A role for activated endothelial cells in red blood cell clearance: implications for vasopathology


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A role for activated endothelial cells in red blood cell clearance: implications for vasopathology

Running title: Pathophysiological role of EC erythrophagocytosis

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Abstract

Background

Phosphatidylserine exposure of red blood cells is acknowledged as a signal that initiates phagocytic removal from the circulation. Several disorders and conditions are known to induce phosphatidylserine-exposure. Removal of phosphatidylserine-exposing red blood cells generally occurs by macrophages in spleen and liver. Previously, however, we have shown that also endothelial cells are capable of erythrophagocytosis. Key players of erythrophagocytosis by endothelial cells appeared to be lactadherin and αv-integrin. Phagocytosis via the phosphatidylserine-lactadherin-αv-integrin pathway is the acknowledged route for removal of apoptotic innate cells by phagocytes.

Design and Methods

Endothelial cell phagocytosis of red blood cells was further explored using a more (patho)physiological approach. Red blood cells were exposed to oxidative stress, induced by tert-butyl hydroperoxide. After opsonisation with lactadherin, red blood cells were incubated with endothelial cells to study erythrophagocytosis and examine cytotoxicity.

Results

Red blood cells exposed to oxidative stress show alterations such as phosphatidylserine-exposure and loss of deformability. When incubated with endothelial cells, marked erythrophagocytosis occurred in presence of lactadherin under both static and flow conditions. As a consequence, intracellular organization was disturbed and shape change (‘rounding up’) of endothelial cells was seen. Increased expression of apoptotic markers indicated that marked erythrophagocytosis has cytotoxic effects.
Conclusions
Activated endothelial cells show significant phagocytosis of phosphatidylserine-exposing and rigid red blood cells under both static and flow conditions. This results in a certain degree of cytotoxicity. We postulate that activated endothelial cells play a role in RBC clearance in vivo. Significant erythrophagocytosis can induce endothelial cell loss, which may contribute to the vasopathological effects as seen, for instance, in sickle cell disease.

Introduction

Shortened survival of RBCs may result from a variety of hereditary and acquired conditions. Premature clearance is often initiated by events causing alteration of RBC membrane integrity. When the induced membrane changes are severe, they result in abrupt intravascular lysis. In case of less acute and milder membrane alterations, RBCs undergo a series of events leading to recognition, ingestion and, ultimately, destruction by phagocytes of the reticulo-endothelial system (RES). Several mechanisms have been described by which RBCs are removed from the circulation. These include oxidation of band 3, leading to naturally occurring antibody-(NAbs) mediated opsonisation followed by Fc-receptor mediated phagocytosis, and programmed or suicidal cell death\(^1\)\(^{-3}\). Because RBCs lack a nucleus and mitochondria, they do not undergo ‘classic’ apoptosis. However, features of programmed cell death have also been proposed for RBCs (‘eryptosis’). These features include: cell shrinkage, decreased cell deformability, vesiculation and membrane phospholipid scrambling by translocation of phosphatidylserine (PS) to the outer leaflet.\(^1\) The pathophysiological conditions initiating extravascular clearance of RBCs are not yet fully elucidated. Loss of membrane phospholipid asymmetry has been recognized as a key trigger that leads to recognition and extravascular removal of senescent, disordered RBCs,
and transfused long-term stored RBCs.\textsuperscript{4-6} This is in agreement with the clearance mechanism for apoptotic nucleated cells.\textsuperscript{7,8} PS-exposure, as a result of membrane scrambling, can be induced on RBCs in multiple ways such as oxidative damage, (inherited) intracorpuscular defects, infection, intravascular drugs or toxic compounds, and mechanical stress.\textsuperscript{4,9-11} Ultimately, this initiates recognition and subsequent removal via phagocytosis by phagocytes in spleen and liver.

Upon PS-exposure by apoptotic nucleated cells or suicidal RBCs, opsonization by lactadherin (also called milk fat globule epidermal growth factor 8 (MFG-E8) or SED1) is essential for macrophage recognition and phagocytosis.\textsuperscript{6,12} Lactadherin is a protein that contains two distinct functional domains: a C-terminal domain that binds to anionic phospholipids such as PS, and an N-terminal domain with two epidermal growth factor (EGF)-repeats.\textsuperscript{13,14} Lactadherin is expressed by subsets of macrophages,\textsuperscript{15} and in and around blood vessels.\textsuperscript{16,17} The second EGF-repeat of lactadherin contains an Arg-Gly-Asp (RGD) motif that binds to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin-expressed on macrophages, epithelial cells, and vascular cells.\textsuperscript{12,13,18} The integrin expression density is dependent on cellular origin and state of activation. Phagocytosis via the PS-lactadherin-$\alpha_v$-integrin pathway is implicated in the removal of apoptotic innate cells but not in the elimination of foreign targets such as pathogens. Clearance and subsequent processing of innate cells should initiate an immunosuppressive effect rather than an immunostimulatory effect, to prevent autoimmune reactions and inflammation.\textsuperscript{19} Since endothelial cells, like macrophages, are equipped with all elements of the immunosuppressive PS-lactadherin $\alpha_v$-integrin pathway\textsuperscript{16,20} it seems reasonable to assume that under certain circumstances they actively engage this pathway. Previously, we have shown that endothelial cells are capable of lactadherin-dependent phagocytosis of RGD-modified RBCs\textsuperscript{16,20}. In the present study we provide evidence for endothelial cell erythrophagocytosis via this pathway by reporting on the interactions of PS-exposing RBCs with endothelium in \textit{in vitro} models mimicking (patho)physiological conditions.
We show that, in presence of lactadherin, PS-exposing RBCs show severely reduced deformability, and are phagocytosed by endothelial cells under both static and flow conditions. This ultimately results in considerable disturbances of the endothelial cell with occasional lethal damage. Our results indicate that the PS-lactadherin-αv-integrin pathway is actively involved in interactions and phagocytosis of RBCs and the endothelium. We postulate that this may have important implications for our understanding of vasopathological events seen in a variety of haematological disorders such as sickle cell disease and malarial infection.

