Procoagulant activity of erythrocytes and platelets through phosphatidylserine exposure and microparticles release in patients with nephrotic syndrome

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Summary
Recent studies showed that an imbalance of prothrombotic and anti-thrombotic factors and impaired thrombolytic activity contribute to the thrombophilia of the nephrotic syndrome (NS). However, it is not clear whether blood cell injury and/or activation is involved in hypercoagulability in NS patients. Our objectives were to study the increase in microparticle (MP) release and phosphatidylserine (PS) exposure on the outer membrane of MP-origin cells in NS patients, and to evaluate their procoagulant activity (PCA). The subjects were patients with membranous nephropathy (MN), minimal change nephrotic syndrome (MCNS) and healthy controls. Analyses of MPs and PS exposure were performed using a flow cytometer. PCA was determined by clotting time and purified coagulation complex assays. We found that lactadherin+ MPs, which derived from red blood cells (RBC), platelet and endothelial cell, increased in NS patients. Moreover, PS exposure on RBCs and platelets in each NS group, especially in MN, are higher than that in controls. MP shedding and PS exposure of RBCs/platelets were highly procoagulant in NS patients. However, blockade of PS with lactadherin inhibited over 90% of PCA while an anti-tissue factor antibody had no significant inhibition effect. Our results demonstrate that the thrombophilic susceptibility of NS may be partly ascribed to MP release and PS exposure of RBCs, platelets and endothelial cells. Lactadherin is a sensitive probe for PS that has high anticoagulant activity.

Keywords
Phosphatidylserine, microparticles, nephrotic syndrome, lactadherin

Introduction
Nephrotic syndrome (NS), is characterised by a heavy urinary protein level exceeding 3.5 g/L.3 m²/day, oedema, hypoalbuminaemia, hyperlipidaemia, and most commonly occurs in association with thromboembolic complications such as renal vein thrombosis (RVT), deep-vein thrombosis (DVT) and pulmonary embolism (PE), which is one of the most serious threats to a NS patient (1). Patients with membranous nephropathy (MN) and heavy proteinuria are at an increased risk of thromboembolism (2–4). There are multiple pathophysiologicals of thrombophilia in NS which seem related to many haemostatic derangements, including platelet activation and aggregation, elevation of factor (F)VIII, FV and fibrinogen levels, reduced antithrombin III, protein C and protein S levels or activity, increased PAI-1 or decreased plasminogen levels (5). Moreover, hypoalbuminaemia, hyperlipidaemia, diuretic or steroid therapy, venous stasis, immunisation and immune complex activation of the clotting cascade all seem to be relevant to the hypercoagulable state of NS (6).

Although many mechanisms explain the hypercoagulable state in NS, there is conflicting data relating to NS and thromboembolism. Many clinical studies have demonstrated hypoalbuminaemia is associated with venous and arterial thromboembolism (7), but serum albumin levels in patients with and without thromboembolic events were not significantly different (5, 6). Decreased, normal or increased levels of antithrombin III have all been found in the NS (8). Therefore, it is very likely that there are additional mechanisms involved in the hypercoagulable state in NS.

Phosphatidylserine (PS), an anionic phospholipid, is usually confined to the inner leaflet of the cell membrane (9). It is externa-
lised to the outer membrane when apoptosis occurs or cells are activated. During this process, vesicles derived from the budding of cellular membranes may be released. These microparticles (MPs) are typically <1 μm in diameter and express PS and membrane antigens that reflect their cellular origin (10). The exposure of PS on the surface of cells and MPs provides binding sites for FXa and prothrombinase complexes and promotes thrombin formation (11). Various studies have demonstrated that increased circulating MP release and PS exposure on red blood cells (RBCs), platelets or acute promyelocytic leukaemia (APL) cells play a critical role in the thrombotic risk of many disorders such as diabetes mellitus, sickle cell disease, uraemia, essential thrombocythaemia and APL (12–16). However, whether the hypercoagulable state in NS is mediated by PS has not been assessed. We hypothesised that MP release and PS exposure on platelets/RBCs would be identified in NS patients and that these would contribute to the thromboembolic events of NS.

