Increased blood cell phosphatidylserine exposure and circulating microparticles contribute to procoagulant activity after carotid artery stenting

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OBJECTIVE Phosphatidylserine (PS) is a major component of the inner leaflet of membrane bilayers. During cell activation or apoptosis, PS is externalized to the outer membrane, providing an important physiological signal necessary for the release of the microparticles (MPs) that are generated through the budding of cellular membranes. MPs express PS and membrane antigens that reflect their cellular origin. PS exposure on the cell surface and the release of MPs provide binding sites for factor Xa and prothrombinase complexes that promote thrombin formation. Relatively little is known about the role of PS exposure on blood cells and MPs in patients with internal carotid artery (ICA) stenosis who have undergone carotid artery stenting (CAS). The authors aimed to investigate the extent of PS exposure on blood cells and MPs and to define its role in procoagulant activity (PCA) in the 7 days following CAS.

METHODS The study included patients with ICA stenosis who had undergone CAS (n = 70), matched patients who had undergone catheter angiography only (n = 30), and healthy controls (n = 30). Blood samples were collected from all patients just before the procedure after an overnight fast and at 2, 6, 24, 48, and 72 hours and 7 days after the CAS procedure. Blood was collected from healthy controls after an overnight fast. Phosphatidylserine-positive (PS+) MPs and blood cells were analyzed by flow cytometry, while PCA was assessed with clotting time analysis, purified coagulation complex assays, and fibrin formation assays.

RESULTS The authors found that levels of PS+ blood cells and PS+ blood cell–derived MPs (platelets and platelet-derived MPs [PMPs], neutrophils and neutrophil-derived MPs [NMPs], monocytes and monocyte-derived MPs [MMPs], erythrocytes and erythrocyte-derived MPs [RMPs], and endothelial cells and endothelial cell–derived MPs [EMPs]) were increased in the 7 days following the CAS procedure. Specifically, elevation of PS exposure on platelets/PMPs, neutrophils/NMPs, and monocytes/MMPs was detected within 2 hours of CAS, whereas PS exposure was delayed on erythrocytes/RMPs and EMPs, with an increase detected 24 hours after CAS. In addition, PS+ platelets/PMPs peaked at 2 hours, while PS+ neutrophils/NMPs, monocytes/MMPs, and erythrocytes/RMPs peaked at 48 hours. After their peak, all persisted at levels above baseline for 7 days post-CAS. Moreover, the level of PS+ blood cells/MPs was correlated with shortened coagulation time and significantly increased intrinsic and extrinsic Xase, thrombin generation, and fibrin formation. Pretreatment of blood cells with lactadherin at their peak time point after CAS blocked PS, resulting in prolonged coagulation times, decreased procoagulant enzyme activation, and fibrin production.

CONCLUSIONS The results of this study suggest that increased exposure of PS on blood cells and MPs may contribute to enhanced PCA in patients with ICA stenosis who have undergone CAS, explaining the risk of perioperative thrombo-
Carotid artery stenosis is known as a main cause for the development of ischemic stroke and therefore became a pivotal target for primary and secondary prevention of stroke. Carotid artery stenting (CAS), a less invasive technique than carotid endarterectomy, has become one of the major treatment modalities for internal carotid artery (ICA) stenosis in recent years. With the use of periprocedural management, including dual antiplatelet therapy and distal protection, rates of thromboembolism following CAS are low. However, compared with carotid endarterectomy, CAS is associated with higher periprocedural complications such as stent thrombosis, restenosis, and transient or permanent neurological deficits due to distal thrombus embolization. These complications can be debilitating and even fatal. Thus, understanding the pathophysiological mechanisms of thrombotic complications is important for early detection and intervention. Recent reports have shown that higher levels of leukocyte-derived MPs and endothelial cell–derived MPs (EMPs) can be used to predict plaque instability. However, the precise molecular and cellular mechanism involved in this hypercoagulable state has not yet been determined.

Phosphatidylserine (PS), a negatively charged phospholipid normally localized on the cytoplasmic face of cell membranes, is preferentially exposed on the cell surface during certain physiological processes, including cell apoptosis and activation. The exposure of PS serves as a scaffold for the activation of clotting factors such as thrombin and factor Xa, thus acting as a key catalyst in the coagulation cascade. Nevertheless, to date, it is still unclear whether PS takes part in the pathophysiological process after CAS. Previous studies have shown that angiographic procedures and angioplasty in peripheral arteries lead to activation and inflammation of the endothelium. Following CAS, increases in P-selectin, von Willebrand factor, and endothelin-1 occur, suggesting the activation of platelets and endothelial cells. However, whether PS is exposed on blood cells in response to CAS and whether increases in PS exposure on these cells contribute to the coagulation cascade remain to be evaluated.

Cell activation is accompanied by the release of microparticles (MPs), which are small vesicles (diameter between 0.1 and 1 μm) that display the specific antigens of their original cells and expose PS because of a lack of membrane asymmetry. MPs released by activated or apoptotic cells (such as endothelial cells, leukocytes, and platelets) have been reported to be sensitive markers of atherothrombotic disease progression and potential effectors of vascular dysfunction. Additional studies have suggested that MPs can be used to measure the severity of damage in vascular diseases. There have been only 3 studies to date that have investigated MPs in relation to carotid artery disease plaques. These reports have shown an increase in platelet-derived MPs (PMPs) in carotid atherosclerosis, with higher levels of annexin V+ MPs associated with advanced carotid disease. They have also suggested that levels of leukocyte-derived MPs and endothelial cell–derived MPs (EMPs) can be used to predict plaque instability. However, the serial changes in levels of circulating MPs of various origins and their role in procoagulant activity (PCA) after CAS are still unknown.