Design and Methods

**Red blood cells**

All RBC (blood group 0, Rhesus D negative) used in this study were acquired from surplus sealed blood bag tubes from banked RBC units that had been used for transfusion. For all experiments, specimens of 3 individual units were pooled and the storage solution SAGM (saline, adenine, glucose, mannitol) was removed upon further usage.

**Cell culture**

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured on EGM™-2 endothelial cell growth medium-2 (Lonza, Verviers, Belgium) consisting of EBM-2 medium supplemented with an EGM-2 bullet kit (containing growth factors, 2% FBS and antibiotics). HUVECs were used as an αvβ3-expressing model for activated endothelium up to a passage number of 7.

**Lactadherin purification**
Bovine lactadherin was purified as previously described. Purity was checked by SDS-PAGE and N-terminal amino acid sequencing and shown to be 97-98%, in two glycosylation forms. Protein concentrations were determined by amino acid analysis based on o-phthalaldehyde derivatization.

**Induction and measurement of PS-exposure**

Two different stimuli were used to induce RBC PS-exposure: calcium ionophore A23187 (Calcimycin, Sigma-Aldrich, St Louis, MO, USA), and tert-butyl hydroperoxide (tBHP, Sigma-Aldrich). Ionophore A23187 incubation was performed according to Kuypers et al. In brief, prior to ionophore A23187 incubation, RBC membrane flippase activity was inhibited by 10 mM N-ethyl maleimide (NEM, Sigma-Aldrich) for 30 minutes, followed by incubation for 1 hour at 4 or 40 µM ionophore in a calcium (1 mM) containing buffer. RBCs were incubated with 1, 2 or 3 mM tert-butyl hydroperoxide for 30 minutes on a tube rocker (no NEM pretreatment). All incubations were performed at room temperature. PS on the outer surface of the RBCs was stained using bovine lactadherin (0.66 mg/ml), followed by a rabbit anti-bovine lactadherin antibody\(^\text{13}\) (1:200) and a swine anti-rabbit-FITC (1:200, F0205 DAKO, Glostrup, Denmark), and finally measured on a Fluorescence Activated Cell Sorter (FACSCanto II™, BD Bioscences, Franklin Lakes, New Jersey, USA).

**Detection of ROS**

Reactive oxygen species (ROS) were measured to indicate the amount of oxidative stress induced by the cellular treatments described above. Control, ionophore and tBHP-treated RBCs were incubated with 5 µM 5- (and 6)-chloromethyl-2’,7’-dichlorodihydro-fluorescein diacetate acetyl ester (CM-H\(_2\)DCFDA, Invitrogen, Carlsbad, CA, USA) at 2% final hematocrit for 30 minutes at
room temperature. Detection of oxidative stress was performed on a Fluorescence Activated Cell Sorter (FACSCanto II™, BD Bioscences).

**RBC deformability**

RBC deformability was analyzed using Laser-assisted Optical Rotational Cell Analyzer (LORCA; Mechatronics, Hoorn, The Netherlands) measurements in which elongation of the cells is measured at increasing shear stress. Ionophore and tBHP stimulated RBC samples were diluted 200 times in 0.14 mol per liter polyvinylpyrrolidone (PVP, Mw 360,000) in PBS (viscosity, 30 mPa/s). One milliliter of the RBC in PVP suspension (37°C) was transferred into the LORCA measuring system and subjected fully automatically to a standardized increase of shear stress. Deformation was expressed as an elongation index, as derived from the resulting ellipsoid diffraction pattern. The deformability curve was obtained by plotting the calculated values for the elongation index versus shear stress (Pa).

**Osmotic fragility**

Red blood cell samples were diluted 100 times using a range of NaCl concentrations (0.1 – 12%) and incubated for 30 minutes at room temperature. After incubation, cells were centrifuged for 5 minutes at 1000 x g. To calculate the amount of free hemoglobin as a result of lysis, the supernatant was measured at a wavelength of 450 nm on a Versamax™ microplate reader (Molecular Devices, Sunnyvale, CA).