Lactadherin is a PS-binding glycoprotein that is secreted by macrophages and contains two lectin-type C domains that are homologous with the PS-binding domains of blood clotting factors FV and FVIII (17). Lactadherin interacts stereoselectively with phospho-L-serine of PS and binds to PS-containing membranes in a proportional manner. In contrast to that of annexin V, another PS-binding protein, lactadherin binding is independent of Ca²⁺ and membrane PE content (18). We have recently reported that lactadherin functions as a more sensitive probe for the detection of PS-positive RBCs, platelets or MPs than annexin V and could act as an anticoagulant by competing with FV and FVIII for membrane-binding sites (19, 20). Therefore, we used lactadherin to detect MP release and PS externalisation on RBCs or platelets of NS patients.

The objective of this study was to examine the PS exposure and MP release of RBC/platelet and evaluate their relevance to hypercoagulable state in NS.

Subjects, materials and methods

Study subjects

Patient samples were from 20 newly diagnosed NS patients (10 MCNS and 10 MN) who were admitted to the First Affiliated Hospital of Harbin Medical University between July 2010 and September 2011. The diagnosis of MCNS or MN was confirmed by renal biopsy before inclusion. Ten healthy subjects were included as normal controls. As risk factors for cardiovascular disease we excluded diabetes and hypertension. Other exclusion criteria were: malignant or systemic disease; pregnancy; iron, folic acid and vitamin B₁₂ deficiency; blood transfusion within the past six months; active or chronic infection; and any drug known to affect haemostasis. Patients were not receiving diuretic, steroids or immunosuppressive therapy before blood samples were obtained. Patients had been followed up for at least three months and only objectively verified symptomatic thromboembolic events were considered. DVT was confirmed by compression ultrasound; PE, by spiral computed tomography; RVT, by venography or Doppler ultrasound. The subjects’ profiles are shown in Table 1. This study was performed with permission from each participant and approval from the ethics committee of Harbin Medical University according to the Helsinki Declaration.

Materials

Calibrated polystyrene latex beads (1.0 μm) were from Sigma (UK). Truecount Tube (Cat. No. 340334), purified CD235a (clone GA-R2), CD31 (clone L133.1), CD41a (clone HIP8), CD142 (clone HFT-1) and mouse IgG1/IgG2a (clone X40/X39) were from Becton Dickinson Biosciences (San Jose, CA, USA), and all monoclonal antibodies were labelled in our laboratory with Alexa Fluoro 647 or Alexa Fluoro 488. Polyclonal antibody against human Tissue Factor (product No. 4502) was from American Diagnostica Inc. (Stamford, CT, USA). Alexa Fluoro 488 and Alexa Fluoro 647-conjugated lactadherin were prepared in our laboratory. Human factors Va, Vili, VIII, IXa, Xa, prothrombin, thrombin were all from Haematologic Technologies (Burlington, VT, USA). Tyrode's buffer containing 1 mM Hepes was from our laboratory and was filtered through a 0.22-μm syringe filter from Millipore (UK). Chromogenic substrates S-2765 and S-2238 were from DiaPharma Group (West Chester, OH, USA).

Protein purification and labelling

Bovine lactadherin was purified as previously described, and was labelled with Alexa Fluoro 488 or Alexa Fluoro 647 according to the package instructions. The ratio of fluorescein to lactadherin was 1.2/1 or 1.1/1 (21, 22).

Blood collecting, preparation of RBCs/platelets/MPs

Blood samples were drawn before therapy with a 21-gauge needle and were collected into a 5-ml tube containing 3.2% citrate (BD, Plymouth, UK). Platelet-rich plasma (PRP) and RBC were prepared within 30 minutes (min) of blood collection by centrifugation for 13 min at 200 x g at room temperature and were analysed immediately after isolation. Platelet-free plasma (PFP) was prepared as previously described (23). Briefly, samples were centrifuged 20 min at 1,500 g, and plasma was then harvested and centrifuged 2 min at 13,000 g to remove all residual platelets. PFP were snap-frozen in liquid nitrogen, and then stored at –80°C until use. In order to isolate the MPs, 250 μl of PFP was thawed on ice for 60 min and then centrifuged for 45 min at 20,000 x g at 20°C (24). Subsequently, 225 μl of supernatant (i.e. MP-free plasma) were removed. The remaining 25 μl MPs pellet was washed once and re-suspended in 75 μl of Tyrode's buffer.
Flow cytometric analysis of MPs phenotype

