Original Paper

Procoagulant Activity of Blood and Endothelial Cells via Phosphatidylserine Exposure and Microparticle Delivery in Patients with Diabetic Retinopathy

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Key Words

Diabetic retinopathy • Factor VIII procoagulant activity • Phosphatidylserines • Cell-derived microparticles • Lactadherin

Abstract

Background/Aims: The mechanisms for thrombosis in diabetic retinopathy (DR) are complex and need to be further elucidated. The purpose of this study was to test phosphatidylserine (PS) exposure on microparticles (MPs) and MP-origin cells from the circulation and to analyze cell-/MP-associated procoagulant activity (PCA) in DR patients. **Methods:** PS-positive MPs and cells from healthy controls (n = 20) and diabetic patients (n = 60) were analyzed by flow cytometry and confocal microscopy. Clotting time and purified coagulation complex assays were used to measure PCA. **Results:** PS exposure on platelets and monocytes was higher in proliferative DR (PDR) patients than in non-PDR patients or controls. The highest levels of MPs (derived from platelets [30%], erythrocytes [13%], leukocytes [28%], and endothelial cells [10%]) were found in patients with PDR. In addition, PS exposure on blood cells and shed MPs in DR patients led to significantly increased FXa and FIIa generation, fibrin formation, and markedly shortened coagulation time. Moreover, lactadherin reduced 70% of PCA by blocking PS, while an anti-tissue factor antibody had a smaller effect. **Conclusion:** Our results confirmed that PCA in DR patients may be partly ascribed to PS exposure and MP release from blood and endothelial cells. Lactadherin may act as an efficient anticoagulant factor in this process.

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Introduction

Vascular complications of diabetes are related to hypercoagulability and increased thrombogenicity [1]. Diabetic retinopathy (DR) is one of the most severe complications of diabetes [2, 3], but the role of procoagulant activity (PCA) and the relevant mechanisms involved in coagulation abnormalities in DR are largely unclear. Ogata et al. reported increased levels of platelet- and monocyte-derived microparticles (MPs) in the plasma of DR patients [4, 5]; however, whether phosphatidylserine-positive (PS⁺) MPs contribute to PCA in DR and the precise role of MPs in prothrombinase and fibrin formation remain poorly investigated. Moreover, the contribution of peripheral blood cells (PBCs) to the procoagulant state in DR is also poorly characterized, although red blood cells (RBCs) and platelets appear to aggregate in blood vessels [6, 7]. Inflammatory responses are closely associated with the pathogenesis of DR, and leukostasis has been found in the retina of diabetic animals [8-10]. Thus, whether leukocytes and leukocyte-derived MPs are involved in hypercoagulability in DR should be elucidated further. Endothelial-derived MPs are implicated in the risk of diabetic microangiopathy and macroangiopathy [11]; thus, we further examined the levels of endothelial-derived MPs in different types of DR.

Normally, PS is located in the inner leaflet of the cell membrane and is inaccessible to blood coagulation proteins. During cell activation and/or apoptosis, PS is exposed on the outside of the cell membrane. These cells release small membrane vesicles, named MPs, which express PS and cell-specific antigens. Several studies have shown that MPs have biological activity, including roles in coagulation, inflammation activation, angiogenesis, and cell-to-cell communication [12-15]. PS on cells and MPs provide a surface for factor Xase and prothrombinase complex formation [16-18]. Our previous studies have shown that elevated circulating levels of PS⁺ cells and MPs play a pivotal role in PCA in nephrotic syndrome and acute promyelocytic leukemia [19, 20]. However, whether the hypercoagulable state in DR is mediated by PS has not been assessed. We assume that PS exposure and MP release may participate in the thromboembolic events of DR.