**Quantification of erythrophagocytosis**

Erythrophagocytosis was quantified using a colorimetric assay described by Gebran et al. This method is based on the pseudoperoxidase activity of hemoglobin. RBCs were initially stimulated
with ionophore or tBHP to induce PS-exposure. Next, 5 μg lactadherin was added to 15 μl (approximately 1.5 x 10^8 cells) of packed RBCs, and incubated for 30 minutes at room temperature. Unbound lactadherin was removed by centrifugation, and 2 μl of packed RBC sample (approximately 2 x 10^7 cells) was subsequently added to HUVECs. HUVECs were seeded in 12 wells plates (7 x 10^4 cells/well) 1 day prior to sample addition. At different time points after incubation with HUVECs at 37°C and 5% CO2 conditions, erythrocyte samples were removed, and 500 μl trypsin/EDTA was immediately added to the wells to detach HUVECs. When detached, 1 ml of PBS containing 5% fetal calf serum (FCS) was added to inactivate trypsin. Samples were collected in 1.5 ml eppendorf tubes and spun down (1500 x g, 5 minutes, room temperature). Supernatant was removed and ery-shock buffer (0.1% NaCl) was added to the pellets for 15 minutes at room temperature to lyse remaining non-phagocytosed erythrocytes. After centrifugation (1500 x g, 5 minutes, room temperature), buffer was removed and remaining pellets were washed 3 times by resuspending the cells in 1 ml PBS. Lysis of HUVECs, to release the hemoglobin content, was achieved by adding 100 μl 0.2 M Tris-HCl buffer containing 6 M urea to the cell pellets, followed by rigorous vortexing and overnight incubation at 4°C. Three freeze-thaw steps were next performed to assure complete lysis of HUVECs. 100 μl 2,7-diaminofluorene (DAF, Sigma-Aldrich) working solution (containing H2O2 and the Tris-HCl/urea buffer) was added to the samples that were transferred to a 96-wells plate. Finally, absorbance was measured at a wavelength of 620 nm. A hemoglobin standard curve was included to calculate hemoglobin concentrations.

**Light microscopy**

One day before sample addition HUVECs (7 x 10^3 cells/well) were seeded in pre-coated 16 well chamber slides (LAB-TEK; Nunc, Rochester, NY, USA). The wells were pre-coated by
incubation with 1% gelatin at 37ºC for 45 minutes followed by glutaraldehyde 0.05% at 37ºC for 30 minutes. After incubation with (lactadherin [1 μg/3 μl packed RBCs] pretreated) 0.5 μl packed RBC sample (approximately 5 x 10⁶ cells), HUVECs were fixed with 2% paraformaldehyde in PBS. Cells were hematoxylin and eosin (H&E)-stained and mounted in depex:xylene (3:1). Images were taken using a Nikon TE2000 microscope equipped with a Nikon Digital Sight DS-2Mv camera.

**Transmission Electron Microscopy**

HUVECs (25 cm² petri dish) were incubated for 4 hours at 37ºC and 5% CO₂, with 12 μl packed (approximately 1.2 x 10⁸ RBCs) tBHP-treated RBCs that were pre-incubated with bovine lactadherin. Cells were fixed directly after incubation by adding an equal volume of 4% paraformaldehyde/0.4% glutaraldehyde to the culture flask. After 15 minutes the fixative/medium was replaced by a 2% paraformaldehyde/0.2% glutaraldehyde solution and stored at 4ºC. After washing with PBS, cells were collected in a 2% agarose in PBS solution by scraping, followed by centrifugation. Samples were postfixed for 2 hours in 1% OsO₄ in 0.1 M cacodylate buffer (pH 7.4), dehydrated by increasing concentrations of ethanol/propylene oxide and embedded in Epon 812. Sections (70 nm) were prepared using a Reichert Ultracut E (Leica, Wetzlar, Germany) and poststained with uranyl acetate and lead citrate. Sections were viewed on a FEI Tecnai T12 transmission electron microscope (FEI, Hillsboro, Oregon, USA), and images were digitally taken using FEI version 3.2 TEM Imaging and Analysis Software.

**Perfusion**

HUVECs were grown on glass coverslips that were coated overnight with chromosulfuric acid 2% and post-fixed with glutardialdehyde 1% and washed with ethanol 70% and PBS. RBC
stimulated with tBHP 3 mM (in presence or absence of lactadherin) were perfused at 37°C in a 5% hematocrit suspension over the HUVECs, at a shear rate of 100 s⁻¹. Association of the RBCs with HUVECs during perfusion was visualized with differential interference contrast microscopy (DIC) using an Axio Observer microscope (Carl Zeiss, Oberkochen, Germany). The perfused coverslips were fixed under flow (4% paraformaldehyde for 5 minutes, 100 s⁻¹), followed by static fixation in paraformaldehyde (1 hour at room temperature, then overnight in 4°C). For confocal imaging, the actin cytoskeleton was stained using phalloidin Alexa 488 (1:100) and the RBCs were stained for band 3 (mouse anti-band 3, 1:100, Sigma) with secondary donkey anti-mouse-Cy3 antibody (1:100, Jackson Laboratories, Suffolk, UK). Fluorescent signals were visualized using a confocal system (Leica TCS-SP2, Leica, Wetzlar, Germany). Excitation of phalloidin 488 labeled actin and Cy3 labeled band 3 was accomplished by confocal argon laser. Samples were viewed using an APO 100x oil objective (zoom 2) and images were acquired using Leica Confocal Software (LAS-AF).