Five μl of MPs-enriched suspension was resuspended in 35 μl Tyrode’s buffer and incubated for 15 min at 4°C in the dark with Alexa Fluor 488-conjugated lactadherin (5 μl); Alexa Fluor 488-conjugated CD41a (5 μl); Alexa Fluor 647-conjugated CD31 (5 μl); Alexa Fluor 488-conjugated CD235a (5 μl)/Alexa Fluor 647-conjugated CD142 (5 μl); Alexa Fluor 488 or Alexa Fluor 647-conjugated IgG2a/IgG1 isotype controls (5 μl) together with beads (1 μm) in a Truecount Tube. Then, the suspension was diluted in 1 ml of Tyrode’s buffer and analysed immediately by flow cytometry (FACS Aria, Becton Dickinson). MPs were initially gated as those particles that were less than 1 μm in size in a plot of side scatter (logarithmic scale; x-axis) and forward scatter (logarithmic scale; y-axis) (25). To distinguish MPs from events due to exosome or noise, MPs were identified by lactadherin positivity. Briefly, these particles were then displayed on a plot of Alexa Fluor 488 fluorescence (logarithmic scale; x-axis) and side scatter (logarithmic scale; y-axis) for further gating. lactadherin-binding was used to numerate PS-expressing circulating MPs (15), whatever their cellular origin. RBC MPs (RMPs)/endothelial cell MPs (EMP)/platelet MPs (PMPs) were defined as smaller than 1 μm and lactadherin-CD235a, lactadherin-CD31/CD41a and lactadherin-CD41a, respectively. MPs exposing TF were lactadherin-CD142+. The number of MPs per μl of each type was calculated by Truecount Tube (with a precise number of fluorescent beads 48678) to determine the number of MPs in a sample) after accumulation of 10,000 gated events (26).

Flow cytometric analysis of PS exposure on RBCs/platelets

The exposure of PS on RBCs/platelets was measured by flow cytometry based on the binding of lactadherin. RBCs/platelets were adjusted to 0.5–1 x 10^6/ml to a final volume of 200 μl in Tyrode’s buffer, and then 5 μl Alexa Fluor 488-conjugated lactadherin was added to the cell suspension and incubated for 10 min at room temperature in the dark. Ten thousand events per sample were acquired and analysed with BD FACSDiva Software.

Confocal microscopy

A volume of 50 μl of RBC suspension (0.5–1 x 10^9/ml) was incubated with 10 μl lactadherin-Alexa Fluor 647 for 10 min at room temperature in the dark. After washing to remove unbound dye, the RBCs pellet was resuspended in 0.5 ml Tyrode’s buffer and analysed immediately. Observation of the PS exposure on platelets and MPs by confocal microscopy was carried out as previously described (20). Samples were excited with 488 or 568 nm emission lines of a krypton-argon laser, and narrow band pass filters were used for restricting emission wavelength overlap. Images were obtained in LSM 510 SYSTEM (Carl Zeiss Jena GmbH, Jena, Germany).

Procoagulant activity and inhibition assays of RBCs/platelets/MPs

PCA of RBCs/platelets/MPs was evaluated by one-stage recalcification time assay in a KC4A-coagulometer (Amelung, Labcon, Heppenheim, Germany). One hundred μl of RBC (1 x 10^8), platelet (1 x 10^7) or MPs-containing suspension (10 μl of MPs-enriched suspension was resuspended in 90 μl Tyrode’s buffer) was incubated with 100 μl of MP-free human plasma at 37°C. After 180 seconds, 100 μl of warmed 25 mM CaCl_2 was added to start the reaction and the clotting time was recorded. All clotting assays were performed in triplicate. For the inhibition assay of coagulation time, 50 μl lactadherin (final concentration 128 nM) or anti-TF (final concentration 40 μg/ml) was incubated with 100 μl cell or MP suspension for 10 min at 37°C. Clotting time was then recorded as above after addition of 100 μl MP-free human plasma and 50 μl of warmed 50 mM CaCl_2 (27).

