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Sickle Red Blood Cell-Derived Extracellular Vesicles Activate Endothelial Cells and Enhance Sickle Red Cell Adhesion Mediated by von Willebrand Factor

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ORIGINAL PAPER

Sickle red blood cell-derived extracellular vesicles activate endothelial cells and enhance sickle red cell adhesion mediated by von Willebrand factor

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Summary

Endothelial activation and sickle red blood cell (RBC) adhesion are central to the pathogenesis of sickle cell disease (SCD). Quantitatively, RBC-derived extracellular vesicles (REVs) are more abundant from SS RBCs compared with healthy RBCs (AA RBCs). Sickle RBC-derived REVs (SS REVs) are known to promote endothelial cell (EC) activation through cell signalling and transcriptional regulation at longer terms. However, the SS REV-mediated short-term non-transcriptional response of EC is unclear. Here, we examined the impact of SS REVs on acute microvascular EC activation and RBC adhesion at 2 h. Compared with AA REVs, SS REVs promoted human pulmonary microvascular ECs (HPMEC) activation indicated by increased von Willebrand factor (VWF) expression. Under microfluidic conditions, we found abnormal SS RBC adhesion to HPMECs exposed to SS REVs. This enhanced SS RBC adhesion was reduced by haeme binding protein haemopexin or VWF cleaving protease ADAMTS13 to a level similar to HPMECs treated with AA REVs. Consistent with these observations, haemin- or SS REV-induced microvascular stasis in SS mice with implanted dorsal skin-fold chambers that was inhibited by ADAMTS13. The adhesion induced by SS REVs was variable and was higher with SS RBCs from patients with increased markers of haemolysis (lactate dehydrogenase and reticulocyte count) or a concomitant clinical diagnosis of deep vein thrombosis. Our results emphasise the critical contribution made by REVs to the pathophysiology of SCD by triggering acute microvascular EC activation and abnormal RBC adhesion. These findings may

Ran An and Yuncheng Man contributed equally to this work.

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help to better understand acute pathophysiological mechanism of SCD and thereby the development of new treatment strategies using VWF as a potential target.

KEYWORDS
adamts 13, endothelial inflammation, extracellular vesicles, sickle cell disease, von willebrand factor

INTRODUCTION

Sickle cell disease (SCD) is a genetically inherited blood disorder, in which a point mutation in the beta globin chain gene replaces A with T at codon 6. This causes a switch from a hydrophilic glutamic acid to a hydrophobic valine, producing abnormal sickle haemoglobin (HbS). HbS polymerises into long and stiff intracellular structures under dehydration and deoxygenation, leading to sickle-shaped, excessively stiff, and adhesive red blood cells (RBCs). Sickle RBCs (SS RBCs) are prone to extra- and intravascular haemolysis.[1] During haemolysis, SS RBCs release damage-associated molecular patterns (DAMPs) including haemoglobin/haeme and extracellular vesicles (EVs).[2–6] The DAMPs cumulatively promote a pro-inflammatory milieu with decreased levels of natural occurring anticoagulant proteins (i.e., protein C and protein S), nitric oxide (NO) bioavailability, and ADAMTS13, and with increased levels of von Willebrand factor (VWF).[7] The decreased of levels of natural anticoagulant proteins may promote endothelial damage,[8,9] the decreased NO bioavailability decreases its effect in impairing blood-endothelial cell (EC) adhesion,[10] and the increased levels of VWF favours RBC adhesion on endothelium.[11] Consequently, these DAMP-associated endothelial damages intermittent vaso-occlusion of the microvasculature in SCD.[2,12–14]

Extracellular vesicles are membrane-bound submicron particles (0.1–1.0 μm) generated from cells under normal and activated conditions with a broad spectrum of size, density, biochemical composition and cellular origins.[15,16] EVs contain various cargos including proteins, lipids and nucleic acids, which may reflect the state of activation of the cells from which they originate[17] and can serve as vehicles for cellular communication.[15] EVs biogenesis were associated with both endosomal sorting complex required for transport (ESCRT)-dependent and -independent pathways. Specifically, RBC-derived EVs (REVs) biogenesis involves alterations in RBC membrane proteins such as band 3[6] and sphingomyelin.[16,18] The level of total circulating EVs (including EVs from platelets, leucocytes, ECs, and RBCs) have been reported to be higher in the bloodstream of patients with SCD at steady state than in healthy controls and increased during SCD vaso-occlusive crises.[19,20] REVs made up the most prevalent subtype, and correlated with haemolysis, oxygen saturation, pulmonary systolic pressure and mortality.[21] REVs in SCD (SS REVs) may be produced through accelerated ageing of RBCs during oxygenation/deoxygenation cycles,[16] under the influence of stress factors,[4,22–24] or during haemolysis in capillary beds. SS REVs are reported to have increased phosphatidylserine (PS) expression on their surface,[12] as well as to contain haeme, Hb, oxidised Hb, ferryl Hb, and microRNAs.[3,25,26] EVs in SCD are known to have a range of pathophysiological impacts on blood–vascular interactions including NO scavenging,[10] immune modulation,[18] coagulation,[27] decrease endothelial monolayer integrity,[28–30] and adhesion of blood cells to the endothelium.[5,28] Garnier et al.[5] showed plasma EV-treated ECs upregulated messenger RNA and protein expression of intercellular adhesion molecule-1 (ICAM-1) at longer terms (4 or 6 h), which led to significantly enhanced neutrophil recruitment to human endothelium in SCD. Gemel et al.[29] and Lapping-Carr et al.[30] reported that plasma EVs isolated from patients with SCD during acute chest syndrome had significantly decreased levels of endothelial vascular endothelial-cadherin protein and disrupted junctions at 48 h. Although these works mainly utilised plasma EVs, the roles of REVs within the plasma EVs were emphasised. Camus et al.[3] demonstrated that in transgenic SAD mice, SS REVs are capable of transferring haeme to ECs, triggering microvascular occlusions, likely through toll-like receptor-4 (TLR-4)-dependent pathways. Importantly, ~45% of the cell-free haeme in plasma is bound to REVs,[3] therefore endothelial activation via cell-free haeme is likely mediated by the transfer of haeme through REVs.[3] However, most of the existing work indicate SS REVs promote endothelial activation at longer terms (>4 h) through transcriptional responses, while the acute (≤2 h) impact of REVs on endothelial activation has not been fully elucidated.

