Enhanced adherence of human uremic erythrocytes to vascular endothelium: Role of phosphatidylserine exposure

MARIO BONOMINI, VITTORIO SIROLLI, FEDERICO GIZZI, SILVIO DI STANTE, ALFREDO GRILLI, and MARIO FELACO

Institute of Nephrology, Department of Medicine, and Biomorphology Department, G. d’Annunzio University, Chieti, Italy

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Background. The exposure of phosphatidylserine (PS) on the outer leaflet of the erythrocyte membrane may have several pathophysiological consequences including increased erythrocyte adherence to endothelial cells, a finding that seems relevant in pathologies with reported vascular injury.

Methods. Because PS externalization increases in erythrocytes from patients suffering from chronic uremia, which is frequently associated with vascular damage, the adherence of uremic erythrocytes to human umbilical vein endothelial cell (HUVEC) monolayers and the role of PS exposure on such cell-cell interaction were studied.

Results. The number of uremic erythrocytes adhering to HUVEC was markedly greater than with normal erythrocytes and significantly correlated ($r = 0.88$) with the percentage of PS-exposing erythrocytes in the population. Adhesion to the monolayers was significantly decreased when uremic erythrocytes were preincubated with either annexin V or PS-containing liposomes, and was strongly greater for PS-positive than PS-negative fluorescence-activated cell sorter (FACS)-sorted uremic erythrocytes. Binding occurred preferentially in the gaps of HUVEC monolayers and was enhanced by matrix exposure. Uremic erythrocytes adhered to immobilized thrombospondin, and binding to endothelial cells was significantly reduced when monolayers were incubated with antibodies to thrombospondin.

Conclusions. These findings suggest that PS externalization may promote increased uremic erythrocyte adherence to endothelium, possibly via a direct interaction with matrix thrombospondin.

The phospholipids of the normal human erythrocyte (RBC) are distributed asymmetrically in the bilayer of the red cell membrane, with the aminophospholipid phosphatidylserine (PS) located exclusively in the inner leaflet [1]. The maintenance of this asymmetry is an energy-requiring process of major physiological importance for the cell [2]. Thus, loss of membrane asymmetry and in particular the appearance of PS at the outer membrane surface is associated with many physiological and pathological phenomena including enhancement of coagulative reactions and cell recognition by phagocytes [reviewed in 2 and 3]. Apoptosis also is accompanied by PS exposure at the cell surface [4]. In addition, it has been demonstrated that PS externalization participates in RBC adherence to the endothelium [5–9], a process that may be relevant in pathologies with reported vascular damage [8].

There is a high prevalence of vascular disease in patients suffering from chronic renal failure (CRF). CRF is also characterized by a number of structural and functional anomalies of the RBC membrane [10–13]. Recently, we found a significant increase in PS-exposing RBC in chronic uremic patients compared with healthy controls [14]. The abnormality seems related to the uremic state regardless of the dialysis treatment, and is most likely caused by inhibition of PS transport from the outer to the inner leaflet of RBC plasma membrane. We also showed that surface-exposed PS promotes the susceptibility of uremic RBC to phagocytosis [15] and thus may be involved in the shortened RBC lifespan of chronic uremia [16].

On the basis of these observations, it is possible to hypothesize that abnormal exposure of outer-leaflet PS in uremic RBC may significantly affect their propensity for adhesion to endothelial cells. The present study was undertaken to examine the adherence of human uremic RBC to human umbilical vein endothelial cells (HUVEC) and to test the role of RBC PS externalization on such cell-cell interaction.

METHODS

Study population

Twenty healthy control subjects (10 women, 10 men; mean age 57 ± 2 years; range 31 to 72) and 20 stable uremic patients on chronic maintenance hemodialysis (10 women, 10 men; mean age 59 ± 2 years; range 30 to 70) were included in this study after giving informed consent.
consent. Patients were not diabetics [17] nor did they receive recombinant human erythropoietin. Other exclusion criteria were: blood transfusion within the past six months; iron, folic acid and vitamin B12 deficiency; uncontrolled hypertension; active infection; use of drugs that might interfere with erythropoiesis (such as theophylline and angiotensin-converting enzyme inhibitors); and malignant or systemic disease. All hemodialysis patients were being dialyzed three times a week with a four-hour dialysis session using bicarbonate dialysate, and none reused synthetic membranes.