Intrinsic, extrinsic FXa and prothrombinase formation and inhibition assays

The formation of intrinsic, extrinsic FXa and prothrombinase in the presence of RBCs/platelets was performed with a two-step amidolytic substrate assay using the following protocols. For the intrinsic FXa formation assay, a total of 2 x 10^6 platelets or 2 x 10^5

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Table 1: Baseline characteristics of patients with NS and healthy subjects at inclusion.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>MCNS</th>
<th>MN</th>
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<tbody>
<tr>
<td>Sex (male/female)</td>
<td>5/5</td>
<td>4/6</td>
<td>3/7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44.8 ± 14.4</td>
<td>44.7 ± 15.3</td>
<td>51.7 ± 5.2</td>
</tr>
<tr>
<td>Proteinuria (g/24 hours)</td>
<td>ND</td>
<td>5.9 ± 2.8</td>
<td>9.4 ± 3.0</td>
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<tr>
<td>Albumin (g/l)</td>
<td>43.1 ± 4.7</td>
<td>23.6 ± 7.3***</td>
<td>20.4 ± 4.6***</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>4.0 ± 0.5</td>
<td>8.9 ± 2.1***</td>
<td>9.0 ± 1.6***</td>
</tr>
<tr>
<td>HDL (mM)</td>
<td>1.5 ± 0.2</td>
<td>1.1 ± 0.5**</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>LDL (mM)</td>
<td>2.4 ± 0.6</td>
<td>7.5 ± 1.1***</td>
<td>7.7 ± 1.4***</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>0.7 ± 0.2</td>
<td>3.0 ± 1.9</td>
<td>3.1 ± 1.5</td>
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<td>Creatinine (μM)</td>
<td>70.5 ± 11.8</td>
<td>87.5 ± 19.0*</td>
<td>78.7 ± 16.4</td>
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<tr>
<td>BUN (mM)</td>
<td>6.1 ± 1.0</td>
<td>7.7 ± 3.0</td>
<td>5.9 ± 2.3</td>
</tr>
<tr>
<td>Platelet count (10^11/l)</td>
<td>235.9 ± 56.3</td>
<td>231.5 ± 84.4</td>
<td>240 ± 55.9</td>
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<tr>
<td>Thrombotic events</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Data are means ± SD, *P < 0.05, **P < 0.01, ***P < 0.001 vs. controls. *P < 0.05 vs. MCNS. HDL, High-density lipoprotein; LDL, low-density lipoprotein; BUN, blood urea nitrogen; ND, not determined.
RBCs were incubated with 1 nM FIXa, 130 nM FX, 5 nM FVIII, 0.2 nM thrombin and 5 mM Ca²⁺ in FXa buffer (1 ml 10x TBS, 200 μl 10% BSA, 8.8 ml ddH₂O) for 5 min at 25°C. The reaction was then stopped by the addition of EDTA to 7 mM final concentration. After the addition of 10 μl S-2765 (0.8 mM) to each reaction, the quantity of FXa formed was determined immediately at 405 nm in kinetic mode on a Universal Microplate Spectrophotometer (PowerWave XS; Bio-Tek, Winooski, VT, USA). Results were evaluated against the rate of substrate cleavage of a standard dilution of FXa. For the extrinsic FXa formation assay, 2 x 10⁴ platelets were incubated with 130 nM FX, 1 nM FVIIa and 5 mM Ca²⁺ for 5 min at 25°C. The reaction was quenched by EDTA and the amount of FXa formation was determined as described above. To measure the production of prothrombinase, platelets (2 x 10⁴) or RBCs (2 x 10⁵) were incubated with 1 nM FVa, 0.05 nM FXa, 1 μM prothrombin and 5 mM Ca²⁺ in prothrombinase buffer (1 ml 10x TBS, 50 μl 10% BSA, 8.95 ml ddH₂O) for 5 min at 25°C. The reaction was stopped using EDTA, and 10 μl S-2238 (0.8 mM) was added to each microplate. Thrombin production was measured against the rate of substrate cleavage from a standard dilution curve of thrombin. Inhibition of coagulation complexes by lactadherin was measured as follows. A total of 2 x 10⁴ platelets or 2 x 10⁵ RBCs were incubated with varying concentrations of lactadherin (0–128 nM) for 10 min at 25°C in Tyrode’s buffer. The mixture was then incubated with the specified clotting factors according to the above protocols. The quantity of thrombin or FXa formation was assessed as previously described (19).

