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ORIGINAL ARTICLE

Homocysteine induces procoagulant activity of red blood cells via phosphatidylserine exposure and microparticles generation

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Abstract Increased homocysteine (Hcy) levels in plasma correlate with the risk of thromboic events. Red blood cells (RBCs), the most abundant blood cells in circulation, also play an active role in the process of thrombus formation. However, the effect of Hcy on procoagulant activity (PCA) of RBCs is unclear. In the present study, RBCs from healthy adults were treated with Hcy (8, 20, 80, 200, 800 μmol/L) for 24 h. Phosphatidylserine (PS) exposure of RBCs and red blood cell-derived microparticles (RMPs) release were detected using Alexa Fluor 488-lactadherin. PCA was assessed by coagulation time and purified clotting complexes testes. We found that Hcy treatment dose dependently enhanced PS exposure and consequent PCA of RBCs. Hcy also elevated the formation of procoagulant RMPs, with statistical significance at 800 μmol/L of Hcy. Moreover, 128 nmol/L lactadherin inhibited about 90 % PCA of RBCs and RMPs. Our data suggest that PS exposure and RMPs shedding are key sources for Hcy-induced PCA of RBCs. Lactadherin could be used to modulate the anticoagulant and procoagulant balance in this process.

Keywords Homocysteine · Red blood cells · Procoagulant activity · Phosphatidylserine · Microparticles · Lactadherin

Introduction

Homocysteine (Hcy), a sulfur-containing amino acid, is a metabolite of the methionine (Currò et al. 2009; Cai et al. 2009). Its physiological level is 5–15 μmol/L in an adult individual (Perła-Kaján et al. 2007). Moderate, intermediate, and severe hyperhomocysteinemia refer to total plasma concentration of Hcy in the range of 16–30, 31–100 and more than 100 μmol/L, respectively (Hankey and Eikelboom 1999). Hyperhomocysteinemia is known as a risk factor for thrombotic events (den Heijer et al. 2007; Dayal et al. 2006), but the precise mechanism still remains to be investigated.

Phosphatidylserine (PS) is mainly restricted to the inner cell membrane. It is externalized to the plasma membrane outer leaflet when a cell undergoes activation or apoptosis (Shi et al. 2006). PS is necessary for assembly of tenase and prothrombinase that results in thrombin generation (Zwaal and Schroit 1997). Approximately, 20–30 trillion RBCs in the adult human body are not only innocent bystanders for coagulation and hemostasis (Andrews and Low 1999; Whelihan and Mann 2013). It was reported that increased PS exposure on RBCs contributes to the hypercoagulability in several diseases (Wahid et al. 2001; Atichartakarn et al. 2002; Setty et al. 2001; Bonomini et al.

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2005). Our prior studies showed that chemotherapeutic agents induce procoagulant activity (PCA) of RBCs via PS exposure (Zhou et al. 2010a). Moreover, red blood cell-derived microparticles (RMPs), the populations of phospholipid vesicles shed from RBCs membrane, are involved in induction of thrombogenic response (Chung et al. 2007; Rubin et al. 2010). Therefore, we speculate that PS of RBCs and RMPs may be related to Hcy-associated thromboembolism.

Lactadherin, also named as MFG-E8, BA-46, PAS-6/7, bovine-associated mucoprotein (Shi et al. 2004), has C-terminus with homology to the PS-binding domains of coagulation factors V and VIII (Stubbs et al. 1990). Lactadherin interacts with phospho-L-serine of PS stereoselectively, and it binds to PS-containing membranes proportionally (Shi et al. 2004). Our previous study showed that lactadherin serves as a sensitive probe to detect PS exposure and microparticles generation of erythrocytes in patients with nephrotic syndrome and polycythemia vera (Gao et al. 2012; Tan et al. 2013). It functions as an efficient anticoagulant via competing with membrane-bound factor V and factor VIII (Shi and Gilbert 2003; Shi et al. 2008). Thus, we utilized lactadherin to measure PS exposure of RBCs, RMPs release and their association with Hcy-induced PCA.

Materials and methods

Materials

Alexa Fluor 488 protein labeling kit and FluoSpheres polystyrene microspheres (1 μm) were purchased from Invitrogen (Carlsbad, CA, USA). Hcy, ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA) were supplied by Sigma-Aldrich (St Louis, MO, USA). RPMI1640 was obtained from Hyclone (Logan, UT, USA). Human factors Va, VIIa, IXa, X, Xa, prothrombin and thrombin were from Haematologic Technologies Inc. (Burlington, VT, USA). Recombinant human factor VIII was obtained from American Diagnostica Inc. (Stamford, CT, USA). The Chromogenic substrates S-2765 and S-2238 were from DiaPharma Group (West Chester, OH, USA). Bovine lactadherin was purified as described previously (Hvarregaard et al. 1996). Alexa Fluor 488-labeled lactadherin was prepared in our laboratory (Shi et al. 2008).