**Materials**

Fluorescein isothiocyanate-labeled annexin V (FITC-AnnV), phosphate-buffered saline (PBS), Hanks' buffered saline solution (HBSS), HEPES buffer, M199 medium, fetal calf serum (FCS), glutamine, heparin, endothelial cell (EC) growth factor, trypsin-edathamil, thrombin, ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetra-acetic acid (EGTA), phosphatidylcholine (PC) from egg yolk, and l-α-phosphatidylserine from bovine brain were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's calcium/magnesium-free PBS was obtained from Mascia Brunelli (Milan, Italy). Anti-thrombospondin mouse monoclonal antibody (clone A4.1) and control monoclonal antibody (trpE) were from Oncogene Research Products (Boston, MA, USA). Endothelial cells (HUVEC) were from Clonetics Corp (San Diego, CA, USA). Thrombospondin from human platelets and fibronectin were purchased from Calbiochem (La Jolla, CA, USA).

**Preparation of cells**

**Erythrocytes.** Blood was drawn by venipuncture into evacuated tubes containing ethylenediaminetetraacetic acid (EDTA). After centrifugation at 700 × g for five minutes at 4°C, the plasma anduffy coat were removed, the RBC pellet was washed three times with PBS, and then resuspended to 1% hematocrit in the same medium for flow cytometry assay or in M199 medium for adherence assay.

**Endothelial cells.** Endothelial cells (HUVEC) were cultured at 37°C in 5% CO2 in 75-cm² flasks (Nunc, Naperville, IL, USA). The culture medium was M199 supplemented with 20% (vol/vol) heat-inactivated FCS, 2 mmol/L l-glutamine, penicillin/streptomycin (100 U/mL and 100 µg/mL), 100 µg/mL heparin and 100 µg/mL EC growth factor. The medium was changed under sterile conditions every two days until the cells reached confluence (3 to 4 days). Cultures grown to confluence, after two washes with calcium/magnesium-free PBS, were harvested using 0.01 trypsin edathamil, split 1:3, and further cultured in new flasks. Confluent monolayers from passage 2 up to passage 6 were used in adherence assays, within two days of HUVEC reaching confluence.

**Preparation of phospholipid vesicles**

Liposomes (small unilamellar vesicles) were prepared by sonication as previously reported [18]. Phospholipids were suspended in chloroform/methanol (90/10), dried under nitrogen and resuspended in PBS by vortexing. The mixtures were then sonicated at 4°C for five minutes at 30 W using a Fisher Sonic Dismembrator model 300. Liposomes contained either 70 molar percent phosphatidylcholine (PC) and 30 molar percent PS (PC/PS liposomes) or only phosphatidylcholine (PC liposomes).

**Endothelial adherence assay**

Confluent EC monolayers were washed twice with PBS to remove traces of serum, covered with RBC suspensions, and incubated for 45 minutes at 37°C while being mechanically agitated. Nonadherent erythrocytes were removed by washing five times with PBS. The RBC that had adhered to the EC were videotaped via an inverted phase-contrast microscope (Leica DM IRB, Leitz, Germany) equipped with a charge-coupled device (CCD) video camera (Model CoolSnap; RS Photometric, Tucson, Arizona, USA). Adherent RBC were counted in 25 random microscope fields ranging over the entire endothelial cell monolayer and reported as the number of adherent cells per 1000 HUVEC.

For studies on the influence of subendothelial matrix exposure on RBC adherence, HUVEC were pretreated for five minutes at 37°C with 0.1 U/mL thrombin in HBSS, 1% bovine serum albumin (BSA), 50 mmol HEPES ph 7.4 (HAH) or with HAH alone as control. These working conditions of thrombin exposure have been shown to be associated with maximal adhesogenic effect [19].