**Statistical analysis**

Numerical variables were tested for normal distribution with the Kolmogorov-Smirnov test. Data are expressed as mean ± standard deviation (SD), and statistical analysis was made by t-test or ANOVA as appropriate. Categorical variables were compared using the Chi²-test. Linear regression analysis was used to detect any relation between MP levels and serum albumin, cholesterol and triglyceride. P < 0.05 was considered statistically significant.

**Results**

**Subject characteristics**

Clinical characteristics of NS patients and controls are shown in Table 1. NS patients had significantly higher cholesterol, triglycerides and lower serum albumin. A significant difference also existed in proteinuria between the two NS groups (Table 1). There was a trend toward a higher number of thromboembolic events in patients with MN; however, this did not reach statistical significance (p = 0.5 across groups).

**Number of MPs**

We first tested the total number of MPs and their phenotypic characterisation. A known count of larger beads (TrueCount beads, Becton Dickinson) acted as an internal standard and enabled us to calculate the absolute number of MPs per analysed volume of specimen (Fig. 1A). For most study subjects, more than 90% of events gated on P1 bound to lactadherin (Fig. 1B). The number of lactadherin+ MPs was significantly higher in each NS group [membranous nephropathy (MN, 4,191 ± 485/μl) and minimal change nephrotic syndrome (MCNS, 3,023 ± 337/μl)] than that in the controls (1,629 ± 252/μl). Furthermore, the number of MPs in MN patients is significantly higher than that in MCNS (p < 0.001; Table 2). The phenotypic characterisation of MPs in MN patients (Fig. 1) showed that the lactadherin+ MPs originated from platelets, endothelial cells (Fig. 1C) and RBCs (Fig. 1D). The number of RMPs/EMPs/PMPs in both NS groups was significantly higher than that in the controls. In addition, patients with MN also had significantly increased RMPs/EMPs/PMPs than those with MCNS (p < 0.001 for all; Table 2). Few MPs with coexpression of both PS and TF (tissue factor) could be identified in either NS patients or healthy controls (Fig. 1D, Table 2).

**Association of MPs with components of NS**

By linear regression analysis, the total amount of MPs, correlated with total cholesterol (r = 0.527, p < 0.05) and albumin (r = −0.633, p < 0.01) (Table 3). Following adjustment for these variances by analysis of covariance, the MPs counts were still significantly increased in NS compared with controls (p < 0.001).

**Measurement of lactadherin+ RBCs/platelets in healthy subjects and NS patients**

Based on the results from the MPs analysis, lactadherin was utilised to quantify and locate PS exposure of RBCs and platelets in each study group by flow cytometry and confocal microscopy. Consistent with the above MPs results, significantly increased PS exposure on RBCs/platelets was found in patients with NS compared with healthy subjects (Fig. 2). The absolute number of lactadherin+ RBC was significantly higher in MN and MCNS patients compared with healthy subjects [MN: 43.1 ± 11.7 x 10⁵/μl (p < 0.001 vs. controls), MCNS: 22.1 ± 8.9 x 10⁵/μl (p < 0.001 vs. controls), and controls: 2.2 ± 1.0 x 10⁵/μl, respectively]. In addition, lactadherin+ RBCs in MN was significantly higher than that in MCNS (p < 0.01) (Fig. 2A). In both NS groups, the absolute number of lactadherin+ platelets was significantly higher than that in healthy subjects [MN: 25.9 ± 6.3 x 10⁴/μl (p < 0.001 vs. controls), MCNS: 17.2 ± 7.5 x 10⁴/μl (p < 0.05 vs. controls), and controls: 9.6 ± 3.3 x 10⁴/μl, respectively]. Furthermore, patients with MN also had significantly increased lactadherin+ platelets than those with MCNS (p < 0.05) (Fig. 2B). To observe PS on the outer membrane of cells, RBCs/platelets of a control subject and an MN patient were incubated.
with Alexa Fluor 647 or 488-lactadherin and imaged on a confocal laser-scanning microscope. As shown in Figure 3A and C, almost no staining by Alexa Fluor 647 or 488-lactadherin could be detected on RBC or platelet membranes in healthy subjects, whereas a light red fluorescence on RBC (Fig. 3B) and a light green fluorescence on platelet accompanying vesiculation formation (Fig. 3D) indicated lactadherin binding in NS patients. These results further confirmed that there is increased MP release and PS exposure on RBCs/platelets in NS patients.