**Cytotoxicity**

HUVECs were seeded in 96 well plates (1 x 10⁴ cells/well) 24 hours prior to RBC sample addition. **Tert**-butyl hydroperoxide stimulated RBC samples were prepared as described above (see ‘Quantification of erythrophagocytosis’) and incubated with HUVECs at 37°C and 5% CO₂ conditions. After 4 hour, RBC samples were gently removed and replaced by plain EGM-2 medium. Twenty four hours after start incubation, HUVECs were detached using trypsin/EDTA. Cells were stained to detect (early) apoptosis with annexin V-FITC (BioVision) and propidium iodide (PI, Sigma) in a buffer containing calcium (2.5 mM) and 5% FCS. Finally, (double) staining of HUVEC was detected using a FACSCanto™ II flow cytometer (BD Biosciences).
Statistical analysis

Data were statistically analyzed by nonparametric 2-way ANOVA with bonferroni post test using GraphPad Prism 5 for Mac OS X (GraphPad Software, San Diego, CA).

Results

A critical first step for cellular phagocytosis is PS externalization. RBCs were stimulated with ionophore, after pre-treatment with NEM, or with tBHP to induce PS externalization. PS-exposure was detected by annexin V-staining using flow cytometry. Untreated control RBCs and NEM-treated RBCs showed marginal PS-exposure (Figure 1A). Incubation with 4 and 40 µM calcium ionophore A23187, however, resulted in 99.9 ± 0.1% and 99.9 ± 0.0% of the total population of RBCs showing PS-exposure. Incubation of RBCs with the oxidative damage-inducing compound tert-butyl hydroperoxide25, showed a dose-dependent PS-exposure, ranging from 4.9 ± 1.9% for 1 mM, via 42.2 ± 18.7% for 2 mM, to 93.4 ± 6.2% for 3 mM tBHP. Calcium ionophore treatment, particularly 40 µM, showed considerably higher geo mean fluorescence intensity (geo MFI) values, indicating that the number of PS molecules per RBC was higher (Figure 1B-H and Table 1). Furthermore, calcium ionophore-treated RBCs demonstrated reduced forward scatter (geo FSC) and crenated, dehydrated, appearance indicating reduced RBC sizes (Table 1 and Supplemental figure 1).

Next to PS-exposure, membrane rigidity is a critical determinant of cell phagocytosis20. RBC deformability was measured using a LORCA, which measures elongation of the cells at increasing shear stress. The lower the elongation index, the more rigid and less deformable RBCs are. Compared to untreated control RBCs, 1 mM tBHP-treated RBCs were only slightly less deformable. In contrast, ionophore-, and 2 and 3 mM tBHP-treated RBCs showed a severe
reduction in RBC deformability (Figure 2A and B). NEM-treated RBCs showed only marginally reduced deformability. Another feature representing RBC membrane integrity is the ability to withstand osmotic shock. 40 μM calcium ionophore-stimulated RBCs showed somewhat increased sensitivity for hypertonic salt concentrations as determined by the osmotic fragility test (Figure 2C, D and Table 2). At hypotonic NaCl concentrations, 2 and 3 mM tBHP-treated RBCs showed decreased lysis (50% lysis at 5.1 and 6.5 g/L NaCl, respectively, versus 4.6 g/L for control RBCs), indicating that the induced membrane changes altered the osmotic response of these cells. Altogether, these results indicate that both ionophore and tBHP induce PS externalization and affect the RBC’s membrane integrity. To determine the fraction of RBCs that showed oxidative stress upon treatment with either calcium ionophore or tBHP, we determined the amount of ROS using a membrane-permeant fluorescent probe (CM-H$_2$DCFDA), as previously described. Hundred percent of red blood cells treated with either 1, 2 or 3 mM tBHP were positive for ROS. Instead, ROS levels in both 4 and 40 μM ionophore-treated cells were lower (approximately 10%), similar to untreated control cells (see Figure 2E).