In this study, we utilised our previously developed endothelium-on-a-chip platform[31] to analyse RBC adhesion to REV-activated human pulmonary microvascular ECs (HPMECs) under physiological flow conditions. SS REVs were generated by increasing the intracellular calcium concentration in SS RBCs. After short-term (2 h) REV activation of microvascular ECs, which mimics the pro-inflammatory phenotype of microvascular endothelium during acute haemolysis in SCD, we analysed RBC adhesion to the HPMECs using clinical blood samples from patients with homogeneous SCD followed in our clinic. Here, we report SS RBC adhesion on REV-activated HPMECs and its clinical associations in SCD. The aim of the present study was to demonstrate the acute impact of REVs generated from SS RBCs on microvascular EC phenotype, focusing on abnormal RBC adhesion.

PATIENTS AND METHODS

Blood sample collection

All experiments were performed in accordance with approved study protocol by the Institutional Review Board (IRB) committee (IRB 05-14-07C). Blood samples from de-identified adult patients with HbSS were collected in EDTA-containing vacutainer tubes at the outpatient clinic at

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University Hospitals Cleveland Medical Center (UHCMC) in Cleveland, Ohio. Informed consent was obtained from all study participants, and all blood samples were collected from participants with HbSS at clinical baseline (steady state). Baseline was defined as steady state for at least 2 weeks before blood collection (no crises as identified by day hospital or emergency department visit, or hospital admission for pain). Healthy donor (HbAA) blood samples were collected in EDTA-containing vacutainer tubes from biorepository at Case Western Reserve University. There were no known medical issues with any of the healthy donors in this study. Hb composition of SCD samples was identified by high-performance liquid chromatography (HPLC) at the Core Laboratory of UHCMC. Clinical variables of the study patients with SCD were obtained from the medical record.

Microfluidic channel endothelialisation

Microfluidic channels were fabricated as previously described.[32–36] Briefly, the microchannels were fabricated by assembling three layers, including a glass slide as the bottom layer, double-sided adhesive (DSA) film as the middle layer, and polymethyl methacrylate (PMMA) as the top layer. Microchannel geometry was determined by the middle DSA layer and channel inlet and outlet was determined on the top PMMA layer (Figure S1). The microchannels were incubated with 0.2 mg/ml fibronectin for 1 h at 37°C. HPMECs were seeded into the microchannels and cultured with 5% CO_2 at 37°C under 100 µl/min flow for 48–72 h until a confluent monolayer over the microchannel bottom surface was formed (Figure S1).

Red blood cell-derived extracellular vesicle (REV) generation and characterisation

Pooled blood samples from 10 patients with HbSS and five healthy donors were used to derive HbSS REV and HbAA REV respectively. RBCs were isolated from either individual or pooled blood samples by centrifuging for 5 min at 500 × *g* at room temperature. Plasma, buffy coat, and the near-plasma portion of the RBC layer were carefully removed. The isolated RBCs were washed twice with phosphate-buffered saline (PBS) and were re-suspended in Hank's balanced salt solution modified with calcium and magnesium (Hank's buffer) at 20% haematocrit. The RBC suspensions were then stimulated with 2 µM calcium ionophore (A23187) for 1 h at 37°C. To isolate generated REVs, cell suspensions were subject to differential centrifugation, initially at 1500 × *g* for 15 min followed by the second-step centrifugation at 3000 × *g* for 15 min. Finally, the supernatants were centrifuged at 25000 × *g* for 2 h at 4°C to harvest REV pellets. REVs were re-suspended in either cell culture media (for EC activation) or PBS (for REV characterisation), split into 20 µl aliquots and stored in −80°C before using. The size distribution and concentration of the generated REVs were measured using ZetaView® particle tracking analyser (Particle-Metrix). For measuring haeme content, REVs were lysed using 0.5% Triton X-100 in deionised water. The lysed solutions were then characterised using Haeme Assay Kit (Sigma-Aldrich). Equal total number of SS REVs and AA REVs were used for haeme content measurement.

Red blood cell-derived extracellular vesicle endothelial cell activation

For all endothelial activation experiments without haemopexin, stock REVs were thawed at 4°C and brought to 37°C in the cell culture incubator. To test the impact of haemopexin on REV-mediated HPMEC activation, REVs were incubated with 2 µM human haemopexin (Sigma-Aldrich) for 1 h at room temperature. For all experiments, cultured HPMECs (LONZA) were washed with fresh culture medium and were then incubated with HbSS REVs or HbAA REVs for 2 h at 37°C. The activated HPMECs were then washed with fresh culture medium and used for adhesion experiments. In control experiments, SS REVs and AA REVs were reconstituted to the same concentration.

Haeme endothelial cell activation

Haeme solution was used to activate ECs in order to compare the activation effect of REVs and purified haeme. Briefly, haeme stock solution was prepared by dissolving bovine haemin in 0.1 M NaOH solution to obtain a final haeme concentration of 40 mM. Next, the stock solution was diluted using RPMI-1640 (FBS and antibiotics free) to the final working concentrations equivalent to the haeme content in REVs determined using the protocol described in Section 2.3.

Fluorescent labelling of von Willebrand factor on endothelial cells

Following REV activation, HPMECs were rinsed with fresh culture medium and fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed HPMECs were rinsed with PBS twice and blocked with 2% bovine serum albumin for 1 h at room temperature. After washing with PBS, HPMECs were incubated with sheep polyclonal anti-human VWF antibody (Abcam) conjugated with fluorescein isothiocyanate (FITC, 1:100 v/v dilution) for 1 h at room temperature in the dark. Fluorescent images were then acquired at multiple locations throughout the microchannel of REV activated HPMECs at ×10.