Blood collection

The study was approved by the Ethics Committee of Harbin Medical University consistent with the Helsinki Declaration. After informed consent, peripheral vein blood collected from each 16 healthy volunteers using a 21-gage needle was anticoagulant with 3.8 % sodium citrate (1:9, v/v). These healthy donors were 19–32 years old, sex matched, non-smokers, and they had not taken any drugs for at least 2 weeks.

Preparation of microparticle-free plasma

Platelet-rich plasma was harvested by centrifugation at $200\times g$ for 15 min. Then, we centrifuged platelet-rich plasma at $3{,}500\times g$ for 15 min to get platelet-poor plasma at room temperature. Microparticle-free plasma was obtained by further centrifugation of platelet-poor plasma at $100{,}000\times g$ for 1 h at 4 °C using ultracentrifuge (HITACHI GX SERIES HIMAC CS 120 GXL, Japan) (Taube et al. 1999).

RBCs isolation and treatment

RBCs were isolated by centrifugation at $200\times g$ for 15 min at room temperature. The supernatant platelet-rich plasma and buffy coat were removed carefully. RBCs were washed three times with Tyrode's buffer (137 mmol/L NaCl, 11.9 mmol/L NaHCO3, 5.5 mmol/L glucose, 5 mmol/L HEPES, 2.7 mmol/L KCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 0.42 mmol/L NaH2PO4, and 0.35 % BSA, pH 7.4). Under a 5 % CO_2 humidified atmosphere at 37 °C, RBCs (1×10^6/mL) were maintained in 10 mL RPMI 1640 supplemented with 5 % autologous microparticle-free plasma in T-25 flasks and exposed to varying concentrations of Hcy (8, 20, 80, 200, 800 μmol/L) for 24 h.

Isolation of RMPs

RBCs in every T-25 flasks were collected by centrifugation ($3{,}000\times g$, 15 min). The supernatants containing RMPs were harvested. As previously described, RMPs were pelleted by ultracentrifugation ($100{,}000\times g$, 1 h, 4 °C) of the supernatants (Chung et al. 2007) and resuspended in 100 μL Tyrode's buffer.

Flow cytometry

RBCs were washed three times in Tyrode's buffer, adjusted to 1×10^6/ml to a final volume of 200 μL in Tyrode's buffer. Then, 5 μL Alexa Fluor 488-lactadherin was added to the RBCs suspension at room temperature, shielded from light (Gao et al. 2012; Tan et al. 2013). After 10 min incubation, the RBCs were analyzed immediately by FACSAria flow cytometry (Becton–Dickinson, USA). BD FACSDiva Software was used to analyze the results.

Microparticles were PS-bearing phospholipid vesicles and smaller than 1 μm (Fu et al. 2010; Rubin et al. 2008). RMPs-containing buffer (100 μL) was stained with 5 μL

Alexa Fluor 488-lactadherin for exactly 30 min in the dark. After addition of 400 μL Tyrode's buffer including a 5×10^5 of 1 μm yellow-green fluorescent microspheres, counts of RMPs were determined by FACS Aria flow cytometer.

Confocal microscopy

RBCs were harvested and washed in Tyrode's buffer. A volume of 5 μL Alexa Fluor 488-lactadherin was incubated with 200 μL RBCs (1×10^6/ml) for 10 min at room temperature in the dark. Cells were washed to remove the unbound dye. The fluorescent-labeled RBCs were resuspended in 0.5 mL Tyrode's buffer. After excitation with 488 nm emission line of a krypton–argon laser and narrow bandpass filters were utilized to restrict emission wave length overlap, the samples in 35-mm glass-bottom culture dish plates (MatTek Corp., Ashland, MA, USA) were observed with a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss Jena GmbH, Jena, Germany).

Scanning electron microscopy

RBCs were fixed with 2.5 % glutaraldehyde-phosphate fixative at 4 °C for 1 h, and allowed to attach onto Formvar-coated coverslips for 30 min at room temperature. After washing with 0.1 mol/L Na-cacodylate HCl buffer, samples were post-fixed with 1 % OsO_4 and dehydration. Then, 10 nm thick platinum was coated before we viewed the images on a S-3400 N scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

Coagulation time and inhibition assay

Coagulation time was measured by a one-step recalcification time assay (Zhou et al. 2010b). Briefly, 100 μl autologous microparticle-free plasma was incubated with 100 μl RBCs (1×10^7 cells in Tyrode's buffer) or 100 μl RMPs for 180 s at 37 °C. After addition of 100 μl of warmed $CaCl_2$ (25 mmol/L), plasma clotting time was the time to the fibrin strand generation by an Amelung KC4A coagulometer (Labcon, Heppenheim, Germany). In addition, 128 nmol/L lactadherin was used to inhibit the coagulation reaction.