Subsequently, we investigated whether lactadherin could bridge RBCs to the endothelium to facilitate their phagocytosis. Cultured human endothelial cells (HUVECs) were used to phagocytose calcium ionophore- and tBHP-stimulated RBCs in presence or absence of lactadherin. Lactadherin was added in excess of exposed PS molecules (Supplemental figure 2). Erythrophagocytosis was quantified by measuring the (pseudo)peroxidase activity of internalized hemoglobin. Ionophore-treated RBCs pre-incubated with lactadherin showed erythrophagocytosis that increased with dose and time. In absence of lactadherin no intracellular hemoglobin could be detected (Figure 3A). The highest concentration of ionophore (i.e. 40 μM) showed levels of erythrophagocytosis comparable to the previously studied RGD-modified RBCs. Induction of oxidative damage induced by tBHP treatment showed a similar dose-
time-dependent increase in erythrophagocytosis in presence of lactadherin (Figure 3B). At the highest concentration, 3 mM tBHP-treated RBCs are phagocytosed to a larger extent after 3 or 4 h incubation, compared to RBCs treated with 40 µM calcium ionophore (2.5- and 4-fold, respectively). 1 mM tBHP-stimulated RBCs did not show erythrophagocytosis, even in presence of lactadherin. Taken together, these results show that both ionophore and tBHP predispose RBCs for uptake by endothelial cells via the PS-lactadherin αv-integrin pathway. Since oxidative stress is a well-known (patho)physiological condition we used tBHP-treated RBCs in subsequent experiments as a model for oxidative stress to erythrocytes.

Erythrophagocytosis of tBHP-treated RBCs by HUVECs, in presence of lactadherin, was visualized by microscopy. Control RBCs and 1 mM tBHP-stimulated RBCs are not internalized by HUVECs (Figure 4A). In contrast, HUVECs show marked erythrophagocytosis of RBCs treated with 2 mM and 3 mM tBHP. This uptake increased with time (Figure 4A). Similar to our RGD-modified RBC model,16,20 multiple RBCs are phagocytosed per endothelial cell. After treatment with 3 mM tBHP Between 5-30 RBCs could be seen per HUVEC (Figure 4B). Notably, some of the RBCs have an altered, echinocyte-like, morphology. To be able to study erythrophagocytosis by HUVECs in more detail, transmission electron microscopy (TEM) was performed. Multiple intracellular RBCs could be detected after erythrophagocytosis of 3 mM tBHP-treated RBCs. This internalization was accompanied by shape change (‘rounding up’) of HUVECs (Figure 4C) and indentations of their nuclei (Figure 4D and E).

Next we investigated whether erythrophagocytosis by the endothelium also occurs under flow. For this we performed perfusion of 3 mM tBHP-treated RBCs over a HUVEC monolayer. Flow rate was set to 100 s⁻¹, which is comparable to the velocity of blood in capillaries and small venules. During this experiment, DIC images (Figure 5), were taken throughout the perfused area. In absence of lactadherin, the association of tBHP-treated RBCs with HUVECs was mostly
unstable (Figure 5A). In contrast, pre-incubation with lactadherin caused increased and stable association of RBCs with HUVECs (Figure 5B). At higher shear rate (300 $s^{-1}$), comparable to large venular velocity, similar results were obtained (Supplemental figure 3). To investigate whether the adherence was followed by internalization we performed confocal microscopy. As shown in Figure 5, phagocytosis of RBCs did not occur in absence of lactadherin (Figure 5C), whereas in presence of lactadherin several intracellular RBCs could be detected (Figure 5D).

To investigate whether erythrophagocytosis had cytotoxic effects on HUVECs we double stained HUVECs with propidium iodide (PI; cell death marker) and annexin V-FITC (PS-exposure; apoptosis marker), after 24 hours incubation with tBHP-treated RBCs. HUVECs incubated with 2 mM tBHP, but mainly 3 mM tBHP-treated RBCs, in presence of lactadherin, showed significant cytotoxicity (Figure 6).

**Discussion**

We previously showed that RBCs modified with RGD peptides coupled to the outer surface, a model mimicking lactadherin opsonization, induced marked erythrophagocytosis by HUVECs.$^{16,20}$ To further explore the role of endothelial cells in the removal of lactadherin-opsonized PS-exposing RBCs, we induced different pathophysiological cellular changes upon them, and subsequently examined the interaction with endothelial cells *in vitro* under both static and flow conditions. In both conditions, RBCs that expose PS and demonstrate loss of membrane integrity and/or deformability induced by either oxidative damage or calcium ionophore, are prone to erythrophagocytosis by endothelial cells. Erythrophagocytosis of increasing numbers of RBCs causes endothelial cell disturbances and morphological changes. Increased expression of
apoptotic markers on endothelial cells indicates that, eventually, this may lead to endothelial cell death.

In order to mimic RBC (oxidative) damage \textit{in vivo}, RBCs were treated with either calcium ionophore 23187 or the oxidative damage-inducing agent \textit{tert}-butyl hydroperoxide (tBHP). Calcium ionophore, widely used to induce PS-exposure leads to a variety of cellular changes, including extensive vesiculation and K$^+$ release. The latter results in cell shrinkage.\textsuperscript{27} \textit{Tert}-butyl hydroperoxide induces oxidative damage by membrane lipid peroxidation and hemoglobin degradation\textsuperscript{28} and represents an established model for oxidative stress.\textsuperscript{29,30} Upon incubation with either calcium ionophore or tBHP, a large fraction of the RBC population exposes PS. This exposure is accompanied by a strong decrease in cellular deformability. Indeed marked changes were observed in the capacity of RBCs to withstand osmotic stress, both after calcium ionophore and tBHP treatment. This may be another indication of altered membrane integrity. In particular, RBCs exposed to oxidative stress were found to be resistant to hypotonic lysis. This likely reflects increased rigidity of the RBC.