The purpose of this study was to investigate the role of PS exposure on blood cells and MPs in the pathogenesis of the hypercoagulable state in patients with ICA stenosis who have undergone CAS. We explored the changes in PS exposure on blood cells and MPs following CAS and the resulting PS-dependent PCA. We used lactadherin to detect PS externalization on cells and MPs in patients who had undergone CAS. Lactadherin binds stereospecifically to phospho-δ-serine, independent of Ca²⁺ concentration and membrane phosphatidylethanolamine content. Studies have shown that lactadherin functions as a more sensitive probe for the detection of PS-positive (PS+) cells or MPs than annexin V and acts as an anticoagulant by competing with coagulation factor V and factor VIII for membrane binding sites.

**Methods**

**Patients**

We enrolled 70 patients with ICA stenosis who had undergone CAS between February 2015 and February 2016. As controls, we also included 30 age-matched healthy subjects and 30 matched patients who had undergone digital subtraction angiography (DSA) but not stenting. All patients were initially evaluated at the First Hospital of Harbin Medical University. After admission, all patients received antiplatelet therapy and routine care. CAS was performed in asymptomatic patients with > 70% stenosis and in symptomatic patients with > 50% stenosis. Patients with prolonged baseline activated partial thromboplastin time (aPTT), malignant tumors, infectious diseases, connective tissue diseases, severe renal insufficiency (creatinine > 2 mg/dl), or acute inflammatory diseases or those who had undergone prior CAS or carotid endarterectomy procedures were excluded. Peripheral blood was collected in healthy controls after an overnight fast and from patients undergoing CAS just before the procedure after an overnight fast and at 2, 6, 24, 48, and 72 hours and 7 days after stent implantation; blood was collected from matched patients before and at 2, 6, and 24 hours after DSA only. The procedure protocol was approved by the institutional ethical committee.
local ethics committee of Harbin Medical University according to the Helsinki declaration, and written informed consent was obtained from all study participants.

Materials

Calibrated polystyrene latex beads (1.0 μm) were purchased from Sigma-Aldrich. The Trucount Tube, purified CD235a (clone GA-R2), CD31 (clone L133.1), CD41a (clone HIP8), CD14, CD142 (clone HFT-1), CD66b (clone G10F5), CD3 (clone 1F4), CD19 (clone A3-B1), and mouse IgG1/IgG2a (clone x40/X39) were from Becton Dickinson Biosciences, and all monoclonal antibodies were labeled in our laboratory with Alexa Fluor 647 or Alexa Fluor 488. Polyclonal antibody against human tissue factor was from American Diagnostica Inc. Alexa Fluor 488– and Alexa Fluor 647–conjugated lactadherin were prepared in our laboratory. Human factors Vα, VIIα, VIIIα, IXα, Xα, prothrombin, and thrombin were all from Haematologics Technologies. Tyrode’s buffer, containing 1 mM HEPES, was prepared in our laboratory and filtered through a 0.22-μm syringe filter from EMD Millipore. Chromogenic substrates S-2765 and S-2238 were from DiaPharma Group. Percoll cell separation medium was from GE Healthcare.

Protein Purification and Labeling

Lactadherin was purified from bovine milk and labeled with Alexa Fluor 647 or Alexa Fluor 488, as previously described. The ratio of fluorescein to lactadherin was 1:2.1 or 1:1.

Blood Collection and Preparation of Platelets, Leukocytes, Red Blood Cells, and MPs

Blood samples were drawn with a 21-gauge needle and collected into a 5-ml tube containing 3.2% citrate. Platelet-rich plasma was prepared by centrifugation (200g, 13 minutes, room temperature) within 20 minutes of blood collection and was analyzed immediately after isolation. Leukocytes were immediately separated using a differential centrifugation gradient (with Percoll/Ficoll), as described elsewhere. To prepare platelet-free plasma, samples were centrifuged first for 20 minutes at 1500g and then again for 2 minutes at 13,000g to remove all residual platelets. Platelet-free plasma samples were snap frozen in liquid nitrogen and then stored at −80°C until use. To isolate MPs, 250 μl of platelet-free plasma was thawed on ice for 60 minutes and then centrifuged for 45 minutes at 20,000g at 20°C. The MP-free supernatant (225 μl) was removed, and the remaining 25 μl of MP pellet was washed once and resuspended in 75 μl of Tyrode’s buffer.