Intrinsic factor Xa, extrinsic factor Xa assays and inhibition tests

For intrinsic factor Xa assay (Fu et al. 2010), a total of 10 μL RBCs (1×10^6 cells in Tyrode's buffer) and 10 μL RMPs were incubated with 0.2 nmol/L thrombin, 5 nmol/L factor VIII, 1 nmol/L factor IXa, 130 nmol/L factor X and 5 mmol/L $CaCl_2$ in factor Xa buffer (1 mL 10× TBS, 200 μL 10 % BSA, 8.8 mL ddH_2O). For extrinsic tenase production, cells and RMPs were incubated with 130 nmol/L factor X, 1 nmol/L factor VIIa and 5 mmol/L $CaCl_2$. EDTA (7 mmol/L) was added to stop the reaction. Factor Xa generation was estimated in the presence of 0.8 mmol/L of 10 μL S-2765 at 405 nm by a BioTek Microplate Reader (Winooski, VT, USA). Different concentrations of lactadherin (0–128 nmol/L) were used to inhibit factor Xa.

Prothrombinase assay and inhibition test

The activation of prothrombin was carried out as follows: 1×10^6 RBCs (10 μL) and 10 μL RMPs were added to prothrombinase buffer (1 mL 10 × TBS, 50 μL 10 % BSA, 8.95 mL ddH_2O) containing 1 μmol/L prothrombin, 0.05 nmol/L factor Xa, 1 nmol/L factor Va and 5 mmol/L $CaCl_2$. Thrombin formation was evaluated at 405 nm using the microplate reader after addition of S-2238 (0.8 mmol/L). Varying doses of lactadherin (0–128 nmol/L) were utilized to inhibit thrombin generation.

Results

Quantitation of PS exposure on RBCs and RMPs number

Lactadherin binds to PS with high affinity (Lin et al. 2007), we utilized Alexa Fluor 488-lactadherin to detect the level of PS exposure on RBCs by flow cytometer (Fig. 1a). In the absence of Hcy, RBCs had a low extent of lactadherin binding. Hcy increased the lactadherin binding of cells in a concentration-dependent manner. Statistical significance was observed in 80 μmol/L Hcy-treated RBCs ($p < 0.05$), 200 μmol/L and 800 μmol/L Hcy-treated RBCs ($p < 0.01$). After the 800 μmol/L Hcy treatment, the amount of lactadherin-binding RBCs was about 5 %.

RMPs have diameters smaller than 1 μm. The PS-positive RMPs are binding to fluorescent-labeled lactadherin. Using flow cytometry, Hcy concentration dependently elevated the counts of RMPs, with statistical significance at 200 and 800 μmol/L Hcy ($p < 0.01$, Fig. 1b).

Observation of PS on RBCs

To observe the loss of PS asymmetry on the RBCs surface directly, RBCs were incubated with Alexa Fluor 488-lactadherin and examined using confocal microscopy. Hcy-untreated RBCs appeared biconcave disks, and almost no labeling by fluorescence-labeled lactadherin could be viewed on these RBCs (Fig. 2a). As shown in Fig. 2b, after 800 μmol/L Hcy treatment for 24 h, partial RBCs diffusely

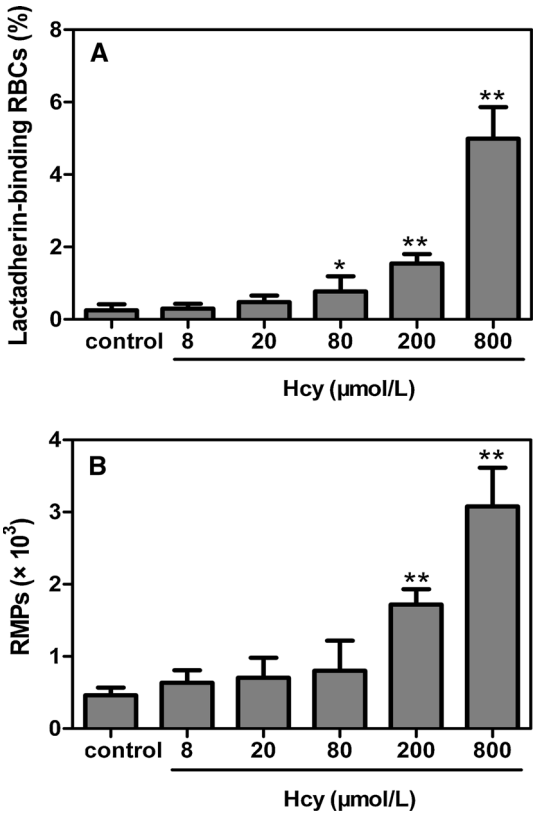

Fig. 1 Flow cytometric analysis of PS exposure on RBCs and RMPs number. RBCs from healthy volunteers were incubated with increasing concentrations of Hcy (8, 20, 80, 200, 800 μmol/L) for 24 h. Hcy-untreated RBCs were utilized as control. $*p < 0.05$ and $**p < 0.01$ indicate significant differences from the control group. **a** RBCs were labeled with Alexa Fluor 488-lactadherin. Lactadherin-binding percent of RBCs was evaluated by flow cytometry. **b** RMPs from 10 mL RBCs culture supernatants were harvested, stained with Alexa Fluor 488-lactadherin. The counts of RMPs were examined using flow cytometry