When compared to our model of lactadherin-opsonized RGD-modified RBCs\textsuperscript{16,20}, RBCs treated with 40 μM calcium ionophore were internalized by HUVECs to a similar degree. However, when exposed to oxidative stress there was a 4-fold increase in erythrophagocytosis. Since the rigidity of such RBCs was substantially increased, these data are in agreement with our previous findings and those of other groups, that increased rigidity of (PS-exposing) targets is crucial for efficient phagocytosis.\textsuperscript{20,31}

The highest PS-exposure and most severe loss of deformability (ionophore 4, 40 μM and tBHP 2, 3 mM) showed the most pronounced erythrophagocytosis, indicating that high levels of PS-exposure and a loss of membrane integrity both play an important role. Importantly however, it
can not be excluded that, in addition to PS, other induced RBC membrane changes may play a role in erythrophagocytosis. Because \textit{in vivo} RBCs likely do not only interact with the endothelium under static conditions we also studied these interactions during flow. Perfusion experiments at a velocity rate comparable to that in microvasculature confirmed the interaction and subsequent erythrophagocytosis by HUVECs of lactadherin-opsonized RBCs exposed to oxidative stress. Notably, endothelial cell erythrophagocytosis occurs only for RBCs that expose PS. In addition, there appears to be an absolute requirement for lactadherin. Because PS (scavenger)-receptors, described to be involved in phagocytosis of PS-exposing cells by macrophages, do not require opsonins like lactadherin\textsuperscript{32}, these receptors appear to be not involved in endothelial cell erythrophagocytosis. Rapid clearance of PS-exposing apoptotic cells is crucial to prevent tissue damage resulting from inflammation or autoimmune responses against intracellular antigens released from the dying cells.\textsuperscript{33-35} In tissues, apoptotic cell recognition, removal and processing, usually occurs within a few hours.\textsuperscript{36} With regard to RBC clearance initial studies seem to confirm this. A pioneering study by Alan Schroit and co-workers showed that artificial insertion of PS on RBCs led to rapid clearance by Kupffer cells and splenic macrophages, with maximum clearance occurring within the first 60 minutes.\textsuperscript{37} Importantly, RBC deformability in this study was likely to be unaltered since PS was artificially inserted into the RBC membranes rather than brought about by physiological mechanisms. This may have delayed clearance. We show that PS-exposing RBCs with increased rigidity are very efficiently recognized and cleared. This makes it very difficult to detect these cells in the circulation. Therefore, the numbers of PS-exposing RBCs measured in several studies may in fact reflect an underestimation of the true amount of PS-exposing cells. In addition, it is also possible that, before being removed and eliminated, PS is shielded by
molecules such as lactadherin. For these reasons it is very difficult to detect PS-exposing RBCs and study characteristics required for opsonization and subsequent phagocytosis. Endothelial cells harboring RBCs exposed to oxidative stress showed no clear immediate signs of degradation. The number of engulfed RBCs may be important because large numbers of internalized RBCs are likely to (sterically) disturb intracellular processes and/or induce intracellular release of oxidized free heme. This leads to cellular dysfunction and, eventually, cell death. Our results indicate that after 24 hours only a fraction of endothelial cells involved in erythrophagocytosis show signs of apoptosis. This may indicate that, up to a certain level, endothelial cells are well capable of internalizing and subsequent processing of damaged or aberrant RBCs. Similar to erythrophagocytosis by macrophages, endothelial cells also have shown to be able of recycling iron upon activation by increasing the expression of key enzymes in this pathway such as heme oxygenase-1 (HO-1), ferroportin and ferritin. RBC characteristics that may affect the fate and rate of processing could include cellular features such as membrane integrity, rigidity and viscosity. The phagocytes as present in liver and spleen are considered to be the major sites of clearance of defective and senescent RBCs. Notably, hepatic sinusoidal endothelial cells (HSECs) were recently shown to play a role in the sequestration of PS-exposing damaged RBCs in the liver. In particular the phagocytic capacity of Kupffer cells in the liver was found significantly enhanced by HSECs, mediated by stabilin-1 and stabilin-2. We show here that endothelial cells themselves also exert phagocytic properties, in both static and flow conditions. It is conceivable that especially under conditions of strongly increased demand for RBC clearance, the endothelium becomes involved. In agreement with this, erythrophagocytosis by sinus endothelial cells of the spleen has been described previously in several cases of hemolytic anemia.
propose a role of the activated endothelium in the removal of PS-exposing lactadherin opsonized RBCs under (patho)physiological conditions in vivo, such as sickle cell disease.