Lactadherin is confirmed as a ligand for PS [21-25]. We demonstrated that lactadherin can be used as a sensitive probe and an effective anticoagulant to localize and block PS [19, 26, 27]. Thus, we utilized lactadherin to explore PS externalization and MP release in DR patients as well as to define the relationship between PS and PCA. We hypothesized that PCA in DR patients is a result of PS-exposing platelets, erythrocytes, leukocytes, and cell-associated MPs. Defining the extent of PS exposure on released MPs and blood cells, as well as on endothelial cells, and evaluating their roles will enable a better understanding of the development of thrombotic pathogenesis in DR. Our results revealed the pivotal role of PS⁺ cells and MPs in PCA and may potentially help to identify optimal targets and effective therapies for preventing a pathologic hypercoagulable state in DR patients.

Materials and Methods

Participants

The study population comprised 80 participants and was separated into four cohorts: 20 healthy controls (a) and three groups of type 2 diabetes mellitus (T2DM) patients with or without DR consisting of 20 T2DM patients without DR (NDR) (b), 20 T2DM patients with non-proliferative DR (NPDR) (c), and 20 T2DM patients with proliferative DR (PDR) (d). Glycemic status in patients with T2DM was evaluated using HbA1c%. Ambulatory patients with T2DM on oral anti-diabetic medications were considered for this study. Patients on insulin therapy were excluded. DR patients were examined by an ophthalmologist and graded according to the proposed clinical grading system. The diagnosis was based on optical coherence tomography and fluorescein angiography. Exclusion criteria were coronary artery disease, malignancy, pregnancy, active or chronic infection, autoimmune disorders, liver dysfunction, blood transfusion within the past 6 months, other microvascular complications such as nephropathy (urinary microalbumin > 300 mg/day), thrombotic events, glaucoma, Eales disease, and branch retinal venous occlusion. Before blood samples were obtained, all

subjects were instructed not to take anticoagulants, antiplatelet agents, oral contraceptives, hypolipidemic drugs, and so on. Written informed consent was obtained from all study participants. The characteristics of all subjects are shown in Table 1.

Study approval

All experiments were conducted in accordance with and approved by the Ethics Committee of Harbin Medical University according to the Helsinki Declaration.

Subjects and methods

Megamix beads (mix of 0.5-, 0.9-, and 3.0- μ m beads) were from Biocytex (Marseille, France). Trucount Tubes (Cat. No. 340334), propidium iodide (PI), purified anti-CD235a (clone GA-R2), anti-CD31 (clone L133.1), anti-CD41a (clone HIP8), anti-CD66b (clone G10F5), anti-CD14 (clone M5E2), anti-CD19 (clone HFT-1), anti-CD142 (clone HFT-1), and anti-CD3 (clone 1F4) antibodies and mouse IgG1/IgG2a (clone X40/X39) were from Becton Dickinson Biosciences (San Jose, CA, USA). Trypsin-EDTA was from Gibco (Grand Island, NY, USA). The monoclonal antibodies were labeled with Alexa Fluor 647 or Alexa Fluor 488 (Invitrogen) in our laboratory.

Protein purification and labeling

Bovine lactadherin, free of lipid and protein contaminants, was purified and labeled with Alexa Fluor 647 or Alexa Fluor 488 [23, 24].

Blood collection and preparation of blood cells and MPs

Venous blood samples were harvested and collected into a 5-mL tube containing 3.2% sodium citrate. Platelet-rich plasma and RBCs were prepared within 30 min of collection by centrifugation for 13 min at $200 \times g$ at room temperature and were analyzed immediately after isolation. To prepare platelet-free plasma, platelet-rich plasma was centrifuged for 20 min at $1,500 \times g$, and plasma was harvested and centrifuged again for 2 min at $13,000 \times g$ to remove all residual platelets [19]. Platelet-free plasma samples were snap-frozen in liquid nitrogen, and then stored at -80°C until use. Leukocytes were isolated using gradient centrifugation with Percoll [19, 20]. To isolate MPs, 250 μ L platelet-free plasma was thawed on ice for 60 min and then centrifuged for 45 min at $20,000 \times g$, as in a previous report [28].