To examine the effect of different components on the binding of uremic RBC to endothelial cells, RBC suspensions from uremic patients were incubated for five minutes with FITC-AnnV (100 nmol/L) to mask RBC surface-exposed PS [15] or with liposomes (final concentration 5 mmol/L phospholipids) composed of either PC or of a mixture PC/PS, before being layered over HUVEC. In another set of experiments, confluent HUVEC monolayers were washed in HAH and incubated for three hours at 37°C with a monoclonal antibody recognizing thrombospondin (TSP) [20] at the concentration of 50 µg/mL or with control antibody at an equivalent concentration. An adherence assay was then performed as described above.

**Adherence to immobilized proteins**

Multiwell slides were coated with 50 µg/mL of purified protein (TSP, FN, or BSA) and incubated at 37°C for one hour in 95% humidity. The slides were then blocked with 1% BSA in HBSS for one hour under the same conditions. RBC suspensions of 1% Hmt in HBSS were placed on protein substrates to an amount sufficient to
cover the entire slide. After incubation for 45 minutes at 37°C, the slides were washed three times with PBS and the adherent RBC were counted in a minimum of 20 fields via an inverted phase contrast microscope equipped with a CCD video camera, as described above.

**Measurement of PS exposure in the RBC population**

The percentage of PS-exposing cells in erythrocyte samples was measured by a flow cytometric assay based on FITC-AnV labeling [14]. Briefly, isolated RBCs were diluted 1:100 (3 to 5 × 10^7 RBC/mL) to a final volume of 0.25 mL in a binding buffer consisting of 10 mmol/L of HEPES-Na (pH 7.4), 136 mmol/L of NaCl, 2.7 mmol/L of KCl, 2 mmol/L of MgCl_2, 1 mmol/L of NaH_2PO_4, 5 mmol/L of glucose, 5 mg/mL of BSA, and 2.5 mmol/L of CaCl_2. After addition of 100 mmol/L of FITC-AnV and incubation for 15 minutes at room temperature in the dark, an aliquot of the sample was directly aspirated into the flow cytometer (Epics Elite; Coulter Electronics, Hialeah, FL, USA). The RBC population was defined by size in forward and side scatter plots: gated cells were counted as annexin positive if they had a mean fluorescence of at least 1.0. The flow cytometer software was used to calculate the percentage of annexin V-positive cells.

For some experiments, FITC-AnV RBC from uremic patients were FACS-sorted to obtain either a PS-positive or PS-negative fraction. Sorted cells were collected into tubes containing 1 mL of PBS and washed once with saline solution. PS-expressing RBC were next incubated with EGTA (2.5 mmol/L) for 30 minutes to remove annexin and washed once with saline solution. Cells (either PS-expressing or non-PS-expressing RBC) were then incubated with HUVEC monolayers for static adherence assay.

**Results**

In recent studies we had observed greater PS exposure in RBC from uremic patients than in healthy controls [14, 15]. Because PS exposure might play its part in RBC adherence to endothelium [5–9], the present study was designed to investigate whether the abnormal PS exposure in uremic RBC might influence their binding to endothelial cells (HUVEC). The number of PS-exposing red cells was determined by annexin V labeling and flow cytometry, and the adherence to confluent HUVEC monolayers by a static adherence assay. Adhesion to non-activated HUVEC by RBC from chronic uremic patients on maintenance hemodialysis was significantly greater (P < 0.001) than adhesion by normal RBC (Fig. 1). The percentage of PS-exposing RBC was also significantly greater in uremic patients than in healthy subjects (3.43 ± 0.18 vs. 0.65 ± 0.03% annexin V-positive RBC, P < 0.01). A strong correlation (r = 0.88, P < 0.0001) was found between the number of uremic RBC adherent to HUVEC and the percentage of PS-exposing RBC in the suspension (Fig. 2).

The latter results suggested that RBC adherence might depend on the percentage of PS-exposing cells in the population. To clarify this possibility, we first FACS-sorted annexin V-positive uremic RBC to obtain either PS-expressing or non-PS-expressing red cells. PS-positive RBC showed a markedly greater propensity for adherence to HUVEC (341 ± 90 RBC/10^3 HUVEC) than...
Fig. 3. Effect of PS competitors on uremic erythrocyte adherence to endothelial cell monolayers. Erythrocyte suspensions from hemodialysis patients (N = 10) were incubated for five minutes with one of the following before the static adherence assay with HUVEC: buffer (Control), annexin V, PS vesicles (30% PS, 70% PC, 5 mMol/L lipid), PC vesicles (100% PC, 5 mMol/L lipid). Error bars depict the standard error. *Significantly different from the control.