Adhesion experiments

For all RBC adhesion experiments without VWF cleaving protease ADAMTS13, RBCs were isolated from individual

blood samples by centrifugation for 5 min at 500 × *g* at room temperature. The isolated RBCs were washed twice with PBS and were re-suspended in fresh basal medium supplemented with 10 mM HEPES at 20% haematocrit. To test the impact of ADAMTS13 on RBC adhesion, freshly isolated RBCs in basal medium supplemented with 10 mM HEPES at 20% haematocrit were incubated for 30 min at 37°C with gentle shaking with 5 μg/ml ADAMTS13 (R&D Systems), and this RBC suspension in HEPES-ADAMTS13 was used for adhesion experiments.[37,38]

For all RBC adhesion experiments, a total sample volume of 15 μl was perfused into the microchannel at the shear rate of 1 dyne/cm^2, corresponding to a typical value observed in human post-capillary venules. After blood perfusion, the microchannels were rinsed with fresh basal medium supplemented with 10 mM HEPES to remove non-adherent RBCs. Visual confirmation of adhesion results confirmed negligible contamination by platelets or white blood cells.

Mice

All animal experiments were approved by the University of Minnesota's Institutional Animal Care and Use Committee. In this study, we utilised male and female HbSS-Townes on a 129/B6 mixed genetic background. The SS mice were created by knocking in human α and $^{A}\gamma\beta^{S}$ globins into the sites where murine α-globin and β-globin were knocked out.[32] SS mice have severe anaemia and an SS RBC half-life of 2.5 days (normal half-life span in mice ~22.6 days).[32] All mice were monitored daily including weekends and holidays for health problems, food and water levels and cage conditions. Littermates were randomly assigned to different treatment groups. All mice were included in each end-point analysis and there were no unexpected adverse events that required modification of the protocol. The mice were aged 11–14 weeks.

Statistical methods

Mean ± standard deviation (SD) of the mean were reported for acquired data in this study. Minitab 18 Software (Minitab Inc.) was used to perform all statistical analyses. Data normality was initially analysed. For comparison, normally distributed data were analysed by two groups *t*-test. Statistical significance was set at 95% confidence level for all tests ($p < 0.05$).

RESULTS

Characterisation of red blood cell-derived extracellular vesicles

We characterised and compared calcium ionophore-derived AA REVs, generated from the pooled blood samples from five healthy donors, and calcium ionophore-derived SS REVs, generated from the pooled blood samples from five patients with SCD (Figure 1). The nanoparticle tracking analysis results demonstrated that derived AA and SS REVs had similar diameters (10–700 nm with >95% of them between 80–400 nm, Figure 1A,B, mean size [SD]: AA REV = 205.4 [223.7] vs. SS REV = 181.7 [200.2] nm). However, per RBC volume, SS RBCs generated significantly more REVs than did AA RBCs (Figure 1C, mean [SD] concentration: SS REV = 3.17×10^7 [3.91×10^6] vs. AA REV = 6.35×10^6 [5.68×10^5]/mL, $p = 0.011$, two-sample *t*-test). The Haeme Assay Kit determined that the generated SS REVs contained a significantly higher concentration of haeme than did the same number of AA REVs (Figure 2D, mean [SD] concentration: SS REV haeme = 17.3 [1.1] vs. AA REV haeme = 6.2 [0.3] μM).

SS REVs increase endothelial von Willebrand factor expression

We incubated HPMECs with derived SS or AA REVs for 2 h. VWF expression was significantly increased on HPMECs incubated with derived SS REVs (Figure 2A,B) compared to VWF expression on HPMECs incubated with derived AA REVs (Figure 2C, $p = 0.012$, two-sample *t*-test).

HbSS RBC adhere specifically to HPMECs incubated with HbSS REV

We first verified the in vitro approach by examining the interactions between SS RBC, AA RBC, and HPMECs treated with SS REVs and AA REVs. HPMEC laminated microchannels were treated with derived SS REV (Figure 2D,F) or derived AA REV (Figure 2E). SS RBC suspensions were then perfused into the first two channels (Figure 2D,E), and AA RBC suspension was perfused into the third (Figure 2F, per the methods section on adhesion experiments). No visible injury or morphological changes were observed on HPMECs treated with SS REV or AA REV. More adherent SS RBCs were observed on HPMECs activated with SS REVs than with AA REVs (Figure 2G, mean [SD] 157 [42] vs. 19 [12], $p < 0.05$) and AA RBC on HPMECs treated with SS REVs (Figure 2G, mean [SD] 16 [12], $p < 0.05$), as well as AA RBC on HPMECs treated with AA REVs (Figure 2G, mean [SD] 5 [4], $p < 0.05$).

REV-mediated RBC adhesion to HPMECs is attenuated by haemopexin

To demonstrate that the observed RBC adhesion was induced by REV-mediated HPMEC activation, we investigated the impact of the haeme-binding protein haemopexin on REV-mediated RBC adhesion. Pre-incubating REVs with haemopexin reduced the HPMEC activation indicated by reduced number of adherent SS RBCs (Figure 2G, mean [SD] 157 [42] vs. 14 [8], $p < 0.05$).

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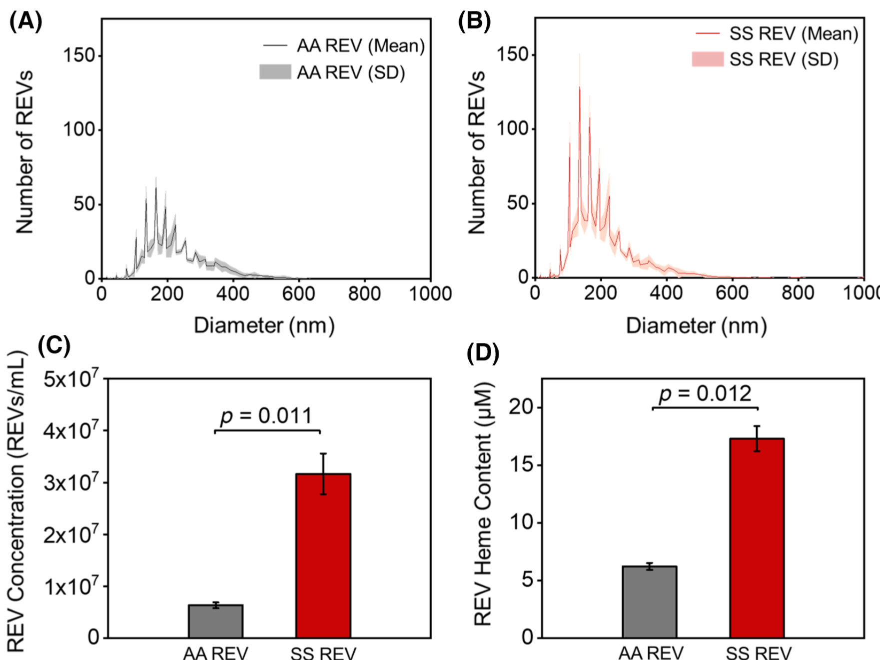