Flow Cytometric Analysis of MPs

MPs were identified as previously reported. Red blood cell (RBC)–, (RMPs), platelet- (PMPs), neutrophil- (NMPs), monocyte- (MMPs), T or B lymphocyte–, and endothelial cell–derived MPs (EMPs) were defined as events smaller than 1 μm and as lactadherin+ CD235a+, lactadherin+ CD41a+, lactadherin+ CD66b+, lactadherin+ CD14+, lactadherin+ CD3/19+, or lactadherin+ CD31+ CD41a–, respectively. MPs exposing tissue factor were identified as those with coexpression of lactadherin and CD41a. The amount of each type of MP per microliter was calculated using a Trucount Tube (containing 48,678 beads) after an accumulation of 10,000 gated events. The number of MPs per microliter of plasma (n) was calculated using the following formula: 

\[ n = \frac{C \times \text{beads}_{\text{added}}}{\text{beads}_{\text{counted}}} \]

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Zhao and colleagues present their study’s Class I status, these hypotheses require, respectively, well-designed, prospective, diagnostic, and therapeutic trials in CAS patients before clinicians can act upon these results.
Flow Cytometric Analysis of PS Exposure on Blood Cells

Lactadherin was used as a probe to measure exposed PS on blood cells by flow cytometry. The concentration of platelets, leukocytes, or RBCs was adjusted to 0.5–1 × 10^6 cells/ml and diluted to a final volume of 200 μl in Tyrode’s buffer. Alexa Fluor 488–conjugated lactadherin (5 μl) was then added to the cell suspension and incubated for 10 minutes at room temperature in the dark. Ten thousand events per sample were acquired and analyzed with BD FACSDiva software.

PCA and Inhibition Assays of Platelets, Leukocytes, RBCs, and MPs

The PCA of platelets, leukocytes, RBCs, and MPs was evaluated using a 1-stage recalcification clotting time assay in a KC4A coagulometer. A total of 100 μl of platelets (10^4), leukocytes (10^5), RBCs (10^6), or MP-containing suspension (10 μl of MP-enriched suspension diluted in 90 μl of Tyrode’s buffer) was incubated with 100 μl of MP-free human plasma at 37°C. After 3 minutes, 100 μl of warmed 25-mM CaCl₂ was added to start the reaction and the clotting time was recorded. All clotting assays were performed in triplicate. Inhibition assays were performed using samples from the time point at which the number of MPs peaked (2 hours for platelets and 48 hours for the other cell types) based on a previous analysis. For those assays, 50 μl of lactadherin (final concentration 128 nM) was incubated with 100-μl cell or MP suspension for 10 minutes at 37°C. The clotting time was then recorded as above, after the addition of 100 μl of MP-free human plasma and 50 μl of warmed 50-mM CaCl₂.

Intrinsic and Extrinsic Factor Xa, Prothrombinase Formation, and Inhibition Assays

We performed factor Xase and prothrombinase activity assays on all samples and inhibition assays at the peak time point. The formation of intrinsic factor Xa in the presence of cells or MPs was performed as follows. A total of 10^4 platelets or leukocytes, 10^5 RBCs, or 10 μl of MP-enriched suspension was incubated with 1 nM factor IXa, 130 nM factor X, 5 nM factor VIII, 0.2 nM thrombin, and 5 mM Ca²⁺ in factor Xa buffer (10 ml of 1× Tris-buffered saline [TBS] with 0.2% bovine serum albumin [BSA]) for 5 minutes at 25°C. The reaction was stopped by the addition of EDTA to a final concentration of 7 mM. The amount of factor Xa was determined by measuring absorbance at 405 nm using a Universal Microplate Spectrophotometer (PowerWave XS, BioTek Instruments Inc.) in kinetic mode immediately after the addition of 10 μl of the chromogenic reagent S-2765 (0.8 mM). The results were evaluated against the rate of substrate cleavage for a standard dilution of factor Xa. Extrinsic factor Xa formation was measured in the same way except that cells or MP-enriched suspension was incubated with 130 nM factor X, 1 nM factor VIIa, and 5 mM Ca²⁺. For the prothrombinase assay, the samples were mixed with 1 nM factor Va, 0.05 nM factor Xa, 1 μM prothrombin, and 5 mM Ca²⁺ in prothrombinase buffer (10 ml of 1× TBS with 0.05% BSA), and the amount of thrombin produced was measured using the chromogenic substrate S-2238. The concentration of thrombin was calculated from the absorbance per minute measurements using a standard dilution curve. To test the inhibition of coagulation complexes by lactadherin, the various cell types (platelets, leukocytes, RBCs, and MPs) were pre-incubated with lactadherin (128 nM) at their peak time point after CAS for 10 minutes 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 factor Xa formation was assessed as previously described.

Fibrin Formation Assays

Fibrin formation was quantified by turbidity, as previously described. Briefly, isolated MPs and blood cells were added to recalcified (10 mM, final) MP-depleted plasma (88% MP-depleted plasma, final) in the absence or presence of 128 nM lactadherin. Fibrin production was measured by turbidity at 405 nm in a SpectraMax 340PC plate reader.

Confocal Microscopy

Confocal microscopy was used to localize PS on the different cell types. Platelets, leukocytes, and RBCs from patients with ICA stenosis were incubated with Alexa Fluor 488–lactadherin (final concentration of 4 nM) for 10 minutes at room temperature in the dark. Cells were then washed to remove unbound dye and were imaged immediately. Observation of PS exposure on platelets and MPs was performed as previously described. To observe the location of coagulation factor binding sites, MPs were co-stained with factor Va-fluorescein-maleimide and factor Xa-EGRck-biotin (complexed to Alexa Fluor 647–streptavidin). To observe the contribution of MPs to fibrin formation at 48 hours, MP-containing suspensions (25 μl of MPs, washed twice and resuspended in 75 μl of Tyrode’s buffer) were incubated with plasma (platelet-poor plasma, centrifuged at 106,000 g for 1 hour at 4°C) and 3 mM calcium. Fibrin networks were imaged using Alexa Fluor 647–conjugated anti-fibrin. Background signal was calculated using a similarly labeled, isotype-matched control antibody. In addition, fibrin formation was quantified by turbidity as described. Isolated MPs were added to recalcified (10 mM, final) MP-depleted plasma (88% MP-depleted plasma, final) in the absence or presence of 128 nM lactadherin. Fibrin formation was measured by turbidity at 405 nm in a SpectraMax 340PC plate reader.