Flow cytometric analysis of the MP phenotype

MPs expressing phosphatidylserine were identified [28]. Erythrocyte, platelet, leukocyte, granulocyte, monocyte, T/B lymphocyte, and endothelial cell-derived MPs were gated as events with a 0.1 to 1.0 μ m diameter and characterized as lactadherin⁺ CD235a⁺, lactadherin⁺ CD41a⁺, lactadherin⁺ CD45⁺, lactadherin⁺ CD66b⁺, lactadherin⁺ CD14⁺, lactadherin⁺ CD3/19⁺, or lactadherin⁺ CD31⁺ CD41a⁺, respectively [19]. MPs

Table 1. Baseline characteristics of patients with DR and healthy subjects at inclusion. Abbreviations: NDR, diabetes mellitus (DM) patients without diabetic retinopathy; NPDR, DM patients with non-proliferative diabetic retinopathy; PDR, DM patients with proliferative diabetic retinopathy; BMI: body mass index; FBG: fasting blood glucose; HDL: high density lipoprotein; LDL: low density lipoprotein; RBC: red blood cell; WBC: white blood cell; PT: prothrombin time; APTT: activated partial prothrombin time. Data are means $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. controls. # $P < 0.05$, ## $P < 0.01$ vs. NDR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NPDR

	Controls	NDR	NPDR	PDR
Sex (male/female)	12/8	10/10	10/10	8/12
Age (years)	49 $\pm$ 14	52 $\pm$ 10	54 $\pm$ 9	59 $\pm$ 11
Duration of disease (years)	-	12 $\pm$ 5	13 $\pm$ 6	14 $\pm$ 6
BMI (Kg/m ²)	22 $\pm$ 3	26.6 $\pm$ 3.6	27.5 $\pm$ 4.1	28.1 $\pm$ 2.9
Glycated hemog (%)	5.4 $\pm$ 0.3	6.8 $\pm$ 0.3**	7.4 $\pm$ 0.4***	8.65 $\pm$ 0.8***##
FBG (mmol/L)	5.6 $\pm$ 0.3	6.9 $\pm$ 0.6**	6.8 $\pm$ 0.4**	8.25 $\pm$ 0.3***##
Albumin (g/l)	37.6 $\pm$ 1.4	41.7 $\pm$ 1.4*	42.2 $\pm$ 2.7*	44.4 $\pm$ 4.1*
Total cholesterol (mM)	3.9 $\pm$ 0.4	4.1 $\pm$ 0.2	4.3 $\pm$ 0.2	4.9 $\pm$ 1.1
HDL (mM)	1.3 $\pm$ 0.1	1.1 $\pm$ 0.1*	1.2 $\pm$ 0.1	1.2 $\pm$ 0.2
LDL (mM)	3.0 $\pm$ 0.1	3.1 $\pm$ 0.2	3.3 $\pm$ 0.2	3.6 $\pm$ 0.9
Triglycerides (mM)	1.8 $\pm$ 0.2	2.0 $\pm$ 0.1	1.8 $\pm$ 0.3	1.6 $\pm$ 0.7
RBC count (10 ¹² /l)	4.8 $\pm$ 0.3	5.5 $\pm$ 0.6	4.9 $\pm$ 0.2	4.4 $\pm$ 0.8
Platelet count (10 ⁹ /l)	181.5 $\pm$ 30.6	197.3 $\pm$ 14.3	279.1 $\pm$ 62.8*#	200.4 $\pm$ 73.5
WBC count (10 ⁹ /L)	7.4 $\pm$ 0.6	5.9 $\pm$ 0.4**	7.3 $\pm$ 1.9	6.9 $\pm$ 3.2
PT (s)	10.8 $\pm$ 0.4	10.3 $\pm$ 0.3	11.1 $\pm$ 0.2**	10.4 $\pm$ 0.4*
APTT (s)	26.3 $\pm$ 0.9	26.9 $\pm$ 1.1	27.3 $\pm$ 2.6	22.5 $\pm$ 2.9**
Fibrinogen (g/l)	2.4 $\pm$ 0.4	2.8 $\pm$ 0.2	3.1 $\pm$ 0.9	3.0 $\pm$ 0.9
D-dimer (mg/ml)	0.4 $\pm$ 0.1	0.2 $\pm$ 0.1**	0.2 $\pm$ 0.08*	0.9 $\pm$ 0.8**
Cardiovascular events	0	2	2	4