Procoagulant activity of RBCs, platelets and MPs in NS

To find the contribution of PS externalisation to hypercoagulable state in NS patients, PCA of RBCs/platelets/MPs from healthy individuals and NS patients was assessed by recalcification-time assays. The results showed that with identical numbers of platelets or RBCs, the coagulation time was significantly reduced in NS patients (p < 0.01) compared with controls with shorter coagulation time in MN than in MCNS (p < 0.05). Similar results were obtained using equal volumes of MPs (Fig. 4A). In order to explore the contribution of PS and TF to the PCA of RBCs/platelets/MPs in NS patients, we performed coagulation inhibition assays. PS and TF were blocked with lactadherin and anti-TF antibody respectively. PCA of RBCs/platelets/MPs was almost entirely inhibited by 128 nM lactadherin, whereas 40-μg/ml anti-TF did not affect the coagulation time of RBCs/platelets/MPs significantly (Fig. 4B).

Table 2: MPs numbers per microliter of plasma in MCNS, MN and controls.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>MCNS</th>
<th>MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total MPs (µl)</td>
<td>1,629 ± 252</td>
<td>3,023 ± 337***</td>
<td>4,191 ± 485###</td>
</tr>
<tr>
<td>MPs (µl) positive for:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD41a+</td>
<td>1,113 ± 168</td>
<td>2,238 ± 300***</td>
<td>3,413 ± 390###</td>
</tr>
<tr>
<td>CD235a+</td>
<td>23 ± 5</td>
<td>170 ± 19***</td>
<td>306 ± 29###</td>
</tr>
<tr>
<td>CD31+ CD41a-</td>
<td>11 ± 2</td>
<td>122 ± 19***</td>
<td>169 ± 15###</td>
</tr>
<tr>
<td>CD142+</td>
<td>2.4 ± 2.0</td>
<td>3.0 ± 2.2</td>
<td>2.9 ± 2.4</td>
</tr>
</tbody>
</table>

Corrected for the number of events with isotype controls, ***P < 0.001 vs. controls. ###P < 0.001 vs. MCNS. Microparticle, MP; MN, membranous nephropathy; MCNS, minimal change nephrotic syndrome.

Table 3: Relationships between level of MPs and blood parameters in NS patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>0.527</td>
<td>0.017</td>
</tr>
<tr>
<td>Albumin</td>
<td>-0.633</td>
<td>0.003</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.036</td>
<td>0.882</td>
</tr>
</tbody>
</table>

Values for r and P were obtained by the Pearson correlation test.

We further investigated the PCA of RBCs or platelets using intrinsic, extrinsic FXa and prothrombinase assays (Fig. 5A). The production of the three procoagulant enzyme complexes was increased in the NS groups and more so in MN (p < 0.001) compared...
with controls. Inhibition assays of procoagulant enzyme complex formation of RBCs/platelets were also performed. Data were normalised to facilitate comparison of the inhibition effect of lactadherin in the different assays. The amount of FXa or thrombin was significantly reduced by lactadherin in a dose-dependent manner, with lactadherin ultimately inhibiting production of the three procoagulant enzyme complexes up to 90% (Fig. 5B).

**Discussion**

In the present study, we first investigated the plasma level of circulating MPs in NS patients and found that circulating MPs levels are augmented as previously reported for individuals with coronary artery disease (28) or systemic lupus erythematosus (29). More important, we demonstrate here that circulating MPs in NS patients (either MCNS or MN) mostly derived from platelets, RBCs, and endothelial cells. When measuring the PS exposure on RBC and platelet plasma membranes, we found an elevated level of PS on RBCs and platelets, which has not been previously observed in NS. In addition, the increased PS exposure in RBCs/platelets supports the assembly of prothrombinase, intrinsic and extrinsic FXa.