Statistical Analysis

Numerical variables were tested for normal distribution with the Kolmogorov-Smirnov test. Data are expressed as the mean ± standard deviation (SD) and were statistically analyzed for significance using a Student t-test or ANOVA as required. Categorical variables were compared using the chi-square test. Line correlation analysis was used to detect any relation between PS+ blood cells/MPs and stenosis degree and d-dimer. A p value < 0.05 was considered statistically significant.
Results

Subject Characteristics

Clinical characteristics in patients just before (pre-CAS) and 48 hours after (post-CAS) the CAS procedure, in matched controls who underwent DSA only, and in healthy controls are shown in Table 1. The 3 groups were homogeneous with respect to major baseline demographics and clinical or laboratory characteristics. Levels of total cholesterol, LDL, fibrinogen, and d-dimer were significantly higher post-CAS than just before the CAS procedure or in the DSA group. Compared with controls, patients with ICA stenosis had no significant difference in clinical and laboratory characteristics.

PS Exposure on Peripheral Blood Cells

We used flow cytometry to measure the extent of PS exposure in cells from healthy controls and patients for 7 days following CAS. At baseline (0 hours), PS exposure on platelets, neutrophils, and monocytes was significantly higher for patients who had undergone CAS than in control subjects (p < 0.001, respectively; Fig. 1A and B). Following CAS, PS exposure increased on platelets, neutrophils, and monocytes (p < 0.001, respectively) at 2 hours as compared with baseline, while PS exposure on RBCs did not significantly increase until 24 hours. PS exposure on platelets decreased after 2 hours, whereas PS on neutrophils, monocytes, and RBCs all peaked 48 hours after the CAS procedure. However, the level of PS exposure remained significantly greater than baseline at Day 7 (p < 0.001). Additionally, the changes in the absolute number of PS+ blood cells were similar to the percentages of PS exposure (Table 2). We also found that cellular PS levels at corresponding time points within 24 hours (all p < 0.05) were markedly higher in patients who had undergone CAS than in those who had undergone DSA only (Fig. 1H).

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics of patients with ICA stenosis undergoing CAS, matched controls undergoing DSA, and healthy subjects at study inclusion</th>
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<tr>
<td>Characteristic</td>
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<tr>
<td>Age in yrs</td>
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<tr>
<td>M/F sex</td>
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<tr>
<td>Total cholesterol (mmol/L)</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
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<tr>
<td>LDL (mmol/L)</td>
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<tr>
<td>HDL (mmol/L)</td>
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<tr>
<td>CRP (mg/L)</td>
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<tr>
<td>Erythrocytes (×10^12/L)</td>
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<tr>
<td>Leukocytes (×10^9/L)</td>
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<tr>
<td>% Neutrophils</td>
</tr>
<tr>
<td>% Monocytes</td>
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<tr>
<td>Platelets (×10^10/L)</td>
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<tr>
<td>PT (sec)</td>
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<tr>
<td>aPTT (sec)</td>
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<tr>
<td>Fibrinogen (g/L)</td>
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<tr>
<td>d-Dimer (mg/L)</td>
</tr>
<tr>
<td>Smoker</td>
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<tr>
<td>Diabetes mellitus</td>
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<tr>
<td>Hypercholesterolemia</td>
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<td>Hypertension</td>
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<td>Stenosis degree</td>
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<td>Cerebrovascular disease</td>
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<tr>
<td>Asymptomatic disease</td>
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<tr>
<td>Transient ischemic attack</td>
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</table>

CRP = C-reactive protein; HDL = high-density lipoprotein; LDL = low-density lipoprotein; n = number of subjects; NA = not available; p = pre-CAS versus control; p = post-CAS versus pre-CAS; p = post-CAS versus DSA group; post-CAS = postprocedural (48 hours after CAS); pre-CAS = preprocedural (just before CAS); PT = prothrombin time.

Data expressed as the means ± SD, unless indicated otherwise. p < 0.05 was considered statistically significant.
sue factor–positive (TF+) blood cells showed no significant increase as compared with baseline (data not shown).