FIGURE 1 SS RBCs generate increased level of REVs than AA RBCs and SS REVs carry higher concentration of haeme than AA REVs. (A, B) size distribution of AA REVs and SS REVs generated from RBCs in pooled-samples from healthy donors or pooled-samples from patients with SCD. Black and red lines indicate the mean number of AA and SS REVs from five individual tests and grey and light-red shaded area indicate the standard deviation (SD). AA REV and SS REV share similar size distribution (mean [SD] size: AA REV = 205.4 [223.7] vs. SS REV = 181.7 [200.2] nm). (C) Concentration of AA REVs and SS REVs generated in vitro per microlitre of purified RBCs in samples from either healthy donors (grey) and patients with SCD (red). SS RBCs generated statistically significantly higher quantity of REVs than AA RBCs (mean [SD] concentration: SS REV = 3.17×10^7 [3.91×10^6] vs. AA REV = 6.35×10^6 [5.68×10^5] ml, $p = 0.011$, two-group t-test). (D) Haeme concentration carried by equal number of AA REVs (grey) and SS REVs (red). SS REVs carried statistically significantly higher concentration of haeme than AA REVs (mean [SD] concentration: SS REV = 17.3 [1.1] vs. AA REV = 6.2 [0.3] μM, $p = 0.012$, two-group t-test). RBC, red blood cell; REVs, RBC-derived extracellular vesicles.*$p = 0.002$; **$p = 0.001$; not significant (NS), $p = 0.058$.

REV-mediated RBC adhesion to HPMECs is attenuated by ADAMTS13

To demonstrate that the observed RBC adhesion were mediated by VWF on REV-activated HPMECs, we investigated the impact of the VWF-specific protease ADAMTS13 on REV-mediated RBC adhesion. The addition of ADAMTS13 significantly reduced the adhesion of SS RBCs to SS REV-activated HPMECs (Figure 2G, mean [SD] 157 [42] vs. 19 [10], $p < 0.05$). These results indicate that RBC adhesion was dependent on HPMEC VWF, because ADAMTS13 dramatically inhibited RBC adhesion, reaching a level that was close to SS RBC on HPMECs treated with AA REVs.

Haeme is comparable to REVs in mediating RBC adhesion to HPMECs

To compare the impact of REVs and haeme on activating HPMECs, we examined the SS RBC adhesion on HPMECs that are activated with 20 μM haeme, which is comparable to the determined SS REV mean (SD) haeme concentration of 17.3 (1.1) μM. The numbers of adherent SS RBCs on SS REV activated HPMECs and on 20 μM haeme-activated HPMECs demonstrated no statistical difference, although a slightly higher adhesion profile was observed on HPMECs activated with SS REVs (Figure 2G, mean [SD] 157 [42] vs. 140 [49], $p = 0.058$).

Haeme- or SS REV-induced vaso-occlusion in SS Townes mice that was inhibited by ADAMTS13

As ADAMTS13 reduced RBC–EC adhesion and RBC adhesion to endothelium is believed to be involved in vaso-occlusion in SCD, we used Townes SS mice and a dorsal skin-fold chamber model to determine if ADAMTS13 would reduce microvascular stasis in response to haemin or SS REVs. Townes SS mice were surgically implanted with a dorsal skin-fold chamber. After chamber implantation, 20–21 subcutaneous flowing venules were selected at baseline using intravital microscopy. After selection and mapping of flowing venules, SS mice were infused via the tail vein with haemin (3.2 μmol/kg) or SS REVs at time zero to induce vaso-occlusion.[3] Then, 20 min later, mice were infused with ADAMTS13 (1 mg/kg) or sterile saline (10 ml/kg). The same venules selected at baseline were re-examined for stasis (no flow) at 1, 2, 3, and 4 h after haemin or SS REV infusion. In SS mice, the levels of haeme- and REV-mediated vaso-occlusion (stasis) at 1, 2, 3, and 4 h were similar (Figure 3A,B). Additionally, ADAMTS13 administered intravenously