exposing tissue factor (TF) were identified as those coexpressing lactadherin and CD142. The number of each type of MP per μL was calculated by using a TruCount Tube (with a precise number of 48678 fluorescent beads to determine the number of MPs in a sample) after the accumulation of 10,000-gated events. The number of MPs per microliter of plasma (n) was calculated using the following formula: $n = (C \times \text{Beads}_{\text{added}}) / (\text{Beads}_{\text{counted}} \times \text{sample volume})$, where C represents the number of positive events with the background signal subtracted.

Flow cytometric analysis of PS exposure on PBCs

We added $0.5\text{--}1.0 \times 10^6/\text{mL}$ RBCs/platelets/white blood cells (WBCs) from each of the patients to a final volume of 200 μL Tyrode's buffer, and then the cell suspension was added to 5 μL Alexa Fluor 488-conjugated lactadherin after incubation [29–33]. Ten thousand events per sample were acquired in a flow cytometer and analyzed with BD FACSDiva Software.

Confocal microscopy

RBCs/platelets/WBCs were incubated with the indicated concentrations of FITC-lactadherin and PI. PS exposure on platelets and MPs was observed using an LSM 510 SYSTEM (Carl Zeiss Jena GmbH, Jena, Germany) [34, 35].

Coagulation time and inhibition assays

A recalcification time assay was used to assess PCA in a KC4A coagulometer [28]. All clotting assays were performed in triplicate. For the inhibition of coagulation time assay, the effect of lactadherin or an anti-TF antibody on the PCA of cell or MP suspensions was tested as follows. Cell or MP suspensions (100 μL) were preincubated with 50 μL lactadherin (final concentration 128 nM) or anti-TF antibody (final concentration 40 $\mu\text{g}/\text{mL}$) for 10 min at 37 °C. Then, the mixtures were added to 100 μL MP-free plasma and 100 μL of 50 mM CaCl_2 . Clotting time was recorded as above [35].

FXa, prothrombinase, and fibrin formation assays and inhibition tests

A total of 2.0×10^4 platelets/WBCs, 2.0×10^5 RBCs, or 10 μL MP-enriched suspension was mixed with 5 nM FVIII, 1 nM FIXa, 130 nM FX, 0.2 nM thrombin, and 5 mM Ca^{2+} in FXa buffer (Tris-buffered saline containing 0.2% bovine serum albumin) and incubated for 5 min at 25°C. The results were measured against a standard dilution of FXa. To measure prothrombinase production, the cells were incubated with 1 nM FVa, 0.05 nM FXa, 1 μM prothrombin, and 5 mM Ca^{2+} in prothrombinase buffer for 5 min at 25°C. EDTA and S-2238 were added to each microplate, and thrombin production was assessed as described previously [28]. To evaluate the role of MPs in fibrin formation, MP-containing suspensions (25 μL MP pellet was washed twice and resuspended in 75 μL Tyrode's buffer) were incubated with prewarmed MP-depleted plasma (15%) in the presence of 100 mM Ca^{2+} . The effect of lactadherin inhibition on coagulation was tested as follows. Different kinds of cells were incubated with 128 nM lactadherin in Tyrode's buffer at 25°C [35].

Statistical analysis

Numerical variables were tested for normal distribution with the Kolmogorov-Smirnov test. Data are expressed as the mean $\pm$ standard deviation for at least 3 replicates, and statistical analysis was made by a t-test or analysis of variance, as appropriate. $P < 0.05$ was considered to indicate statistical significance.