To visualize PS exposure, samples of platelets, leukocytes, and RBCs were incubated with Alexa Fluor 488–lactadherin or propidium iodine (PI) (for leukocytes) and imaged using a confocal laser scanning microscope. As

FIG. 1. Flow cytometry and confocal microscopy of PS exposure on blood cell membranes. Comparison of PS exposure on blood cells from patients who had undergone CAS or DSA only and in healthy subjects. Cells were incubated with Alexa Fluor 488–lactadherin in the dark for 10 minutes at room temperature before flow cytometric analysis. Changes in the percent of lactadherin binding for various cell types were measured in healthy subjects (n = 30) and in ICA stenosis patients after CAS (n = 70): platelets (PLT) and RBCs (A) and polymorphonuclear leukocytes (PMN) and mononuclear cells (MNC) (B). Confocal microscopy of PS exposure on the plasma membrane of blood cells (C–G). Platelets, RBCs, or white blood cells (WBCs) from healthy subjects or ICA stenosis patients at 2 hours (platelets), 24 hours (RBCs), or 2 hours (WBCs) after the CAS procedure were incubated with Alexa Fluor 488–lactadherin or PI in the dark for 10 minutes at room temperature. Cells were then washed very gently to remove unbound dye. Cell membranes display green fluorescence when labeled with lactadherin, and nuclei display red fluorescence. Lactadherin staining (green) is observed on platelet membranes and MPs (white arrowheads; white arrow indicates increased degree of PS staining) (D) and on RBCs (yellow arrowhead) (F) and WBCs (G), but there is no staining in healthy subjects (C and E). Bar = 5 mm. Percent changes in lactadherin binding were measured in blood cells from patients in the DSA group (n = 30) and in those who had undergone CAS (H). Data displayed as mean ± SD. *p < 0.001 versus controls; **p < 0.05 versus 0 hours; #p < 0.05, CAS versus DSA group. Ctr = healthy control; h = hours.
shown in Fig. 1C–G, Alexa Fluor 488–lactadherin staining was not detected on platelet or RBC membranes from the healthy subject, while robust staining was observed on platelets, particularly on extended pseudopods and shed MPs, in the ICA stenosis patient 2 hours post-CAS. Light green fluorescence was also observed around the RBC membrane at 24 hours post-CAS. Notably, leukocytes also exhibited co-staining with lactadherin and PI 2 hours after the CAS procedure. These results confirmed that there is increased PS exposure on blood cells in patients who have undergone the CAS procedure.

### Number and Cellular Origin of MPs

Flow cytometry was used to measure the total number of MPs and their phenotypic characteristics. Our analysis showed that MPs could be derived from platelets (PMPs), endothelial cells (EMPs), monocytes (MMPs), neutrophils (NMPs), and RBCs (RMPs). Our data show the time

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Erythrocyte (%10^10/L)</th>
<th>Platelet (%10^9/L)</th>
<th>Neutrophil (%10^8/L)</th>
<th>Monocyte (%10^8/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>1.77 ± 0.66</td>
<td>1.2 ± 0.14</td>
<td>2.78 ± 0.13</td>
<td>0.10 ± 0.02</td>
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<tr>
<td>CAS patients: time after procedure</td>
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<tr>
<td>0 hrs</td>
<td>3.13 ± 0.61</td>
<td>4.16 ± 0.44*</td>
<td>5.48 ± 0.27*</td>
<td>0.32 ± 0.01*</td>
</tr>
<tr>
<td>2 hrs</td>
<td>3.34 ± 1.34</td>
<td>24.66 ± 0.63†</td>
<td>6.84 ± 2.60†</td>
<td>0.46 ± 0.14†</td>
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<tr>
<td>6 hrs</td>
<td>4.22 ± 0.10</td>
<td>20.42 ± 0.90†</td>
<td>8.22 ± 3.11†</td>
<td>0.61 ± 0.18†</td>
</tr>
<tr>
<td>24 hrs</td>
<td>9.73 ± 0.49†</td>
<td>16.62 ± 1.58†</td>
<td>11.93 ± 2.73†</td>
<td>0.70 ± 0.20†</td>
</tr>
<tr>
<td>48 hrs</td>
<td>19.49 ± 5.57†</td>
<td>13.49 ± 1.68†</td>
<td>13.31 ± 4.86†</td>
<td>0.81 ± 0.24†</td>
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<tr>
<td>72 hrs</td>
<td>9.3 ± 0.14†</td>
<td>6.18 ± 2.22†</td>
<td>13.01 ± 2.98†</td>
<td>0.64 ± 0.19†</td>
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<tr>
<td>7 days</td>
<td>8.17 ± 0.45†</td>
<td>5.98 ± 0.90†</td>
<td>8.67 ± 1.99†</td>
<td>0.52 ± 0.13†</td>
</tr>
</tbody>
</table>

* p < 0.05 versus control.
† p < 0.05 versus 0 hours.

**FIG. 2.** Flow cytometric analyses of PS exposure on various cells from healthy individuals (white bars) and their dynamic changes within 7 days following CAS (gray bars). The number of total PS+ MPs (A) and MPs derived from various origins were calculated: platelets (B), neutrophils (C), monocytes (D), erythrocytes (E), and endothelial cells (F). Data are shown as the mean ± SD. *p < 0.05 versus controls; **p < 0.001 versus 0 hours.
course of MP release during the week following CAS in patients with ICA stenosis. Total PS+ MPs increased significantly at 2 hours after CAS (p < 0.0001), peaked at 48 hours, and remained elevated for 1 week (p < 0.0001; Fig. 2A). Interestingly, we found that the changes in PS+ PMPs, NMPs, MMPs, and RMPs are consistent with the percentage of PS+ platelets, neutrophils, monocytes, and RBCs (Fig. 2B–E). Elevation of EMPs was not seen until 24 hours (p < 0.05, compared with 0 hours) and continued to increase over the time course (Fig. 2F). Compared with controls, patients at baseline (0 hours) had higher numbers of total PS+ MPs, PMPs, NMPs, and MMPs. The numbers of CD142+ TF+ MPs, T cell (CD3+)–derived MPs, and B cell (CD19+)–derived MPs showed no significant changes (data not shown). Additionally, the number of PS+ MPs of all types was higher after CAS than DSA (p < 0.05; Fig. 3).