Fig. 4. Uremic (□) and normal (■) RBC adherence to immobilized proteins. Multiwell slides were coated with 50 μg/mL of indicated proteins and then blocked with 1% BSA for 60 minutes. A washed suspension of RBC (1% Hmt in HBSS) was placed on the immobilized proteins for 45 minutes, followed by three washes with PBS. *Significantly different between normal and uremic RBC adherence to TSP (P < 0.001, N = 10).

Fig. 5. Effect of monoclonal antibody to thrombospondin (TSP) on uremic RBC adherence to endothelial cell monolayers. HUVEC monolayers were incubated with monoclonal antibody to TSP or a control monoclonal antibody (trpE) before being layered in a static assay with uremic RBC suspensions. Results are expressed as percentage of the buffer-treated control adherence, which is set to 100%. Error bars represent SEM calculated from the absolute values from 8 experiments and normalized relative to the adherence of the buffer-treated control. *Significant differences from the control.

did PS-negative uremic RBC (25 ± 12 RBC/10³ HUVEC; P < 0.02, N = 6). Next, RBC from hemodialysis patients were incubated with FITC-annexin V to mask surface-exposed PS before they were added to HUVEC monolayers. Preincubation with annexin V significantly reduced RBC adherence to HUVEC (P < 0.001; Fig. 3), indicating that annexin V shields PS on the RBC from interacting with endothelial cells. Uremic RBC adherence was also significantly inhibited (by 76%) by the presence of phospholipid vesicles containing PS, whereas the presence of vesicles containing PC alone had no inhibitory effect on adherence (Fig. 3). In the aggregate, these data indicate a major role for a PS-rich domain in the outer hemileaflet of the uremic RBC membrane in causing RBC adherence to endothelial cells.

Ultrastructural observation of RBC-HUVEC binding showed that the adherence of uremic RBC to endothelial cells occurred preferentially to the edges of cells and the gap between cells (data not shown), as previously reported for calcium ionophore-treated PS-exposing RBC [8]. Since such a finding would indicate the importance of subendothelial matrix in uremic RBC-HUVEC binding, we decided to evaluate whether uremic RBC endothelial adhesion could be further enhanced by increasing matrix exposure. To generate exposure of the matrix, HUVEC were pretreated with agents such as thrombin, which cause the cells to retract [19]. Treatment of endothelial cells with thrombin significantly increased uremic RBC adherence. Expressed as an adherence ratio (RBC adherence with thrombin pre-treatment divided by RBC adherence without thrombin pretreatment), this adherence-promoting effect was 2.11 ± 0.25 with a range of 1.20 to 3.50 (N = 8; P < 0.005).

We next examined the adherence of erythrocytes to immobilized adhesive proteins of the endothelial matrix (Fig. 4). There was markedly greater adherence by uremic erythrocytes to immobilized TSP than to FN or to BSA (non-specific control). In comparison with normal RBC, RBC from uremic patients showed a significantly increased adherence to TSP, while exhibiting no statistical difference in their adherence to either FN or BSA (Fig. 4). The ability of uremic RBC to adhere to immobilized TSP was inhibited by 72% when red cells were preincubated with annexin V (76 ± 12 RBC/10³ HUVEC vs. 300 ± 22 RBC/10³ HUVEC for untreated RBC, P < 0.001, N = 10).

In additional experiments, HUVEC monolayers were incubated with a monoclonal antibody recognizing TSP [20] or a control monoclonal antibody, before static adherence assay. As shown in Figure 5, uremic RBC adher-
ence proved significantly decreased (P < 0.001) when HUVEC had been pretreated with anti-TSP antibody, whereas irrelevant antibody had no effect on adherence.

