SUPPLEMENTAL MATERIAL

Cell lysis, immunoblotting, immunoprecipitation and immunofluorescence

Cell harvest and immunoblotting has been described previously[23]. Tissue factor monoclonal (American diagnostica), Pancadherin and actin (Santa Cruz) antibodies were used. For TF neutralizing assay, goat-antihuman anti-TF antibody (American Diagnostica, catalogue # 4501) was used. An isotype antibody served as control. The TF bands were normalized to Pancadherin and quantified using ImageJ (NIH). Immunoprecipitation using tissue factor antibody (American diagnostica) followed by agarose (A+G plus) beads (Santa Cruz) were performed as described previously[29]. The vSMCs and HCAECs grown in chamber slides (Lab-Tek) were fixed with and processed as described previously[22].

TF stability assay, ubiquitination, activity assay, ELISA and RT-PCR

The vSMCs treated with 20 μM emetine for different time intervals were harvested for stability assay. The ubiquitination of TF was examined by pretreating vSMCs with proteasome inhibitor MG132 (Boston Biochem) at 10 μM for 6 hours followed by immunoprecipitation as described above. vSMCs cells lysates treated with uremic serum or solutes for 24 hours were lyzed using 50mM Tris buffered saline (pH 8.0) with 1% Triton X-100 and centrifuged at 14000g for 20 min. TF activity was measured using human tissue factor chromogenic activity kit® (Assaypro) using manufacturer's instructions. The amount of factor Xa generated is measured by its ability to cleave highly specific chromogenic substrate for factor Xa normalized to number of vSMCs.

RNeasy mini kit (Qiagen) was used to extract total RNA from VSMCs. 300ng of total RNA were converted to cDNA using Sensiscript RT kit® (Qiagen) followed by RT-PCR using human TF Taqman probe (Applied Biosystems). Human actin Taqman probe (Applied Biosystems) served as loading control. Levels of tissue factor mRNA were

determined using comparative Ct method. TF levels were measured in serum using TF ELISA from American Diagnostica per manufacturer's instructions.

Uremic solutes and vSMC injury model

All uremic solutes (Sigma) were used at concentrations seen in CRF/ESRD patients (Table 1)[20]. Urea, creatinine, oxalic acid, indole-3-acetic acid (IA), indoxyl sulfate (IS), homocysteine were dissolved in water. UA being water insoluble was dissolved in 1M NaOH and p-cresol was dissolved in DMSO. Human serum albumin (HSA) was purchased from Lee Biosolutions (St. Louis, USA) and used with HSA at 4 gm/dl final concentration[20].

One million vSMCs seeded on culture plates with 9mm molded grid (BD Bioscience) were treated with uremic solutes for 24 hours. The cells were then injured with pipette tip corresponding to the grid. Cells washed and incubated without uremic solutes for 2 hours prior to harvest.

Data Supplement Figure 1. TF expression is highest in vSMCs among different vascular cell-types.

(A) Relative abundance of TF in endothelial, vascular and THP-1 cells. HCAECs, vSMCs, and THP-1 were lyzed and were immunoblotted with TF and actin antibodies. Densitometry analysis performed on TF bands with Image J using actin to normalize the signal. Representative blot from two experiments is shown.

(B) Expression of TF in HCAECs and vSMCs were fixed and stained for TF. Immunofluorescence was performed using FITC staining.

(C) vSMC imposes highest thrombogenicity in flow-loop model. The silastic tube, coated with fibronectin or EC or vSMCs were subjected to flow-loop model. A representative visual examination of clot after stopping the system is shown.

Data Supplement Figure 2. Uremic serum does not later TF message

No increase in TF mRNA with uremic serum. vSMCs exposed to pooled control or non-diabetic or diabetic uremic serum were harvested for total RNA. Quantitative RT-PCR in two steps, was performed after extracting RNA and synthesing cDNA using random hexamers and oligo dT primers. qRT-PCR reactions were run in triplicates for each sample, and in the same tube beta actin was quantified using real time PCR and labeled taqman probes (VIC) and for detecting TF mRNA (FAM). The Ct values were utilized to generate delta-delta values between normal and uremic patients to calculated TF expression, normalized by beta acting levels. Error bars = SEM.