**PS+ Blood Cells and MPs in Asymptomatic and Symptomatic Patients Undergoing CAS**

As shown in Table 3, we found that the levels of PS+ platelets, polymorphonuclear leukocytes (PMNs), mononuclear cells (MNCs), and MPs were higher in symptomatic patients than in asymptomatic patients at baseline (p < 0.05). However, levels of PS+ blood cells and MPs were not significantly different between asymptomatic and symptomatic patients post-CAS, suggesting the CAS procedure has the same effect on PS in symptomatic and asymptomatic patients.

As anticipated, PS+ blood cells and MPs of patients with peri-procedural complications (occurring within 48 hours after CAS) were all higher than those in patients without complications (Table 4). Relationships between PS+ blood cells/MPs and stenosis degree and D-dimer were analyzed. We found that stenosis degree had a significant positive correlation with levels of PS and that d-dimer had a positive correlation with PS+ platelets, RBCs, and MPs (Table 5).

**PCA of Platelets, Leukocytes, RBCs, and MPs**

We found levels of PS in the DSA group to be lower than those in patients who had undergone CAS and that levels are reduced to baseline levels within 24 hours. We assessed coagulation time following CAS by using a recalcification clotting time assay. At all time points, patient platelets, leukocytes, and MPs showed significantly shorter coagulation times compared with baseline (p < 0.001; Fig. 4A). In contrast, RBC coagulation times did not decrease until after 24 hours post-procedure (p < 0.001). Platelet coagulation time was lowest at 2 hours while the coagulation time of leukocytes, RBCs, and MPs was lowest at 48 hours,

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**FIG. 3.** Flow cytometric analyses of MPs in patients who had undergone DSA only or CAS. The number of total PS+ MPs (A) and MPs derived from various origins was calculated: platelets (B), neutrophils (C), monocytes (D), erythrocytes (E), and endothelial cells (F). Data are shown as the mean ± SD. *p < 0.05 versus 0 hours, DSA group; #p < 0.05, CAS versus DSA group.
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thus inversely correlating with the percentage of PS+ cells
seen by flow cytometry. Additionally, the coagulation
times of CAS patients’ platelets, leukocytes, and MPs at
baseline were lower than those found in healthy controls
(p < 0.001; Fig. 4B). We further investigated PCA using
intrinsic factor Xa, extrinsic factor Xa, and prothrombinase
assays. The activity of procoagulant enzyme complexes
on platelets, leukocytes, and MPs at baseline was signifi-
cantly higher than that in controls (p < 0.001). The changes
in intrinsic/extrinsic factor Xase and prothrombinase activi-
ties of blood cells and MPs were consistent overall with
the changes of PS exposure after CAS (Fig. 5A–D).

Inhibition assays were performed using the time point
at which procoagulant activity peaked for each cell type (2
hours for platelets and 48 hours for leukocytes, RBCs, and
MPs). Lactadherin (128 nM) prolonged the coagulation
times of all cell types (p < 0.001, respectively; Fig. 4B).
Inhibition assays of intrinsic/extrinsic factor Xase complex
and thrombin formation were also performed (Fig. 5E).
For blood cells and MPs, lactadherin blocked activity of
the procoagulant enzyme complexes up to 80%, confirm-
ing the role of PS in supporting PCA.

Fibrin Deposition and Formation Assays

In healthy controls, platelets and leukocytes supported
rapid formation of clots, whereas initial clot formation in
RBCs and MPs was delayed longer than 20 minutes (Fig.
6). When analyzed by time points after CAS, the final fi-
brin levels of leukocytes, RBCs, and MPs increased steadi-
ly from baseline and peaked at 48 hours after CAS, while
platelets peaked 2 hours after stenting, and all remained
higher than baseline after 7 days (Fig. 6A–D). The trend
of fibrin production on blood cells and MPs was consistent
with the number of PS+ cells and MPs. Additionally, base-
line fibrin formation in platelets, leukocytes, and MPs in
patients having undergone CAS was higher than in healthy
controls. To determine whether PS inhibition alters fibrin
formation, we treated peak samples with lactadherin prior
to measuring fibrin formation. The addition of lactadherin
significantly prolonged the onset of clot formation and re-
duced the final fibrin level by 65%.

To examine whether PS exposure relates directly
to local PCA by facilitating prothrombinase assembly,
confocal microscopy was used to look at the binding of
fluorescence-conjugated factor Va and factor Xa to MPs
from patients 48 hours post-CAS. A significant fraction of
bound factor Va and Xa appeared to be co-localized on
MPs, indicating that MPs are able to offer a biological sur-
face for binding of coagulation factors, most likely through
externalized PS (Fig. 6E). Because cellular PCA dictates
fibrin clot formation, we sought to explore whether MPs
had a similar effect. MPs were incubated with recalcified
MP-depleted plasma, resulting in the formation of a dense
fibrin network (Fig. 6F). We observed abundant fibrin for-
mation spread around MPs, with the network density nega-
tively correlated with the distance from MPs, suggesting
that MPs may dictate fibrin formation through exposed PS.