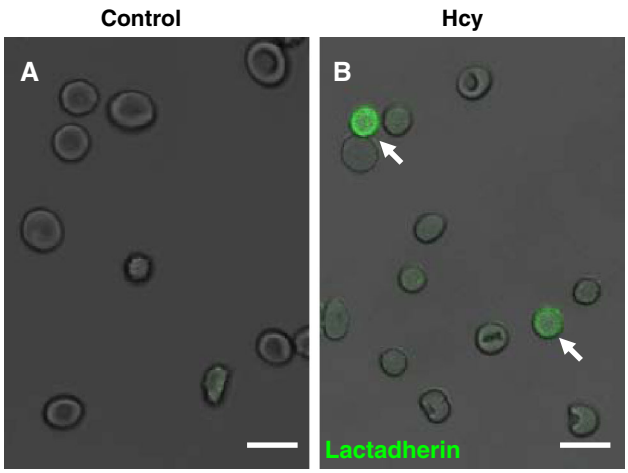

Fig. 2 PS exposure on RBCs using confocal microscopy. RBCs were stained with Alexa Fluor 488-lactadherin in the dark at room temperature. **a** Hcy-untreated RBCs were used as control. **b** RBCs were treated with 800 μmol/L Hcy for 24 h. The outer membrane of some RBCs appeared green when stained with fluorescent-labeled lactadherin (*arrows*). *Bars* represent 10 μm

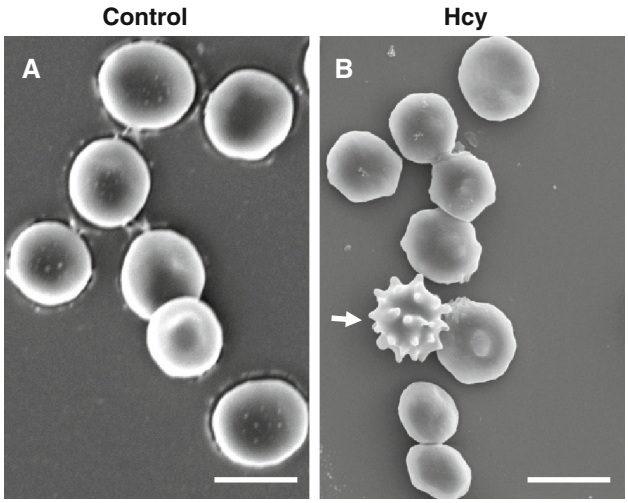

Fig. 3 Scanning electron microscopy of RBCs. The ultrastructure of RBCs was examined by scanning electron microscopy. **a** Shape of RBCs from healthy volunteers in the absence of Hcy. **b** RBCs were exposed to 800 μmol/L Hcy for 24 h, the morphological changes were viewed. An echinocyte (*arrow*). *Bars* represent 6 μm

bound green fluorescence-labeled protein on the outer membrane. The results indicated that PS exposure was increased on RBCs treated with 800 μmol/L Hcy.

Ultrastructure of RBCs

We next used scanning electron microscope to view the surface topography of RBCs. As described in Fig. 3a, RBCs without any treatment had biconcave shape and a disk diameter of about 7 μm. After exposure to 800 μmol/L Hcy for 24 h, most of the RBCs were smaller in size, the membrane of some cells is characterized by many thorny projections (Fig. 3b). Through transmission electron microscope, we confirmed the crenate cells and the concave cells are all red blood cells and they lack a cell nucleus (data not shown).

Coagulation time and complexes of RBCs and RMPs

To investigate the PCA of RBCs and RMPs, recalcification time assay was carried out. Incremental PCA was exhibited by reductive clotting time. Hcy-untreated group was used as control (Fig. 4a). Hcy enhanced the PCA of RBCs and RMPs in a concentration-dependent fashion. For RBCs, the reduction of coagulation time was significant at treatment with 200 and 800 μmol/L Hcy ($p < 0.01$). And for RMPs,