Moreover, blocking of PS with lactadherin inhibits activity of pro-
Figure 4: Coagulation time and inhibition assay. A) Coagulation times of 100 μl RBCs (1 x 10^8)/platelets (1 x 10^7)/ MPs (10 μl of MPs-enriched suspension was resuspended in 90 μl Tyrode’s buffer) in each group were evaluated. RBCs/platelets from NS patient (especially MN) had more procoagulant activity than that from healthy donors. B) Coagulation times of RBCs/platelets/MPs of MN patients were detected in the absence or presence of 128 nM lactadherin or 40 μg/ml anti-TF antibody. Lactadherin almost entirely inhibited PCA supported by RBCs/platelets/MPs, whereas anti-TF antibody had no effect. Data are displayed as mean ± SD for triplicate samples of independent experiments (n = 10). P-values <0.05 were considered significant. NS, non-significant; MCNS, minimal change nephrotic syndrome; MN, membranous nephropathy; TF, tissue factor.

Figure 5: Formation and inhibition assays of procoagulant enzyme complexes. A) FXa and thrombin production of 2 x 10^5 RBCs or 2 x 10^4 platelets in each group are shown. Intrinsic FXa formation was measured in the presence of FIXa, FVIII and thrombin. Extrinsic FXa production was assessed in the presence of FVIIa. Thrombin generation was investigated in the presence of FXa and FVa. Results displayed are mean ± SD for triplicate samples of independent experiments (n = 10). P-values <0.05 were considered significant. B) The extrinsic Xa (○), intrinsic Xa (●) and thrombin (△) production of RBCs or platelets were measured in the presence of indicated concentrations of lactadherin. Each point represents mean ± SD for triplicate samples of independent experiments (n = 10). Lactadherin decreased activity of procoagulant enzyme complexes up to 90%. Data were normalised to the amount of formation without lactadherin for comparison of the extent of inhibition. MCNS, minimal change nephrotic syndrome; MN, membranous nephropathy.
coagulant enzyme complexes and consequently decreases the PCA of RBCs/platelets/MPs. These findings demonstrated that the hypercoagulability of NS was due to exposed PS on RBCs, platelets and MPs.

Circulating MPs and PS exposure indicate cell activation or apoptosis, both of which are present in NS. Several studies have shown increased platelet activation and aggregation (30), decreased RBC deformability, increased RBC aggregation and impairment (31). Our present findings of increased MP release and PS exposure on RBCs/platelets in NS patients seem to support the above opinions. We believe further studies are necessary to evaluate circulating MPs origin from leukocyte and especially kidney (e.g. podocyte MPs) in NS.

Dyslipidaemia is associated with endothelial cell injury, dysfunction and EMP release (32, 33). Moreover, blood cells are vulnerable to injury in hypoproteinaemia (31, 34). In our study, when considered in isolation, both dyslipidaemia and hypoalbuminaemia increased the level of MPs. However, we found that patients with NS have a higher level of MPs compared with healthy subjects, even after adjustment for these important clinical variables. These indicate that other mechanisms specific for NS itself (e.g. immune complexes) may contributes to the increased MPs because NS is a disease characterised by increased levels of immune complexes, which may contributes to cell immunologic injury (35, 36). The mechanisms responsible for the increased MPs in NS require further study and elucidation. However, we detected the PS exposure on RBC/platelet membrane in NS patients and found a significantly higher PS exposure than healthy subjects. Because exposure of PS on RBCs/platelets seems to be required for MP release (37), our subsequent findings of increased PS on RBCs/platelets could further explain the elevated PMPs or RMPs in NS patients.