Discussion

First, we first found the percentage of PS+ blood cells
and the number of MPs (all types) increased after the CAS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0 Hrs</th>
<th>2 Hrs</th>
<th>48 Hrs</th>
<th>7 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lact+ PLT (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS</td>
<td>1.3 ± 0.09</td>
<td>10.5 ± 0.11</td>
<td>5.5 ± 0.27</td>
<td>2.4 ± 0.17</td>
</tr>
<tr>
<td>p Value</td>
<td>0.003</td>
<td>0.210</td>
<td>0.103</td>
<td>0.078</td>
</tr>
<tr>
<td>Lact+ RBC (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS</td>
<td>0.58 ± 0.06</td>
<td>0.67 ± 0.03</td>
<td>3.90 ± 0.31</td>
<td>1.80 ± 0.1</td>
</tr>
<tr>
<td>p Value</td>
<td>0.060</td>
<td>0.238</td>
<td>0.120</td>
<td>0.178</td>
</tr>
<tr>
<td>Lact+ PMN (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS</td>
<td>10.0 ± 0.91</td>
<td>14.2 ± 0.71</td>
<td>28.3 ± 1.40</td>
<td>15.6 ± 0.78</td>
</tr>
<tr>
<td>p Value</td>
<td>0.021</td>
<td>0.171</td>
<td>0.484</td>
<td>0.178</td>
</tr>
<tr>
<td>Lact+ MNC (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS</td>
<td>5.3 ± 0.32</td>
<td>10.8 ± 0.55</td>
<td>19.6 ± 1.56</td>
<td>12.7 ± 1.25</td>
</tr>
<tr>
<td>p Value</td>
<td>0.001</td>
<td>0.125</td>
<td>0.719</td>
<td>0.720</td>
</tr>
<tr>
<td>Lact+ MPs (μL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACS</td>
<td>2000 ± 120</td>
<td>4369 ± 219</td>
<td>4987 ± 249</td>
<td>4100 ± 316</td>
</tr>
<tr>
<td>p Value</td>
<td>0.003</td>
<td>0.713</td>
<td>0.730</td>
<td>0.761</td>
</tr>
</tbody>
</table>

ACS = asymptomatic carotid stenosis; Lact = lactadherin; MNC = monocyte; PLT = platelet; PMN = neutrophil; SCS = symptomatic carotid stenosis.
one possible explanation is that the marker used in
our study to detect PS+ platelets and PMPs is more sensitive. The immediate increase in PS+ platelets and PMPs
following CAS could be attributable to the mechanical forces of stent implantation that may induce apoptotic alterations
of platelet surface membranes. Our data showed that the
percentage of PS+ platelets positively correlated with d-
imer. Increased PS+ platelets and PMPs may be involved in
thromboembolism complications after CAS. Note that
patients usually received dual antiplatelet therapy daily
after CAS. In our results, we found increased platelet activa-
tion following CAS, which peaked at 2 hours. It is pre-
sumed that dual antiplatelet therapy should be considered
for at least 2 hours after CAS. Thus, monitoring platelet
activation could be important in preventing postoperative
complications.

Similar to platelets, the levels of circulating PS+ neu-
rophils/NMPs and monocytes/MMPs were significantly
increased and remained elevated at 7 days following the
procedure, indicating that neutrophils and monocytes play
an active role in the evolution of the hypercoagulant and
inflammatory state and may lead to the development of
in-stent restenosis after CAS. Consistent with our results,
data in other studies have shown that NMPs promote co-
agulation by adhering to and activating endothelial cells
to release inflammatory cytokines. The levels of PS+
monocytes/MMPs were increased at baseline in patients
who had undergone CAS as compared with levels in the
control group, suggesting neutrophils and monocytes are
recruited to the intima of atherosclerotic vessel walls. Our
findings indicate that PS+ neutrophils/NMPs and mono-
cytes/MMPs may be involved in the pathophysiology of
inflammation and PCA after CAS.

A previous study has shown that the erythrocyte sedi-
mentation rate may be a predictor of long-term outcomes
following CAS. However, the role of erythrocyte activa-
tion and/or apoptosis after CAS is largely unknown. Un-
lke the PS+ platelets and PMPs, the PS+ RBCs and R MPs
were not increased until 24 hours and peaked 48 hours
post-CAS, suggesting that they may be released into circu-
ulating blood through mechanical thrombosis rupture.
Indeed, our findings have shown that d-dimer was markedly
increased at 48 hours post-CAS, indicating thrombosis
rupture. Thus, our observations may provide new infor-
mation about procoagulant mechanisms and coagulation
abnormalities after CAS.