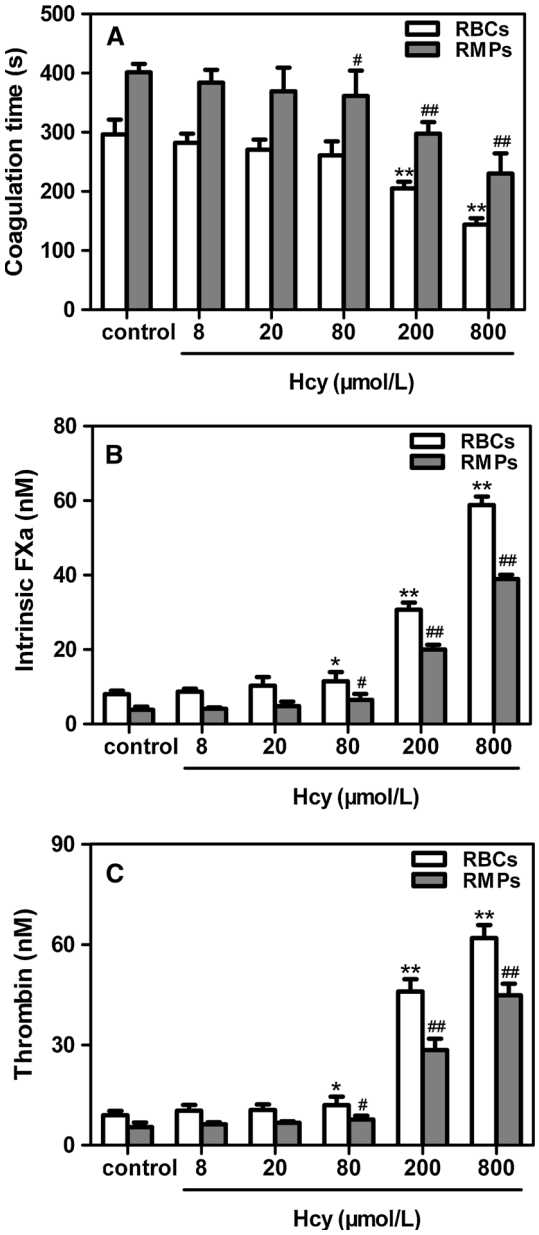

Fig. 4 Recalcification time and enzyme complexes tests. RBCs were treated with different concentrations of Hcys for 24 h, cells and RMPs were collected. Hcy-untreated groups were utilized as control. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences from control RBCs. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with control RMPs. **a** Coagulation time of RBCs (100 μl, 1×10^7 RBCs in Tyrode's buffer) and RMPs (100 μl RMPs prepared from 10 ml of the RBCs supernatants) was estimated. **b** Intrinsic factor Xa generation of RBCs (10 μL, 1×10^6 cells in Tyrode's buffer) and RMPs (10 μL RMPs prepared from 1 ml of the RBCs supernatants) were assessed in the presence of thrombin, factor VIII, factor IXa, factor X and $CaCl_2$ in factor Xa buffer. **c** Thrombin formation of RBCs (1×10^6) and RMPs (10 μL) were determined in the presence of prothrombin, factor Xa, factor Va and $CaCl_2$ in prothrombinase buffer

the reduction of clotting time was significant at treatment with 80 μmol/L Hcy ($p < 0.05$), 200 and 800 μmol/L Hcy ($p < 0.01$).

Next, the capacity of RBCs and RMPs to support the enzyme complexes production that contributes to PCA was explored. We found the Hcy-untreated RBCs or RMPs exhibited little effect on the assembly of prothrombinase complex. Hcy dose dependently increased conversions of factor X to intrinsic factor Xa (Fig. 4b) and prothrombin to thrombin (Fig. 4c) of RBCs and RMPs, respectively, with statistical significance at 80 μmol/L Hcy ($p < 0.05$), 200 and 800 μmol/L Hcy ($p < 0.01$) treatment. But hardly any extrinsic factor Xa production was examined (results not illustrated).

Coagulation inhibition tests of RBCs and RMPs

PS can be blocked with lactadherin (Shi et al. 2004). To detect the relationship between PS exposure and Hcy-induced PCA of RBCs and RMPs, inhibition tests of coagulation were carried out. In the inhibition assay of coagulation time, 128 nmol/L lactadherin efficiently inhibited the PCA of 800 μmol/L Hcy-treated RBCs and RMPs (Fig. 5a).

Moreover, normalized coagulation complexes inhibition data showed that generation of intrinsic factor Xa or thrombin was gradually decreased by increasing concentration of lactadherin, and 128 nmol/L lactadherin inhibited approximately 90 % of intrinsic factor Xa or thrombin production (Fig. 5b, c). These results showed that PS of RBCs and RMPs was important in Hcy-induced PCA.

Discussion

RBCs make up more than 99 % of the total blood cells and live for about 120 days in the circulation. They take part in the process of thrombus formation (Andrews and Low 1999). Many available reports have suggested that the potential reasons include blood viscosity effects, activation of cryptic adhesion receptors of erythrocytes (Andrews and Low 1999) and RBCs–platelet interactions (Santos et al. 1991). Moreover, the RBC surface has the ability to activate coagulation reaction (Kawakami et al. 1995; Whelihan et al. 2012). RMPs are also highly procoagulant (Chung et al. 2007; Rubin et al. 2010). In this study, we demonstrated that Hcy exerted PCA of RBCs via PS exposure and RMPs generation. Externalized PS on RBCs and RMPs supported the intrinsic facter Xa and thrombin formation. In addition, lactadherin worked as the PS blocker to reduce PCA of RBCs and RMPs through inhibition of clotting enzyme complexes.

