophan amino acid, which then undergoes sulfation in liver. Indoxyl sulfate circulates in blood in predominantly albumin-bound form and excreted in kidneys by organic-anionic transporter (OAT) 1 and 3.

Table 1

Different stages of CKD with corresponding IS levels [84–88]

Stage of CKD	GFR ml/min	Average IS (ug/ml)
Normal	>90	0.243#
Stage I*	>90	0.243
Stage II	60–89	0.5
Stage III	30–59	3.2
Stage IV	15–29	5.4
Stage V	<15	19.8
ESRD	<15HD	42.5

Normal kidney function but urine findings or structural abnormalities or genetic trait point to kidney disease