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Authorship and Disclosures

Contribution: M.H.A.M.F. designed studies, performed the experiments, interpreted data, and wrote the paper; R.v.W. designed studies, interpreted data and wrote the paper; G.A. performed the experiments and interpreted data; K.L.v.R. performed the experiments and interpreted data; H.M.D. interpreted data; J.T.R. provided lactadherin and interpreted data on lactadherin; K.d.V. interpreted data; R.M.S. designed experiments, supervised the studies, interpreted data, and wrote the paper. C.A.J.M.G. designed experiments, interpreted data and supervised the project and wrote the paper; W.W.v.S. designed experiments, supervised the studies, interpreted data, and wrote the paper.

References


Tables

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<td>14.0 ± 1.7</td>
</tr>
<tr>
<td>tBHP 1 mM (F)</td>
<td>8 ± 0.8</td>
<td>40.4 ± 1.6</td>
</tr>
<tr>
<td>tBHP 2 mM (G)</td>
<td>58 ± 29.2</td>
<td>36.4 ± 0.9</td>
</tr>
<tr>
<td>tBHP 3 mM (H)</td>
<td>454 ± 169.3</td>
<td>38.6 ± 1.0</td>
</tr>
</tbody>
</table>

Table 1. PS-exposure of RBCs after treatment with calcium ionophore A23187 or oxidative stressor tBHP

<table>
<thead>
<tr>
<th>Sample</th>
<th>Haemolysis 50% (g/L NaCl)</th>
<th>Standard error</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control RBC</td>
<td>4.620</td>
<td>0.087</td>
<td>0.985</td>
</tr>
<tr>
<td>NEM</td>
<td>4.293</td>
<td>0.143</td>
<td>0.956</td>
</tr>
<tr>
<td>Iono 4 μM</td>
<td>4.320</td>
<td>0.094</td>
<td>0.981</td>
</tr>
<tr>
<td>Iono 40 μM</td>
<td>4.109</td>
<td>0.159</td>
<td>0.947</td>
</tr>
<tr>
<td>tBHP 1 mM</td>
<td>4.447</td>
<td>0.110</td>
<td>0.975</td>
</tr>
<tr>
<td>tBHP 2 mM</td>
<td>5.056</td>
<td>0.092</td>
<td>0.983</td>
</tr>
<tr>
<td>tBHP 3 mM</td>
<td>6.518</td>
<td>0.131</td>
<td>0.962</td>
</tr>
</tbody>
</table>

Table 2. Osmotic fragility of RBCs after treatment with calcium ionophore A23187 or oxidative stressor tBHP
Figure legends

**Figure 1. Phosphatidylserine exposure of RBCs induced by calcium ionophore A23187 and oxidative stress (tBHP).** A) Overview of the percentage of PS-exposing RBCs after stimulation with calcium ionophore A23187 or tBHP. Results represent mean ± s.d. of 3 independently performed experiments. Typical examples of FACS plots (dot plots) after lactadherin-Alexa 488 staining of B) control RBCs, C) N-ethyl maleimide (NEM), D) 4 μM ionophore, E) 40 μM ionophore, F) 1 mM tBHP, G) 2 mM tBHP, and H) 3 mM tBHP.

**Table 1. Overview flow cytometry parameters.** The geo mean fluorescent intensity (PS exposure of whole population) and the geo forward scatter (FSC; indication for size) value for all treated red blood cell samples.

**Figure 2. Mechanical alterations of RBCs induced by calcium ionophore and oxidative stress (tBHP).** Changes in RBC deformability analyzed by LORCA measurements. The elongation patterns of 4 μM and 40 μM calcium ionophore stimulated RBCs showed severe loss of deformability (A) while N-ethyl maleimide (NEM) treated RBC were only slightly less deformable than untreated RBCs. RBCs treated with 2 mM and 3 mM tBHP, also showed severe loss of deformability, whereas 1 mM tBHP treated RBCs showed normal deformability (B). Osmotic fragility studies demonstrated increased haemolysis of 40 μM calcium ionophore stimulated RBC at hypertonic salt concentrations (C). At hypotonic NaCl concentrations 2 and 3 mM tBHP treated RBCs showed decreased haemolysis at hypotonic NaCl concentrations, indicating that membrane changes altered osmotic response of these cells (D). Oxidative stress
was measured using a probe for intracellular reactive oxygen species (ROS), CM-H$_2$DCFDA. For each group the percentage of cells that stained positive for oxidative stress is depicted in the graph (E). Red blood cells treated with either 1, 2 or 3 mM tBHP stained 100% of the total population to stain positive for oxidative stress, whereas both 4 and 40 μM ionophore treated cells only showed levels of oxidative stress comparable to untreated control cells.

Table 2. Overview degree of haemolysis. All osmotic fragility data were fitted to a sigmoid curve in order to calculate the NaCl concentration at which 50% of RBCs lysed.