Results

Characteristics of the DR patients and healthy subjects

Table 1 summarizes the clinical characteristics of the DM patients and controls. No difference was found in age or body mass index between the diabetic patients and control subjects. Patients with PDR had significantly higher HbA1c, D-dimer, and shorter activated partial thromboplastin time than did the controls and the NDR and NPDR patients. A significant difference was found in fasting blood glucose between the three DM groups (Table 1). The number of subjects with hypertension was similar among the T2DM groups and was higher in the T2DM groups than in the control group. There were more cardiovascular events

in patients with PDR than in the other groups. Fig. 1 shows the fundus conditions of the DR and control subjects.

Number of MPs in the DR patients

The total number of MPs and phenotypes of the study groups were evaluated. More than 85% of events gated on P1 were connected to lactadherin, indicating PS exposure. MPs derived from platelets (CD41a⁺ PMPs), endothelial cells (CD31⁺/41⁺ endothelial-derived MPs), mononuclear cells (CD14⁺), granulocytes (CD66b⁺), T lymphocytes (CD3⁺), B lymphocytes (CD19⁺), erythrocytes (CD235a⁺ RMPs), and TF (CD142⁺) are shown in Fig. 2A–D. A greater number of MPs (lactadherin⁺ MPs; $P < 0.05$) was observed in patients with DR than in healthy subjects. In addition, patients with PDR also had significantly increased RMPs and leukocyte-derived MPs than those with NDR, as well as mononuclear cell- and granulocyte-derived MPs in the leukocyte-derived MP population. No statistical difference was found in the TF⁺ MP level between the NDR, NPDR, and PDR patients (Table 2). Only a few MPs co-expressing both PS and TF were found in the DM patients and controls (Table 2).

Fig.1. Clinical features of diabetic retinopathy. Normal fundus image can be seen in normal control patient (A). There is exudation in fundus images of NPDR (B). Hemorrhage and proliferative membrane can be seen in fundus image of PDR (C).

Fig. 2. MPs in PDR patients. Total number of MPs and phenotypic characterization in the four study groups were evaluated. More than 85% of events gated on P1 connected with lactadherin which indicates PS exposure. (A) Lactadherin-positive MPs were labeled with Alexa Fluor 488- and Alexa Fluor 647-labeled antibodies: MPs were from RBCs (Alexa Fluor 488-CD235a⁺) and the tissue factor expressing MPs (Alexa Fluor 647-CD142⁺) (Fig. 2A); MPs were from platelets (Alexa Fluor 488-CD41a⁺) and endothelial cells (Alexa Fluor 647-CD31⁺/Alexa Fluor 647-CD41a⁺) (Fig. 2B); MPs were from T lymphocytes (Alexa Fluor 488-CD3⁺) and PMNs (Alexa Fluor 647-CD66b⁺) (Fig. 2C); MPs were from B lymphocytes (Alexa Fluor 647-CD19⁺) and MNCs (Alexa Fluor-488 CD14⁺) (Fig. 2D).

MPs were from RBCs (Alexa Fluor 488-CD235a⁺) and the tissue factor expressing MPs (Alexa Fluor 647-CD142⁺) (Fig. 2A); MPs were from platelets (Alexa Fluor 488-CD41a⁺) and endothelial cells (Alexa Fluor 647-CD31⁺/Alexa Fluor 647-CD41a⁺) (Fig. 2B); MPs were from T lymphocytes (Alexa Fluor 488-CD3⁺) and PMNs (Alexa Fluor 647-CD66b⁺) (Fig. 2C); MPs were from B lymphocytes (Alexa Fluor 647-CD19⁺) and MNCs (Alexa Fluor-488 CD14⁺) (Fig. 2D).