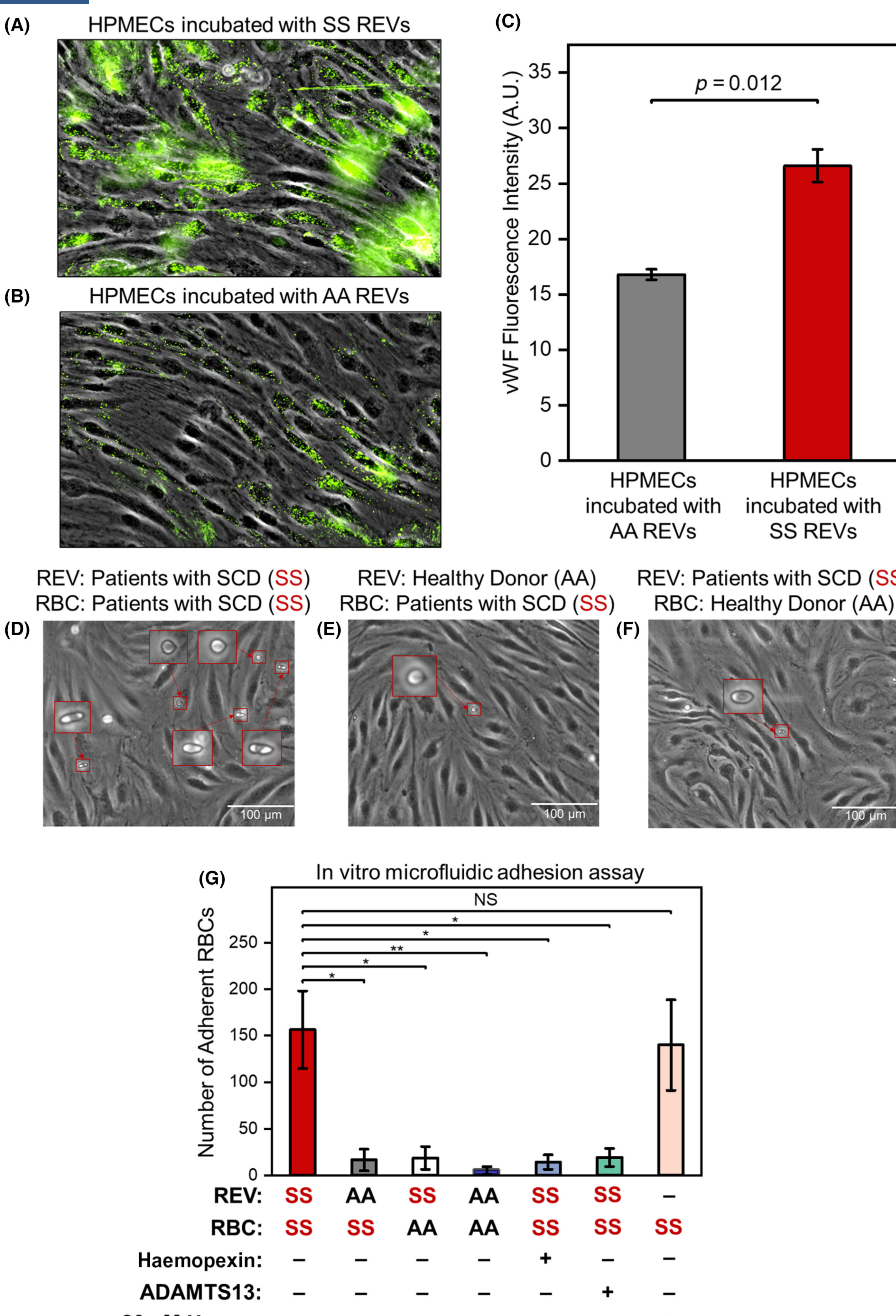

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FIGURE 2 SS REVs activate HPMECs, increase VWF expression and mediates enhanced adhesion of SS RBCs, which can be reduced by haemopexin and ADAMTS13. (A, B) HPMECs were treated with SS REVs (A) and AA REVs (B) for 2 h at 37°C and incubated with fluorescently labelled antibodies against VWF following a fixing step with 4% paraformaldehyde. The SS REV treated HPMECs demonstrated significantly increased VWF expression comparing to HPMECs incubated with AA REVs (C, n = five, p = 0.012, two-sample t-test). (D) SS RBC adhesion on HPMECs activated with SS REVs. (E) SS RBCs adhesion on HPMECs treated with AA REVs. (F) AA RBCs adhesion on HPMECs treated with SS REVs. Insets are closer view of RBCs adhered to HPMECs. (G) Interaction between SS RBC and SS REV activated HPMECs is significantly stronger than the other test groups, and this interaction is reduced by haemopexin and ADAMTS13 (mean [SD]: SS REV-SS RBC = 157 [42], AA REV-SS RBC = 16 [12], AA REV-SS RBC = 19 [12], AA REV-AA RBC 5 [4], SS REV-SS RBC with haemopexin = 14 [8], SS REV-SS RBC with ADAMTS13 = 19 [10], and haeme-SS RBC = 140 [49], $p < 0.05$ for all groups except for haeme-SS RBC, n = five in each group, two-group t-test). HPMECs, human pulmonary microvascular endothelial cells; RBC, red blood cell; REVs, RBC-derived extracellular vesicles; VWF, von Willebrand factor.

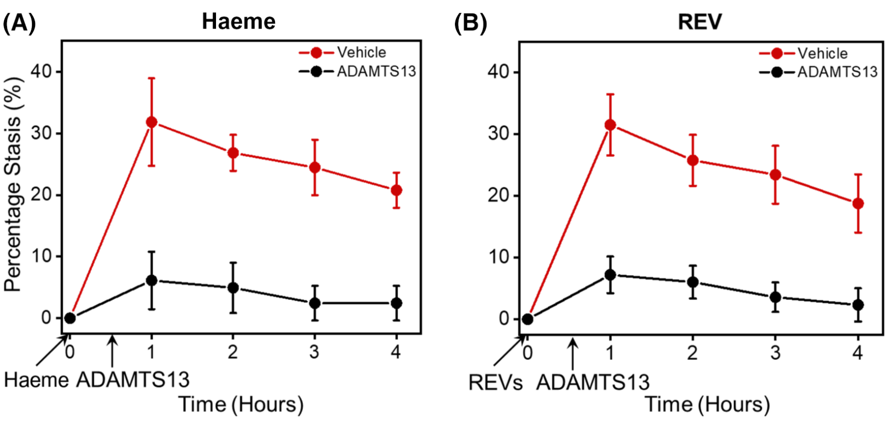

FIGURE 3 Haeme- and REV-induced vaso-occlusion in SS Townes mice are inhibited by ADAMTS13. (A) Haeme-induced vaso-occlusion (measured in percentage of stasis) in vivo in SS Townes mice is inhibited by ADAMTS13 (mean [SD] for 1, 2, 3, and 4 h: Vehicle: 31.8% [7.1]%, 26.8% [2.9]%, 24.5% [4.5]%, 20.8% [2.9]% vs. ADAMTS13: 6.1% [4.7]%, 4.9% [4.1]%, 2.4% [2.8]%, 2.4% [2.8]%, $p < 0.05$ for all time points, n = four for each group at each time point). (B) REV-induced vaso-occlusion in vivo in SS Townes mice is inhibited by ADAMTS13 (mean [SD] for 1, 2, 3, and 4 h: Vehicle: 31.5% [5.0]%, 25.8% [4.1]%, 23.4% [4.7]%, 18.7% [4.7]% vs. ADAMTS13: 7.2% [3.0]%, 6.0% [2.7]%, 3.6% [2.4]%, 2.3% [2.7]%, $p < 0.05$ for all time points, n = four for each group at each time point). REVs, RBC-derived extracellular vesicles.

20 min after haemin or SS REV infusion significantly inhibited stasis at 1, 2, 3, and 4 h post-haemin or post-REV as compared to SS mice that received saline at 20 min (Figure 3A,B).