**DISCUSSION**

The exposure of PS on the external leaflet of the RBC plasma membrane can have several pathophysiological consequences with particular regard to the processes of hemostasis, cell phagocytosis and cell-cell interaction [2, 3]. The present study found not only increased RBC PS exposure, confirming our previous findings [14, 15], but also an enhanced adherence of uremic RBC to endothelial cell monolayers, which represents a new observation in chronic renal failure. Our results suggest that PS on the uremic RBC surface may serve as a recognition site for the HUVEC monolayers. These results also suggest a role for subendothelial matrix factors, possibly TSP, in promoting the abnormal adherence of uremic RBC to endothelial cells.

This study is consistent with the observation previously made in other conditions that loss of membrane phospholipid asymmetry in RBC may lead to RBC-endothelium binding [5, 6, 8, 21, 22]. Several observations argue in favor of a role for surface-exposed PS in the adherence of uremic RBC to endothelial cells. A linear correlation between adherence and the number of exposing PS RBC was found. In addition, a remarkable difference was observed between HUVEC adhesion of PS-exposing or PS-negative uremic RBC. Strong evidence for the involvement of PS on the uremic RBC surface in binding to endothelial cell monolayers is provided by results of experiments with PS competitors. A significant reduction in adherence was observed when uremic RBC were preincubated with annexin V. This has a propensity for binding to the PS domain, which may render it unavailable for other potential receptor-mediated processes [15]. Similarly, PS-containing lipid vesicles decreased uremic RBC binding by competing with PS-exposing RBC for the apparent binding sites on the HUVEC monolayer. Specificity for a PS pathway is demonstrated by the absence of any inhibitory effect by PC only liposomes. Although our data do not exclude the possibility that other factors also may be important, they indicate a major role for externalized PS in the adherence of uremic RBC to HUVEC monolayers.

Regarding the endothelial cell’s participation in uremic RBC-HUVEC binding, though we have not attempted to fully delineate this aspect, both the localized interaction between uremic erythrocytes and HUVEC monolayers and the doubly increased RBC adhesiveness found when matrix exposure had been generated, suggest that an adherence-promoting factor(s) is (are) involved in the subendothelial matrix. Recent studies have shown that the adherence of calcium ionophore-treated PS-exposing RBC to HUVEC monolayers may occur via a direct interaction between PS and TSP in the subendothelial matrix [8]. TSP is a “matricellular” protein [23], capable of functional interactions with a variety of cell types and matrix proteins [24–26]. It is synthesized and secreted by endothelial cells and some other cell types in culture and is incorporated into the extracellular matrix of these cells [27–29]. TSP is trimeric and multifunctional, making it suitable for participation in various and complex adhesive events.

Our results show that uremic RBC adhered preferentially to immobilized TSP as distinct from FN or BSA. Adherence to TSP by uremic RBC was significantly greater than adherence by normal RBC and was markedly inhibited by preincubation of red cells with annexin V, which further suggests a significant adhesogenic effect for PS exposed on the RBC membrane. Furthermore, adherence to endothelial cells of uremic RBC significantly decreased when HUVEC monolayers were pretreated with antibodies directed against TSP. Taken together, these results indicate a possible role for a TSP-mediated mechanism in the abnormal adherence of uremic RBC to vascular endothelium. This issue, however, deserves further investigation, since an inability to completely block adherence under conditions that decrease the availability of TSP suggest the existence of other adherence-promoting factors.

The RBC-endothelial interaction seems especially pronounced in pathologies characterized by vascular damage and a subpopulation of PS-exposing RBC, such as sickle cell anemia, diabetes, and thalassaemia [6, 21, 22]. It has been shown that adhesion of sickle erythrocytes to the vascular endothelium may cause inhibition of endothelial DNA synthesis, up-regulation of endothelin-1 gene expression, decrease in the levels of nitric oxide synthase mRNA and protein, and inhibition of nitric oxide activity [abstract; Phelan et al: *Blood* 86(Suppl 1): 418a, 1995] [30–32].

Many studies point to endothelial dysfunction and reduced nitric oxide bioactivity in chronic uremic patients [33–40], a finding that may play a role in the pathogenesis of vascular disease in these patients. Our present data indicate that increased PS exposure on the surface of uremic erythrocytes causes adhesion of these cells to endothelial cell monolayers. Whether abnormal RBC adhesiveness contributes to endothelial dysfunction in uremia is a concept that still requires exploration.

**REFERENCES**

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