An increased propensity to have exposed PS on the outer membrane leaflet of RBCs/platelets/MPs and the relevance of PCA has been shown in patients with various prothrombotic disorders (12–16). However, the role that exposed PS plays in the NS hypercoagulable state is not clear. Because diuretic, steroids or immunosuppressive agents may participate in the thrombophilia of NS (6), we selected newly diagnosed NS patients. In our study, the increased exposure of PS on the surface of platelets/RBCs/MPs provides binding sites for a number of activated clotting factors including the FXa and prothrombinase complexes, and thus promoting the coagulation cascade reaction and subsequently leading to a dramatic increase in thrombin generation. In addition, elevated levels of FV, FVIII, and fibrinogen have been described in NS patients (38). We think that elevated PS exposure accelerates activation of these coagulant factors and production of thrombin. Furthermore, the increased thrombin may play a critical role in converting elevated fibrinogen to fibrin in NS patients. In our present report, RBCs and platelets of NS patients were pre-incubated with lactadherin, which has a propensity for PS binding and inhibited over 90% of the three procoagulant enzyme activities in a dose-response manner. By blocking PS, lactadherin restored coagulation times of RBCs/platelets/MPs to control levels. We performed experiments to assess whether TF was involved in the increased PCA of RBCs, platelets and MPs. Anti-TF antibody did not affect the procoagulant activity of RBCs/platelets/MPs because RBC do not express TF and platelet expression of TF is still uncertain (39); moreover, we did not find MPs expressing TF by flow cytometry analysis. Therefore, strong evidence demonstrates that the majority of PCA is PS-dependent. Our present study not only showed increased PS exposure on RBCs/platelets but also confirmed an RBC/platelet pathological procoagulant phenotype, which may be a factor inducing a hypercoagulable state in NS.

In our study, we used lactadherin to quantify and localise PS exposure on RBCs/platelets/MPs. Lactadherin is an effective PS-staining reagent because using this reagent more PS-rich platelets/ RBCs/MPs were detected by confocal microscopy and flow cytometry in NS. Additionally, a low but measurable percentage of lactadherin-positive platelets/RBCs from healthy subjects were found in agreement with previous studies (20, 40). Our results also indicate that lactadherin is an efficient anticoagulant that works by blocking PS on RBCs/platelets/MPs to inhibit PCA, as certified in inhibition assays of coagulation. The PS-blocking ability of lactadherin suggests that it may be useful in preventing blood clotting or regulating the balance between procoagulant and anticoagulant phenotypes. It is necessary to further study the possibility of applying lactadherin in thrombotic complications of NS.

In summary, this is the first study to show that loss of RBCs/platelets membrane phospholipid asymmetry with increased PS exposure and MP release contributes to the hypercoagulable state of NS patients, and thus, provides additional evidence for the thrombosis in patients with NS. Alternately, PS appears to be a new marker or predictor of thromboembolic complications in NS.

What is known about this topic?
- Blood cell activation or injury are present in nephrotic syndrome (NS) patients.
- Loss of RBCs/platelets membrane phospholipid asymmetry with increased phosphatidylserine (PS) exposure and microparticles (MPs) release contribute to the hypercoagulable state of NS patients.

What does this paper add?
- The total numbers of circulating MPs is augmented in NS patients compared with that in healthy subjects and mostly derived from platelets, RBCs, and endothelial cells.
- A new mechanism of thrombophilia of the NS patients: Increased RBCs/platelets PS exposure and MPs release supports the assembly of procoagulant enzyme complexes and resulted in increased procoagulant activity (PCA), moreover, blockade of PS decreases the PCA of RBCs/platelets/MPs in NS patients.
- Lactadherin functioned as a novel probe for the detection of PS exposure and MPs release and as an efficient anticoagulant inhibited intrinsic factor Xase, extrinsic factor Xase, and prothrombinase formation through competing for PS binding sites with factor V and VIII.
Abbreviations
NS, nephrotic syndrome; MP, microparticle; PS, phosphatidylserine; PCA, procoagulant activity; MN, membranous nephropathy; MCNS, minimal change nephrotic syndrome; DVT, deep-vein thrombosis; PE, pulmonary embolism; RVT, renal vein thrombosis; TF, tissue factor; RMPs, RBC MPs; EMPs, endothelial cell MPs, PMFs, platelet MPs; BSA, bovine serum albumin; BUN, blood urea nitrogen; ND, not determined; NS, non-significant.

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Conflicts of interest
None declared.

References