Clinical implications of REV-mediated RBC adhesion to HPMECs

To determine the association between clinical phenotypes and adhesion profiles, we examined a haemolytic subpopulation (Group 1, N = six) with distinctly lower lactate dehydrogenase (LDH) levels and absolute reticulocyte counts (ARCs) compared with the second subpopulation (Group 2, N = nine, Figure 4A).[39] Patients in Group 1 had significantly lower LDH levels and ARCs (N = eight, LDH mean [SD]: Group 1: 186 [99] vs. Group 2: 305 [55] iu, p = 0.031, two-group t-test; ARCs mean [SD]: Group 1: 162 [74] vs. Group 2: 401 [122] $\times 10^{10}$, $p < 0.001$, two-group t-test). We then compared the RBC adhesion profile between Group 1 and Group 2 patients and found significantly lower RBC adhesion to HbS REV activated HPMECs in Group 1 patients than in Group 2 patients (Figure 4B, mean [SD] 117 [72] vs. 267 [125], p = 0.012, two-group t-test). Patients in Group 1 also had lower white blood cell (WBC) counts than did patients in Group 2 (Figure 4C, mean [SD] 8.5 [1.2] vs. 11.4 [3.6] $\times 10^9$/L, p = 0.047, two-group t-test). Group 1 also had a suggestion of having lower ferritin values (Figure 4D, mean [SD] 876 [1527] vs. 2841 [2782] μg/L, p = 0.106, two-group t-test). Similar differences of RBC adhesion profiles between the two groups of patients were also observed within the groups distinguished using a single cut-off level of ARC level at 300×10^9/L or using a single cut-off level of LDH level at 250 u/L (Figure S2).

Finally, patients with a documented history of deep vein thromboses (DVT, N = four) had higher RBC adhesion to REV-activated HPMECs compared to those without confirmed DVT (N = 11, Figure 4E, mean [SD] 361 [119] vs. 151 [78], p = 0.033, two-group t-test). None of the six Group 1 patients and four of the seven Group 2 patients (with definitive DVT record) were diagnosed with DVT. DVT records of two patients in Group 2 were not available (DVT diagnosis for one patient was not clear and the DVT record was not accessible for the other patient). Similarly, Group 2 patients with higher RBC adhesion profile had significantly greater possibility of having DVT than patients in Group 1 (Table S1).

DISCUSSION

Two dominant pathophysiological events have been characterised with SCD: abnormal RBC adhesion and intravascular haemolysis. Intravascular haemolysis promotes

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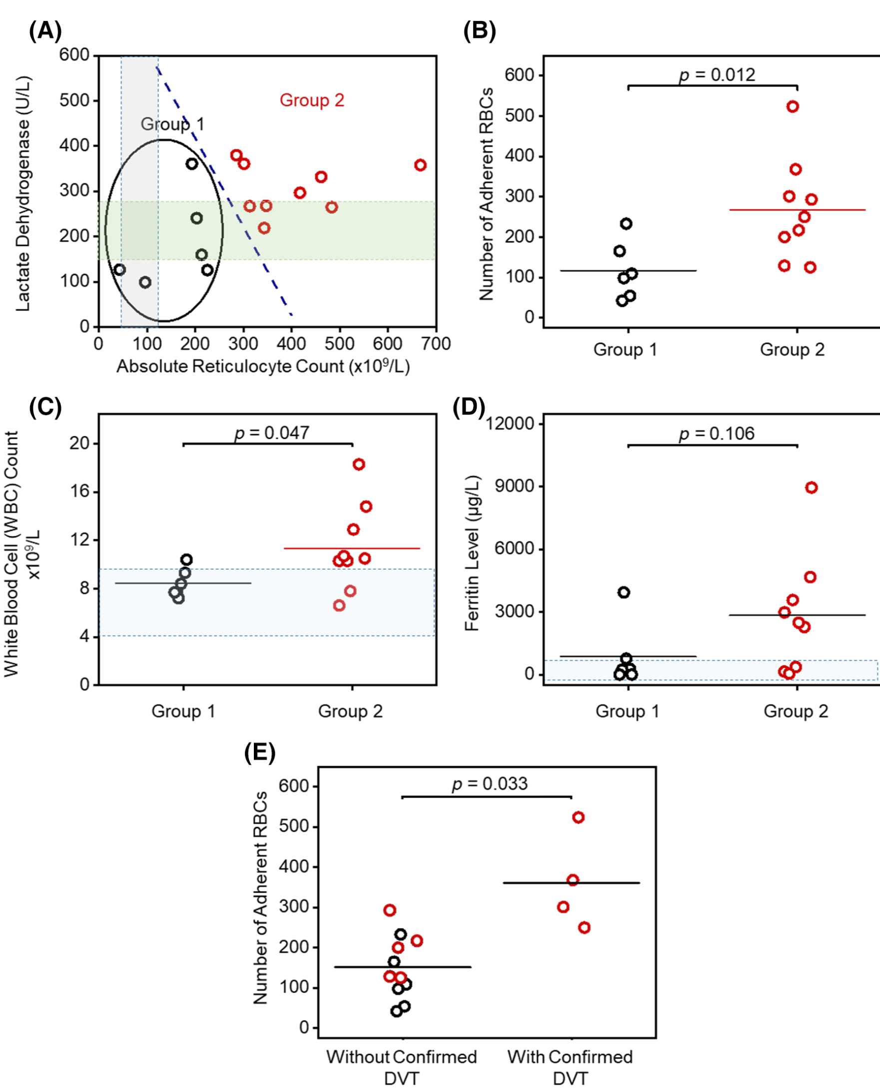