Table 2. Number of microparticles per microliter of plasma in the NDR, NPDR, and PDR patients and controls. Abbreviations: MP, microparticle; NDR, diabetes mellitus (DM) patients without diabetic retinopathy; NPDR, DM patients with non-proliferative diabetic retinopathy; PDR, DM patients with proliferative diabetic retinopathy. Corrected for the number of events with isotype controls. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. controls. # $P < 0.05$ vs. NDR and NPDR

Lactadherin ⁺ MPs (/μL)	Controls	NDR	NPDR	PDR
Total	1628.7 ± 105.5	3455.7 ± 452.6**	3626.7 ± 412.9**	4289.7 ± 506.1***
CD41a ⁺	1236.2 ± 118.8	2837.7 ± 477.6**	3104.5 ± 288.2***	3582.7 ± 394.4***
CD235a ⁺	23.6 ± 4.5	187.3 ± 36.9**	225.4 ± 29.7***	285.3 ± 37.6***#
CD31 ⁺ CD41a ⁺	12.3 ± 2.5	91.7 ± 17.7**	99.5 ± 20.5**	123.3 ± 18.5***
CD66b ⁺	12.7 ± 2.9	59.1 ± 19.2*	64.2 ± 23.0*	97.4 ± 11***#
CD14 ⁺	6.7 ± 1.9	14.5 ± 4.3*	15.8 ± 3.8*	18.7 ± 2.2***#
CD3 ⁺	4.1 ± 1.9	10.1 ± 3.2*	15.4 ± 3.0**	17.6 ± 4.5**
CD19 ⁺	3.3 ± 1.2	9.8 ± 1.5**	14.9 ± 2.3**#	16.2 ± 2.2***#
CD142 ⁺	2.2 ± 1.8	2.8 ± 2.3	2.9 ± 2.1	3.2 ± 2.4

PS exposure on peripheral cells in the DR patients

Consistent with the above MP results, the percentage of lactadherin⁺ RBCs/platelets/polymorphonuclear cells (PMNs)/mononuclear cells (MNCs) in the DR group was significantly increased compared with the healthy controls ($P < 0.001$), and greater in the PDR patients than in the NPDR and NDR patients ($P < 0.01$, Fig. 3). In addition, PS exposure on platelets and MNCs in the PDR patients was $6.6 \pm 0.6 \times 10^9/L$ and $19.7 \pm 0.9 \times 10^9/L$, respectively, which was more than in the NPDR patients (platelets: $4.2 \pm 0.3 \times 10^9/L$, $P < 0.01$; MNCs: $16.5 \pm 0.6 \times 10^9/L$, $P < 0.01$), NDR patients (platelets: $3.4 \pm 0.3 \times 10^9/L$, $P < 0.01$; MNCs: $12.8 \pm 0.1 \times 10^9/L$, $P < 0.01$), and healthy subjects (platelets: $1.5 \pm 0.2 \times 10^9/L$, $P < 0.001$; MNCs: $3.9 \pm 0.3 \times 10^9/L$, $P < 0.001$). Lactadherin⁺ platelets and MNCs were increased in the PDR patients compared with those in the NPDR and NDR patients ($P < 0.05$). RBCs/platelets/leukocytes of the healthy controls and PDR patients were stained with Alexa Fluor 488-lactadherin and PI. As shown in Fig. 4A and C, there was little lactadherin staining detected on the RBCs, platelets, and leukocytes of the control group, whereas significant lactadherin binding was observed on the platelets (arrow, Fig. 4D)/RBCs (arrow, Fig. 4B)/leukocytes (Fig. 4E) of the DR patients. These results indicated an increased number of PS⁺ blood cells in the DR patients.

PCA in the DR patients

The PCA of blood cells and MPs from the control group and DR patients was analyzed. The clotting time of RBCs/platelets/WBCs/MPs isolated from the PDR patients was shortened compared with the NPDR patients, NDR patients, and control subjects (Fig. 5A) ($P < 0.01$). To confirm that the detected PCA of blood cells/MPs in the PDR patients was related to PS or TF, coagulation inhibition assays were conducted. The coagulation time of PBCs and MPs was significantly increased with lactadherin, while the anti-TF antibody (26.5 $\mu g/mL$) did not markedly affect coagu-

Fig. 3. PS exposure on blood cells in different groups. Lactadherin-positive RBCs/platelets/PMNs/MNCs from healthy subjects ($n = 10$), NDR patients ($n = 20$), NPDR patients ($n = 20$), and PDR patients ($n = 20$) were analyzed. Data are expressed as the mean $\pm$ SD (** $P < 0.001$, ## $P < 0.01$).