Figure 3. Erythrophagocytosis by endothelial cells (HUVECs) of PS-exposing RBCs induced by calcium ionophore A23187 and oxidative stress (tBHP). A) Quantification of erythrophagocytosis by HUVECs after 1, 2, 3, and 4 hours of incubation with 4 and 40 μM calcium ionophore treated RBCs in the presence (+) of lactadherin, and RGD-modified RBCs(16). B) Quantification of erythrophagocytosis by HUVECs after 1, 2, 3, and 4 hours of incubation with tert-butyl hydroperoxide in the presence (+) of lactadherin. Each experiment was independently performed three times. Graphs depict the mean ± s.d. of 3 independently performed pseudoperoxidase assays. Statistic analysis was performed in which all groups were compared the ‘RBC +’ group at the same time point. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Figure 4. Imaging erythrophagocytosis by HUVECs of oxidative stress (3 mM tBHP) stimulated RBCs. A) Overview of erythrophagocytosis of lactadherin incubated tBHP stimulated RBCs in time. Large numbers of RBCs are phagocytosed, increasing in time, particularly for 2 and 3 mM tBHP. Hematoxylin & eosin (H&E) stained, scale bars represent 20 μm. Close up of HUVECs incubated for 4 hours with 3 mM tBHP stimulated RBCs pre
incubated with lactadherin showing large numbers of RBCs in each HUVEC. H&E stained, scale bar represents 5 μm. C) Transmission electron microscopy (TEM) micrograph showing the intracellular presence of multiple erythrophagocytosed RBCs making the HUVEC ‘round up’. Scale bar represents 2 μm. D) Transmission electron microscopy (TEM) micrograph showing the intracellular presence of multiple erythrophagocytosed RBCs making the nucleus indented. Scale bar represents 2 μm. E) Close up image D. Scale bar represents 1 μm.

**Figure 5. Perfusion of oxidative stress (3 mM tBHP) stimulated RBCs over HUVEC.** Images taken during and after perfusion (shear rate 100 s⁻¹) of 3 mM tBHP stimulated RBCs over HUVECs, A) in the absence of lactadherin no association was seen and B) in the presence of lactadherin marked endothelial cell association was seen. Differential interference contrast (DIC) images, scale bars represent 20 μm. Confocal images taken after perfusion with 3 mM tBHP stimulated RBCs over HUVECs, C) in absence of lactadherin no association was seen and D) in the presence of lactadherin RBCs were taken up by HUVECs. Z-stack scans through HUVEC were made to show RBCs are internalized by HUVEC. Actin cytoskeleton = green (phalloidin), RBCs = red (band 3), magnification 100x (zoom 2).

**Figure 6. Cytotoxic effect of erythrophagocytosis on HUVECs.** Red blood cells (RBCs) treated with 3 mM tBHP were pre-incubated with lactadherin and incubated with HUVECs for 4 hours. After 24 hours cytotoxicity (apoptosis) was measured by staining the HUVECs with both propidium iodide (PI; cell death marker) and annexin V-FITC (PS exposure; apoptosis marker). Generally, cells incubated with RBCs that were 3 mM tBHP treated, in presence of lactadherin, showed some cytotoxicity. In addition, 2 mM tBHP treated RBCs, also resulting in marked erythrophagocytosis, showed less cytotoxicity and after incubation with 1 mM tBHP treated RBCs no toxicity was seen. Graph depicts mean and error bars represent ± s.d. Statistical
analysis: *, $P < 0.05$ versus same $t$BHP concentration without lactadherin; **, $P < 0.001$ versus same $t$BHP concentration without lactadherin.
Figure 1

A

PS exposure (% of population)

RBCs  NEM  4 µM  40 µM  1 mM  2 mM  3 mM

Ionophore A23187  tert-butyl hydroperoxide

B  C  D  E  F  G  H

DOI: 10.3324/haematol.2011.048694

DOI: 10.3324/haematol.2011.048694
Figure 3

A

B
Figure 5
Figure 6
Figure S1. Red blood cell appearance upon stimulation with calcium ionophore or tert-butylhydroperoxide (tBHP). Differential interference contrast (DIC) microscopy images of untreated control (A), NEM (B), 4 µM ionophore (C), 40 µM ionophore (D), 1 mM tBHP (E), 2 mM tBHP (F), 3 mM tBHP (G). Scale bars represent 20 µM.
Figure S2

Figure S2. Western blot analysis of lactadherin incubated ionophore stimulated RBCs. A) After incubation with ionophore followed by lactadherin (+) or no lactadherin (-), a lactadherin band is seen only in the ionophore treated samples. B) Supernatant of lactadherin incubated RBC samples contains abundant lactadherin, indicating that an excess of lactadherin was added.
Figure S3. Perfusion 3 mM tBHP stimulated RBCs over HUVEC. Images taken during perfusion at a shear rate of 300 s⁻¹ of 3 mM tBHP stimulated RBCs over HUVECs, A) in the absence of lactadherin no association was seen and B) in the presence of lactadherin marked endothelial cell association was seen. Differential interference contrast (DIC) images, scale bars represent 20 µm.