FIGURE 4 RBC adhesion to REV-activated HPMECs correlates with patient clinical phenotype including haemolytic and inflammatory biomarkers. (A) A subpopulation (Group 1, N = six) with distinct haemolysis markers of lactate dehydrogenase (LDH) levels and absolute reticulocyte counts (ARCs) comparing to the rest (Group 2, N = nine) via k-means clustering analysis. RBCs from patients in Group 2, with significantly higher LDH levels and ARCs, have greater adhesion to REV-activated HPMECs compared to the RBCs from patients in Group 1 (B, mean [SD]: 267 [125] vs. 117 [72], $p = 0.012$, two-group t-test). The grey and green shaded areas indicate normal ranges for ARC and LDH respectively. (C) Patients in Group 2 with higher LDH and ARC and enhanced RBC adhesion have significantly higher WBC counts, than those in Group 1 (mean [SD]: 11.4 [3.6] vs. 8.5 [1.2] $\times 10^9$, $p = 0.47$, two-group t-test). Shadowed area: WBC count range from 4.5 to 10×10^9. (D) Patients in Group 2 with higher LDH and ARC and enhanced RBC adhesion have higher ferritin levels, although not statistically significant, than those in Group 1 (mean [SD]: 2841 [2804] vs. 876 [1527] µg/L, $p = 0.106$, two-group t-test). Shadowed area: normal range of ferritin level (2–1000 µg/L) [71, 72]. (E) Patients with confirmed DVT had significantly higher RBC adhesion to REV-activated HPMECs than those without confirmed DVT (mean [SD] 361 [119] vs. 151 [78], $p = 0.033$, two-group t-test). Six out of six patients in Group 1 with lower RBC adhesion, and three out of six patients in Group 2 did not have DVT. Four out of eight patients in Group 2 with higher RBC adhesion had DVT. DVT status of two out of eight patients in Group 2 were not available (one patient diagnosed as 'unclear', one record not accessible). *In (C) two patients had the exact same WBC count at 7.1×10^9/L. DVT, deep vein thrombosis; HPMECs, human pulmonary microvascular endothelial cells; RBC, red blood cell; REVs, RBC-derived extracellular vesicles; WBC, white blood cell.

endothelial activation and causes endothelial dysfunction; and SS RBCs are known to have reduced deformability[33,34] and increased adhesivity to the activated endothelium, leading to vaso-occlusion. These mechanisms likely intersect and contribute to acute painful episodes and chronic organ damage.[35]

The SCD RBC membrane abnormalities include aberrant timing or increased rigidity during maturation, and abnormal activation by 'stress signals', of surface molecules such as very late antigen-4 (VLA-4), CD36, LW (ICAM-4) and basal cell adhesion molecule (BCAM)/Lutheran (Lu).[36,39–43] Cumulative oxidative damage, resulting in excessive PS externalisation on the SCD RBC membrane, causes abnormal adhesion.[44,45] RBC adhesion to activated endothelium has been associated with elevated endothelium adhesion molecules including P-selectin,[46] ICAM-1,[47] vascular cell adhesion molecule-1 (VCAM-1),[48] E-selectin,[49] and VWF,[50] as well as subendothelial proteins such as laminin (LN) under

various physiological conditions. These abnormal interactions between SCD RBC and activated endothelium plays a pivotal role in the initiation and propagation of unpredictable vaso-occlusive crisis episodes, thereby contributing to the pathophysiology of SCD.

Intravascular haemolysis releases free haeme, Hb, and REVs. Haeme induces endothelial activation through TLR-4, resulting in increased expression of VCAM-1, ICAM-1, E-selectin, P-selectin, interleukin (IL)-1, IL-6, IL-8, and tissue factor via a pathway involving the activation of nuclear factor kappa B subunit 1 (NF-κB) phospho-p65, which are known to mediate RBC adhesion.[2,47,49] Importantly, up to one-third of cell-free haeme in plasma is sequestered in circulating REVs.[3] Therefore, we set out to reproduce this important physiological mechanism in vitro. SS REVs can transfer haeme to ECs in annexin-a5-sensitive fashion and have been reported to trigger transcriptional responses from middle- to longer-time periods (4–48 h)[3,5,29,30,51] causing endothelial injury, linking haemolysis to chronic vascular injury in the SCD mouse.[3,4,51] In addition to these long-term transcriptional responses, haeme is also known to activate acute stress through sentinel pathway mediated by TLR-4 signalling that leads to production of reactive oxygen species, triggers Weibel–Palade body (WPB) degranulation and rapid release of VWF to the cell surface, which eventually causes vaso-occlusion.[2,52] It has been shown that VWF multimers released from ECs result in a significant increase in adhesion of SS RBCs to endothelium ex vivo,[11] suggesting an important role in the pathophysiology of sickle cell-induced microvascular occlusion.[11,50] However, the relationship of this finding and SS REVs has not been well-described.

Leveraging the endothelium-on-a-chip technology, we treated HPMECs with calcium ionophore-generated REVs in order to mimic the highly haemolytic plasma milieu that is often typical in patients with HbSS.[14] We demonstrated enhanced VWF expression on HPMECs treated with SS REVs comparing to those treated with AA REVs within 2 h. This demonstrates that REVs are capable of inducing acute endothelial response in the short term, in addition to known long-term effects. Further, VWF is known to mediate SS RBC adhesion independent of platelets[50,53] and plasma levels of VWF may correlate with haemolysis in SCD.[54] We found that SS RBCs demonstrate increased adherence to SS REV-activated HPMECs, compared to the adhesion of SS RBCs to AA REV-treated HPMECs, and to the adhesion of AA RBCs to SS REV-activated HPMECs, and to the adhesion of AA RBCs to AA REV-treated HPMECs. These adhesion events were dramatically mitigated by haeme-binding protein haemopexin or by VWF-specific protease ADAMTS13, reaching levels that were close to SS RBC on HPMECs treated with AA REVs.

Circulating EVs in SCD are known to have a prothrombotic effect due to their surface tissue factor and PS expression. Circulating REVs in SCD are particularly known to contain significant amounts of cell-free haeme and facilitate haeme delivery to ECs.[4,5] Here, we demonstrated the effect of haemopexin in mitigating REV-mediated acute endothelial activation. These results agreed with our previous work regarding the effect of haemopexin in mitigating haeme-mediated vaso-occlusion in SS mice.[2] Furthermore, we compared the adhesion profiles of SS RBCs on HPMECs activated using SS REVs and on HPMECs activated using haeme at the concentration carried by the SS REVs. The adhesion profiles between these two groups were statistically non-significant, despite the fact that slightly higher adhesion profile was observed on HPMECs activated by SS REVs compared to the ones activated by haeme. These results agreed with previous publication by Camus et al.[3] that the effect of REVs in activating endothelium is largely carried via haeme.