Fig. 4. PS exposure on blood cells by confocal microscopy. Platelets, RBCs and WBCs from healthy controls and

PDR patients were labeled with Alexa Fluor 488 -lactadherin and propidium iodide. Cell membrane displayed green fluorescence when labeled with lactadherin and nucleus displayed red fluorescence. There is little staining in RBC and WBC from healthy subjects (Fig. 4-A) Lactadherin staining (green) was observed on platelet membranes and MPs (Fig. 4-B) and RBCs (Fig. 4-C)/WBCs (Fig. 4-E) in PDR patients, but little staining in platelets membrane and MPs from healthy subjects (Fig. 4-D) but little staining was seen in the control group (A, C). The scale bar of Fig. 4-A and Fig. 4-B equals 5 μm . The scale bar of Fig. 4-C equals 10 μm . The scale bar of Fig. 4-D and Fig. 4-E equals 10 μm .

lation time (Fig. 5B). The PCA of blood cells/MPs was evaluated by intrinsic FXa, extrinsic FXa, and prothrombinase assays (Fig. 6A–D). The amount of procoagulant enzyme complexes was increased in the DR groups compared with the controls ($P < 0.001$), and was higher in the PDR patients than in the NPDR and NDR patients ($P < 0.05$ for each kind of cell). Inhibition assays of procoagulant enzyme complex formation were also performed with lactadherin. Lactadherin significantly blocked the generation of procoagulant enzyme complexes by approximately 70% (Fig. 6E).

We also tested the ability of MPs to support fibrin formation. The onset of MPs was shorter and the production of fibrin was higher in the DR group than in the control group. Fibrin formation was inhibited when lactadherin, and not the anti-TF antibody, was added to the plasma (Fig. 6F). These data indicated that MPs trigger PS-dependent fibrin production.

Discussion

In this study, we made some significant observations. First, we found that the plasma levels of PS⁺ MPs derived from platelets, erythrocytes, leukocytes, and endothelial cells were augmented significantly in DR patients (either NPDR or PDR) compared with healthy subjects. Meanwhile, we found PS⁺ blood cells were markedly increased in DR patients compared with those in controls. In addition, the increased exposure of PS on blood cells and MPs supported intrinsic and extrinsic FXa generation and prothrombinase assembly. Moreover, lactadherin competed with factor V and VIII for PS binding sites and consequently

Fig. 5. Inhibition assays for coagulation time in PDR patients. Coagulation time of blood cells and MPs in healthy controls ($n = 10$), NDR patients ($n = 20$), NPDR patients ($n = 20$), and PDR patients ($n = 20$) was detected in the absence (A) or presence (B) of 128 nM lactadherin or 25.6 μ g/mL anti-TF antibody. Data are displayed as the mean $\pm$ SD (* $P < 0.01$, ** $P < 0.001$, # $P < 0.001$).

Fig. 6. Inhibition assays of procoagulant enzyme complexes and fibrin deposition. FXa and thrombin production of RBCs (A), platelets (B), WBCs (C), and MPs (D) of the different groups are shown. Lactadherin inhibited the formation of procoagulant enzyme complexes by 70% (E). Fibrin production of MPs was analyzed in the presence of recalcified MP-depleted plasma (F). Data are displayed as the mean $\pm$ SD (* $P < 0.01$, # $P < 0.001$).

decreased the PCA of PBCs and MPs, while an anti-TF antibody had a less significant inhibition effect. These results lead us to believe that exposed PS on cells and MPs plays an important role in the thrombophilic tendency and microvascular complications in patients with DR.