Hyperhomocysteinemia is a contributing factor in thrombosis (den Heijer et al. 2007; Dayal et al. 2006). Cell membranes with exposed PS form a catalytic surface to accelerate the coagulation cascade. Our prior research

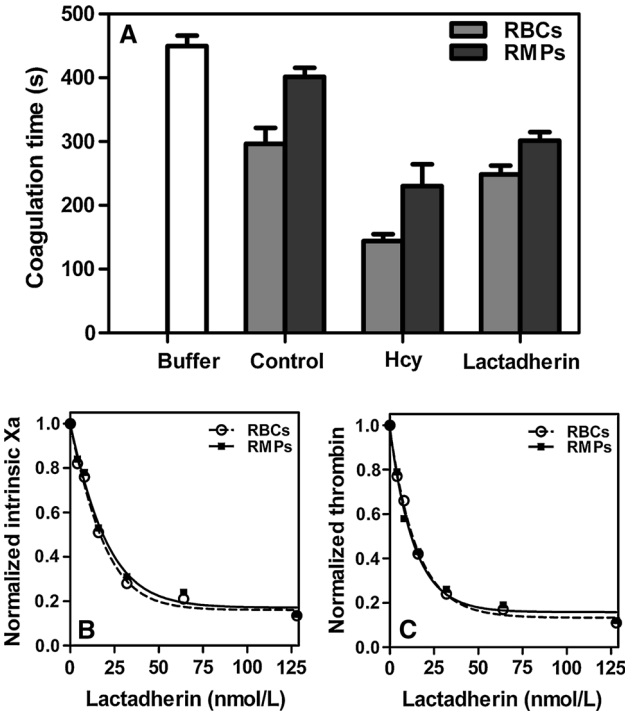

Fig. 5 Inhibition assays of coagulation time and complexes. After exposure to 800 μmol/L Hcy for 24 h, RBCs and RMPs were harvested. **a** Coagulation time of Tyrode's buffer, RBCs and RMPs without Hcy treatment, RBCs and RMPs in the absence or presence of 128 nmol/L lactadherin were determined. **b**, **c** Intrinsic FXa and thrombin production of RBCs/RMPs were assessed with the treatment of increasing doses of lactadherin (0–128 nmol/L). Results were standardized to the amount of formation without lactadherin for comparison of the inhibitive level

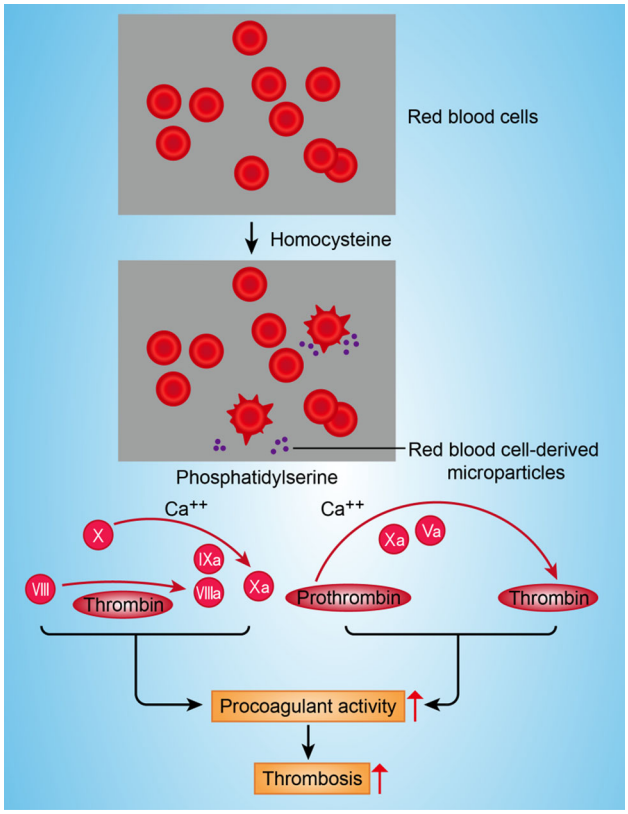

Fig. 6 Schematic diagram for Hcy-induced PCA of RBCs. Hcy-treated RBCs express PS on their outer membrane and generate procoagulant RMPs. Externalized PS on RBCs and PS-bearing RMPs can support conversions of factor X to factor Xa and prothrombin to thrombin, respectively. The increased PCA may contribute to Hcy-associated thrombosis

found that Hcy enhances clot-promoting activity of endothelial cells through PS externalization (Zhu et al. 2012). RBCs make up the largest population of blood cells, but the relationship between Hcy and prothrombotic response of RBCs has not been fully understood. In this study, various doses of Hcy similar to those measured in healthy adults or patients with hyperhomocysteinemia were chosen to co-incubate with RBCs (Hankey and Eikelboom 1999). Using flow cytometry, we found that Hcy increased PS exposure of RBCs surface. Furthermore, Hcy treatment dose dependently decreased clotting time and enhanced coagulation complexes of RBCs.