Implantation of a stent can cause endothelial disrup-
tion and abrasion. Percutaneous transluminal angioplasty
leads to endothelial denuding and mechanical disruption of
the atherosclerotic plaque with exposure of procoagu-
lant plaque components to flowing blood. The increase
in PS+ EMPs following CAS in our study confirms the

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**TABLE 4. Baseline characteristics of patients with periprocedural complications within 48 hours post-CAS**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Data</th>
<th>PLTs</th>
<th>RBCs</th>
<th>PMNs</th>
<th>MNCs</th>
<th>MPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stenosis degree</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age in yrs</td>
<td>yrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stenosis</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**TABLE 5. Relationships between PS+ blood cells/MPs and clinical data at their peak time point**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Data</th>
<th>PLTs</th>
<th>RBCs</th>
<th>PMNs</th>
<th>MNCs</th>
<th>MPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stenosis</td>
<td>r = 0.473</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>degree</td>
<td>p = 0.021</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Dimer</td>
<td>r = 0.437</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p = 0.034</td>
<td>p &lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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*Note: Patient No. 1 = restenosis; Patient No. 2 = TIA; Patient No. 3 = stent-thrombosis; TIA = transient ischemic attack; + = yes; − = no.*
presence of endothelial cell injury. The numbers of EMPs started to increase at 24 hours and remained elevated for 7 days post-CAS. Recent studies have shown that the level of MMP-9, which is exposed on EMPs, was elevated in vitro at 24 hours post-CAS, supporting our in vivo findings.

PS exposure on the outer membranes of blood cells and MPs serves to increase the apparent affinity of the cofactors VIIIa and Va for the enzyme factors IXa and X, respectively. This increases the generation of factor Xa and thrombin, resulting in fibrin production and inducing increased PCA. Based on the role of PS in the coagulation cascade and its dynamic changes in the 7 days after CAS, we further studied its effects on PCA. Increased PS exposure on blood cells and MPs from patients who had undergone CAS increased the activity of the intrinsic and extrinsic Xase complexes and thereby shortened coagula-

FIG. 4. Coagulation time and inhibition assay for platelets, WBCs, RBCs, and MPs. A: Coagulation time in platelets, WBCs, RBCs, and MPs decreased (p < 0.05) after the CAS procedure. Arrows indicate the time point of highest PCA and are used to illustrate panel B. B: Lactadherin was used to block PS and inhibit PCA in platelets, WBCs, RBCs, and MPs at the time indicated by the arrows in panel A. Coagulation time was prolonged in the lactadherin-treated samples. Data are shown as the mean ± SD.

FIG. 5. Formation and inhibition of procoagulant enzyme complexes post-CAS. Factor Xa and thrombin production are shown in platelets (A), WBCs (B), RBCs (C), and MPs (D). Intrinsic factor Xa formation was measured in the presence of factors IXa, VIII, and thrombin. Extrinsic factor Xa production was assessed in the presence of factor VIIa. Thrombin generation was assessed in the presence of factors Xa and Va. The ability of lactadherin (128 nM) to block the formation of procoagulant enzyme complexes on cells and MPs was evaluated (E). In each kind of cell and MPs, lactadherin decreased activity of the procoagulant enzyme complexes by 80%. Data are displayed as the mean ± SD. *p < 0.05 versus controls; **p < 0.001 versus 0 hours. IIa = factor VIIa; Ex-Xa+Lac = extrinsic factor Xa+lactadherin; In-Xa+Lac = intrinsic factor Xa+lactadherin.
FIG. 6. Fibrin deposition, formation, and inhibition on blood cells/MPs and circulating MPs support factor Va/Xa binding and fibrin deposition post-CAS. Recalcified (10 mM, final) normal pooled MP-depleted plasma was added to confluent platelets (A), WBCs (B), erythrocytes (C), and MPs (D) in healthy subjects and in patients who had undergone CAS in the absence or presence of 128 nM lactadherin. Fibrin polymerization was monitored by turbidity at 405 nm. Simultaneous staining (yellow, E) of factor Va (green) and Xa (red) is observed around MPs. Converted fibrin networks were detected around MPs at 48 hours post-CAS in the presence of recalcified MP-depleted plasma (F). Bar = 2 μm (E) and 5 μm (D).
tion time. Blockage of PS with lactadherin inhibited procoagulant enzyme production up to 80%, decreased fibrin formation approximately 65%, and prolonged coagulation time. Our confocal microscopy data also showed that factor Va and Xa appear to be co-localized on patient MPs through externalized PS. Further, MPs mixed with re-calcified MP-depleted plasma became attached to fibrin fibrils, which strongly suggests that MPs were directly incorporated into the fibrin network. Thus, we have demonstrated that increasing levels of PS+ platelets, leukocytes, erythrocytes, and MPs following CAS are associated with high PCA.

The potential limitations of this study include its limited number of subjects and the absence of patient follow-up regarding postoperative complications after 7 days. However, we made observations at 2 time points within 24 hours. Furthermore, we found that 3 of the limited number of patients had thromboembolism within 48 hours post-CAS. The clinical data of these 3 patients may be useful in the future (Table 4). Future large-scale prospective studies are needed to establish whether PS could be used as a non-invasive biomarker to guide treatment decisions in patients who have undergone CAS.

Conclusions
Our results shed new light on the potential mechanisms of coagulation abnormalities after CAS. PS+ blood cells and MPs from activated platelets, neutrophils, monocytes, RBCs, and endothelial cells changed dramatically after CAS. Stenosis degree and MPs from activated platelets, neutrophils, monocytes, and endothelial cells to fibrin network formation, structure, and stability. Blood 114:4886–4896, 2009


Mesri M, Altieri DC: Leukocyte microparticles stimulate


35. Shi J, Heegaard CW, Rasmussen JT, Gilbert GE: Lactadherin binds selectively to membranes containing phosphatidyl-serine and increased curvature. Biochim Biophys Acta 1667:82–90, 2004


Disclosures
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

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