In the current study, we found that MPs in DR patients were not only derived from platelets and monocytes [4, 5] but also from RBCs, PMNs, lymphocytes, and endothelial cells, suggesting that PBCs are comprehensively involved in the genesis of MPs in DR. The total number of MPs in DR patients was markedly increased compared with healthy subjects. In addition, patients with PDR showed the highest level of MPs originating from RBCs and leukocytes (especially PMNs) compared with those with NDR. MPs derived from endothelial cells were also increased in PDR patients in comparison with NDR patients, indicating a greater degree of endothelium damage and a higher risk of a coagulation abnormality in patients with PDR. Previous studies have only focused on the role of platelets in coagulation in DR patients; however, the contribution of erythrocytes and leukocytes to PCA in DR has been largely overlooked [5, 7]. We found that exposed PS on the outer membrane leaflet of RBCs/platelets/PMNs/MNCs in DR patients was increased compared with that in healthy controls, and was greater in PDR patients compared with NPDR and NDR patients. Furthermore, we observed that the PCA of RBCs and leukocytes was significantly enhanced in DR patients. On the basis of the large amount of RBCs in peripheral blood and the important role of leukocytes in DR, it is convincible to propose that RBCs, leukocytes, and platelets contribute cooperatively to the hypercoagulable state of DR patients.

It has been confirmed that PS exposure on platelets or MPs is an essential contributor to the formation of FXa and prothrombinase complexes [30-35]. Our studies have shown that increased exposure of PS on the surface of PBCs/MPs supports the assembly of prothrombinase complexes, thus promoting the coagulation cascade reaction and contributing to thrombin generation. Then, the increased levels of thrombin convert fibrinogen to fibrin, which accounts for the hypercoagulable state of DR patients. In our current report, we found that lactadherin specifically binds to PS and inhibits more than 70% of procoagulant enzyme complex formation, significantly decreasing the PCA of MPs and PBCs in DR patients. In contrast, an anti-TF antibody did not markedly affect the PCA of PBCs/MPs, although TF is an important initiator of coagulation. Therefore, these results suggest that exposed PS on the surface of PBCs and released MPs is responsible for the excessive PCA in DR patients.

PS exposure is usually detected with annexin V [36]. However, annexin V binds to PS at certain Ca^{2+} concentrations and this binding is not dependent on membrane PS content. In the present study, lactadherin was used to probe and localize PS on PBCs/MPs and to inhibit PCA by blocking PS. Our results demonstrate that lactadherin may be used as a sensitive probe and an effective blocker of PS in DR and other procoagulant and prothrombotic conditions.

To prevent a hypercoagulable state, it seems crucial to block PS. PS provides a binding site for FXa and prothrombinase complexes and promotes thrombin formation. Thus, Xa and thrombin inhibitors may be used to reduce Xa and thrombin production, thereby preventing fibrin and thrombosis formation, and consequently lessening local ischemia and injury. More importantly, our results show that lactadherin effectively blocks exposed PS and therefore inhibits PCA, indicating that lactadherin acts as an efficient anticoagulant for preventing blood clotting or modulating the balance between the procoagulant and anticoagulant phenotypes. Further studies are necessary to evaluate the possibility of applying lactadherin as a therapeutic strategy against prothrombotic conditions in patients with DR.

Our observations suggest that PS^+ cells and MPs are involved in the increased blood PCA of DR patients. Accordingly, our results indicate that the procoagulant profiles of PS^+ cells and MPs reflect the development of vascular pathology in DR. Our findings are of potential clinical relevance, reveal new mechanisms involved in the hypercoagulable state, and suggest that the lactadherin-mediated blockade of PS could constitute a novel therapeutic intervention in patients with DR.

Our findings described herein demonstrate that the loss of asymmetry in PBC membrane phospholipids with increased PS exposure on released MPs and their original cells leads to the hypercoagulable state in DR patients and provide evidence for the mechanism underlying

the thrombosis observed in DR. Additionally, PS can be regarded as a diagnostic predictor for the procoagulant state in patients with DR.

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Disclosure Statement

No conflict of interests exists.

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