RMPs could be shed from spiculated plasma membranes of RBCs (Chung et al. 2007). These RMPs express the erythroid cell lineage marker. Using atomic force microscopy, Salzer et al. found that most of the MPs prepared from ionophore A23187-treated erythrocytes are less than 300 nm (Salzer et al. 2002). It was reported that erythrocyte vesiculation helps to clear modified hemoglobin and toxic derivatives, and it associates with elimination of premature removal molecules to protect healthy erythrocytes. Thus, the erythrocyte vesiculation is thought to play a self-protective role (Willekens et al. 2008). RMPs, the submicron expressing PS on their surface, also act as coagulation bombs (Rubin et al. 2010). Using scanning electron microscopy, we found that some RBCs treated by 800 μmol/L Hcy for 24 h changed to echinocytes. Our flow cytometric data showed that Hcy concentration dependently increased counts of RMPs. Moreover, Hcy enhanced PCA of RMPs in a dose-dependent manner. This result is consistent with prior reports that RMPs own thrombogenic activity (Chung et al. 2007; Kozuma et al. 2011).

As the PS probe, lactadherin has more advantages than the widely used annexin V, such as proportional to PS content and independent of Ca^{2+} or PE to bind PS (Shi and Gilbert 2003; Shi et al. 2008). Therefore, lactadherin was utilized to measure PS externalization on RBCs and counts of RMPs in this study. We showed that nearly 90 % thrombin generation and consequent PCA of RBCs and RMPs were inhibited by 128 nmol/L lactadherin. These data indicated that after the treatment with Hcy, PS exposed on cells and RMPs was the important reasons for PCA of RBCs. Lactadherin may act as an efficient

anticoagulant effect and prevent the advance of Hcy-induced blood hypercoagulability.

In summary, we provide new insights into the roles of PS and RBCs in the development of Hcy-related thromboembolism. PS exposure on RBCs and procoagulant PS-bearing RMPs induced by Hcy can support conversions of factor X to factor Xa and prothrombin to thrombin, respectively. Our study suggests that the enhanced PCA may contribute to Hcy-induced coagulopathy (Fig. 6). Lactadherin could be used as an ideal probe for monitoring PS-associated diseases and developed as an attractive therapeutic drug for PS-related hypercoagulable states.

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Conflict of interest Authors declare that they have no conflict of interest.

References

Andrews DA, Low PS (1999) Role of red blood cells in thrombosis. Curr Opin Hematol 6:76–82

Atichartakarn V, Angchaisuksiri P, Aryurachai K, Onpun S, Chuncharunee S, Thakkinstian A, Atamasirikul K (2002) Relationship between hypercoagulable state and erythrocyte phosphatidylserine exposure in splenectomized haemoglobin E/beta-thalassaemic patients. Br J Haematol 118:893–898

Bonomini M, Sirolli V, Merciaro G, Antidormi T, Di Liberato L, Brummer U, Papponetti M, Cappelli P, Di Gregorio P, Arduini A (2005) Red blood cells may contribute to hypercoagulability in uraemia via enhanced surface exposure of phosphatidylserine. Nephrol Dial Transplant 20:361–366

Cai B, Shan L, Gong D, Pan Z, Ai J, Xu C, Lu Y, Yang B (2009) Homocysteine modulates sodium channel currents in human atrial myocytes. Toxicology 256:201–206

Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS, Chung JH (2007) Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. Arterioscler Thromb Vasc Biol 27:414–421

Currò M, Condello S, Caccamo D, Ferlazzo N, Parisi G, Ientile R (2009) Homocysteine-induced toxicity increases TG2 expression in Neuro2a cells. Amino Acids 36:725–730

Dayal S, Wilson KM, Leo L, Arning E, Bottiglieri T, Lentz SR (2006) Enhanced susceptibility to arterial thrombosis in a murine model of hyperhomocysteinemia. Blood 108:2237–2243

den Heijer M, Willems HP, Blom HJ, Gerrits WB, Cattaneo M, Eichinger S, Rosendaal FR, Bos GM (2007) Homocysteine lowering by B vitamins and the secondary prevention of deep vein thrombosis and pulmonary embolism: a randomized, placebo-controlled, double-blind trial. Blood 109:139–144

Fu Y, Zhou J, Li H, Cao F, Su Y, Fan S, Li Y, Wang S, Li L, Gilbert GE, Shi J (2010) Daunorubicin induces procoagulant activity of cultured endothelial cells through phosphatidylserine exposure and microparticles release. Thromb Haemost 104:1235–1241

Gao C, Xie R, Yu C, Wang Q, Shi F, Yao C, Xie R, Zhou J, Gilbert GE, Shi J (2012) Procoagulant activity of erythrocytes and platelets through phosphatidylserine exposure and microparticles release in patients with nephrotic syndrome. Thromb Haemost 107:681–689

Hankey GJ, Eikelboom JW (1999) Homocysteine and vascular disease. Lancet 354:407–413

Hvarregaard J, Andersen MH, Berglund L, Rasmussen JT, Petersen TE (1996) Characterization of glycoprotein PAS-6/7 from membranes of bovine milk fat globules. Eur J Biochem 240: 628–636

Kawakami S, Kaibara M, Kawamoto Y, Yamanaka K (1995) Rheological approach to the analysis of blood coagulation in endothelial cell-coated tubes: activation of the intrinsic reaction on the erythrocyte surface. Biorheology 32:521–536