Haeme is known to rapidly mobilise WPB VWF and P-selectin onto the EC surface and cause vaso-occlusion in SS mice.[2] Belcher et al.[2] previously demonstrated that polyclonal antibodies to VWF blocked stasis induced by haeme in SCD mice with dorsal skin-fold chambers. Here, in addition to the in vitro experimental results, we confirmed the effect of ADAMTS13 in mitigating both haeme- and REV-mediated vaso-occlusion measured in percentage of stasis in vivo using Townes SS mice. Together, both in vitro and in vivo results suggest a specific interaction between these cellular elements that is potentially mediated by HPMEC VWF expression induced by the haeme content in SS REVs (Figure 5). Despite the in vivo and in vitro studies demonstrating comparable impacts of REVs and haeme or haeme-loaded particles by this study and by others,[3] the role of SS REVs in regulating VWF expression were investigated in vitro in this study. Future work will be conducted to confirm this SS REV-regulated mechanism in vivo using the mouse model. Additionally, the ligand VWF on SS RBCs mediating the adhesion events remains unclear therefore future work will be conducted in identifying potential ligands and to correlate the RBC surface expression of these ligands with patient phenotypes.

To analyse the heterogeneity of patient RBC adhesion to REV-activated ECs, we uniformly activated HPMECs with SS REVs generated from pooled blood samples. Therefore, the RBC adhesion quantified in this study only depends on the adhesivity of RBCs in individual patients. Accordingly, we describe two groups, one of which is characterised by elevated LDH levels and ARCs, and had significantly greater RBC adhesion as compared to the other group (Figure 4A,B). LDH and ARC are important in vivo haemolytic biomarkers that have been linked to disease severity.[55,56] Therefore, these results suggest that RBCs are more adhesive in patients with a more severe haemolytic phenotype in SCD. Additionally, we found that patients with a documented history of confirmed DVT (four patients) had higher RBC adhesion to REV-activated HPMECs compared to those without confirmed DVT. We postulate that this is because the higher adhesivity indicates more severe sickling of RBCs, which has been reported to mediate entrapment of these cells within venous clots.[57] Future work will test the associations between REV-mediated RBC

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FIGURE 5 Impact of REVs on microvascular endothelial cell response. SS REVs are capable of promoting HPMEC VWF expression within 2h, likely through the acute stress sentinel pathway of Weibel–Palade body degranulation. SS RBCs adhere specifically to SS REV-activated HPMECs, likely mediated by endothelial VWF. The adhesion is decreased with VWF cleaving protease ADAMTS13, and with haeme-binding protein haemopexin. Enhanced SS RBC adhesivity was observed in patients with elevated biomarkers of haemolysis and inflammation and thrombophilia. HPMECs, human pulmonary microvascular endothelial cells; ICAM-1, intercellular adhesion molecule-1; RBC, red blood cell; REVs, RBC-derived extracellular vesicles; VWF, von Willebrand factor.

adhesion and patient clinical outcomes including haemolysis and record of DVT, and ongoing treatment, in an increased number of patients.

In conclusion, in this study, we have improved our endothelium-on-a-chip approach by activating the endothelial layer with a biologically complex in vitro generated REVs obtained from isolated RBCs. We believe that this is physiologically relevant in comparison to a simpler biochemical signal such as tumour necrosis factor alpha (TNF-α) or haeme. Our findings indicate that both pathological RBCs with higher adhesivity, and an activated endothelium are required for the observed abnormal interaction. The SS RBCs demonstrate enhanced adhesion to HPMECs activated with SS REVs in a patient-specific fashion. This heterogeneity in adhesion solely reflects heterogeneous RBC adhesion to uniformly activated HPMECs (using pooled derived REV). The RBC adhesion profiles associated with haemolytic and inflammatory biomarkers including LDH, ARC, WBC and ferritin levels. Future investigations will re-examine these clinical associations in expanded patient population, as well as examine RBC adhesion on ECs that are activated with patient-specific REVs, which may better reflect the overall disease process in an individual patient. Understanding the important collective interplay between RBCs and REV-activated microvascular ECs will better characterise the multicellular adhesion paradigm for acute and chronic vaso-occlusion in SCD and may enable us to develop more effective treatment paradigms.

AUTHOR CONTRIBUTIONS

Ran An and Yuncheng Man contributed equally to this work. Ran An, Yuncheng Man and Umut A. Gurkan conceived the project. Ran An, Yuncheng Man, Kevin Cheng, Tianyi Zhang, Chunsheng Chen, Fang Wang, and Fuad Abdulla conducted the experiments. Ran An, Yuncheng Man, Kevin Cheng, Tianyi Zhang, Chunsheng Chen, Fuad Abdulla, Erdem Kucukal, William J. Wulftange, Gregory M. Vercellotti, John D. Belcher, and Utku Goreke analysed the data. Ran An, Yuncheng Man, Lalitha V. Nayak, Jane A. Little, Gregory M. Vercellotti, John D. Belcher, and Umut A. Gurkan discussed and interpreted the data. Ran An and Yuncheng Man prepared the figures and table and wrote the manuscript. Ran An, Yuncheng Man, Lalitha V. Nayak, Jane A. Little, Gregory M. Vercellotti, John D. Belcher, and Umut A. Gurkan reviewed and edited the manuscripts. Allison Bode collected patient clinical information from the medical record and blood sample collection. Ran An conducted the experiments and drafted the initial manuscript at Case Western Reserve University, and finished the revisions at University of Houston.

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CONFLICT OF INTEREST

Ran An, Jane A. Little, Umut A. Gurkan and Case Western Reserve University have financial interests in Hemex Health Inc. Jane A. Little, Erdem Kucukal, Umut A. Gurkan and Case Western Reserve University have financial interests in BioChip Labs Inc. Umut A. Gurkan and Case Western Reserve University have financial interests in Xatek Inc. Umut A. Gurkan has financial interests in DxNow Inc. Financial interests include licensed intellectual property, stock ownership, research funding, employment, and consulting. Hemex Health Inc. offers point-of-care diagnostics for haemoglobin disorders, anaemia, and malaria. BioChip Labs Inc. offers commercial clinical microfluidic biomarker assays for inherited or acquired blood disorders. Xatek Inc. offers point-of-care global assays to evaluate the haemostatic process. DxNow Inc. offers microfluidic and bio-imaging technologies for in vitro fertilisation, forensics and diagnostics. Competing interests of Case Western Reserve University employees are overseen and managed by the Conflict of Interests Committee according to a Conflict-of-Interest Management Plan. Gregory M. Vercellotti and John D. Belcher receive research funding from Omeros, CSL Behring, Hilhurst Biopharmaceuticals and Astellas/Mitobridge. JDB is a consultant for Astellas/Mitobridge. GMV is a consultant for Sanofi and Astellas/Mitobridge.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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