Kozuma Y, Sawahata Y, Takei Y, Chiba S, Ninomiya H (2011) Procoagulant properties of microparticles released from red blood cells in paroxysmal nocturnal haemoglobinuria. Br J Haematol 152:631–639

Lin L, Huai Q, Huang M, Furie B, Furie BC (2007) Crystal structure of the bovine lactadherin C2 domain, a membrane binding motif, shows similarity to the C2 domains of factor V and factor VIII. J Mol Biol 371:717–724

Perła-Kaján J, Twardowski T, Jakubowski H (2007) Mechanisms of homocysteine toxicity in humans. Amino Acids 32:561–572

Rubin O, Crettaz D, Canellini G, Tissot JD, Lion N (2008) Microparticles in stored red blood cells: an approach using flow cytometry and proteomic tools. Vox Sang 95:288–297

Rubin O, Crettaz D, Tissot JD, Lion N (2010) Microparticles in stored red blood cells: submicron clotting bombs? Blood Transfus 8(Suppl 3):31–38

Salzer U, Hinterdorfer P, Hunger U, Borken C, Prohaska R (2002) Ca(++)-dependent vesicle release from erythrocytes involves stomatin-specific lipid rafts, synexin (annexin VII), and sorcin. Blood 99:2569–2577

Santos MT, Valles J, Marcus AJ, Safier LB, Broekman MJ, Islam N, Ullman HL, Eiroa AM, Aznar J (1991) Enhancement of platelet reactivity and modulation of eicosanoid production by intact erythrocytes. A new approach to platelet activation and recruitment. J Clin Invest 87:571–580

Setty BN, Rao AK, Stuart MJ (2001) Thrombophilia in sickle cell disease: the red cell connection. Blood 98:3228–3233

Shi J, Gilbert GE (2003) Lactadherin inhibits enzyme complexes of blood coagulation by competing for phospholipid-binding sites. Blood 101:2628–2636

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

Shi J, Shi Y, Waehrens LN, Rasmussen JT, Heegaard CW, Gilbert GE (2006) Lactadherin detects early phosphatidylserine exposure on immortalized leukemia cells undergoing programmed cell death. Cytometry A 69:1193–1201

Shi J, Pipe SW, Rasmussen JT, Heegaard CW, Gilbert GE (2008) Lactadherin blocks thrombosis and hemostasis in vivo: correlation with platelet phosphatidylserine exposure. J Thromb Haemost 6:1167–1174

Stubbs JD, Lekutis C, Singer KL, Bui A, Yuzuki D, Srinivasan U, Parry G (1990) cDNA cloning of a mouse mammary epithelial cell surface protein reveals the existence of epidermal growth factor-like domains linked to factor VIII-like sequences. Proc Natl Acad Sci USA 87:8417–8421

Tan X, Shi J, Fu Y, Gao C, Yang X, Li J, Wang W, Hou J, Li H, Zhou J (2013) Role of erythrocytes and platelets in the hypercoagulable status in polycythemia vera through phosphatidylserine exposure and microparticle generation. Thromb Haemost 109:1025–1032

Taube J, McWilliam N, Luddington R, Byrne CD, Baglin T (1999) Activated protein C resistance: effect of platelet activation, platelet-derived microparticles, and atherogenic lipoproteins. Blood 93:3792–3797

Wahid ST, Marshall SM, Thomas TH (2001) Increased platelet and erythrocyte external cell membrane phosphatidylserine in type 1 diabetes and microalbuminuria. Diabetes Care 24:2001–2003

Whelihan MF, Mann KG (2013) The role of the red cell membrane in thrombin generation. Thromb Res 131:377–382

Whelihan MF, Zachary V, Orfeo T, Mann KG (2012) Prothrombin activation in blood coagulation: the erythrocyte contribution to thrombin generation. Blood 120:3837–3845

Willekens FL, Werre JM, Groenen-Döpp YA, Roerdinkholder-Stoelwinder B, de Pauw B, Bosman GJ (2008) Erythrocyte vesiculation: a self-protective mechanism? Br J Haematol 141: 549–556

Zhou J, Shi J, Hou J, Cao F, Zhang Y, Rasmussen JT, Heegaard CW, Gilbert GE (2010a) Phosphatidylserine exposure and procoagulant activity in acute promyelocytic leukemia. J Thromb Haemost 8:773–782

Zhou J, Zheng Y, Shi J, Lu C, Hou J, Yu H, Qiao X, Qi S, Gilbert GE (2010b) Daunorubicin induces procoagulant response through phosphatidylserine exposure in red blood cells. Thromb Res 125:178–183

Zhu J, Xie R, Piao X, Hou Y, Zhao C, Qiao G, Yang B, Shi J, Lu Y (2012) Homocysteine enhances clot-promoting activity of endothelial cells via phosphatidylserine externalization and microparticles formation. Amino Acids 43:1243–1250

Zwaal RF, Schroit AJ (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood 89:1121–1132