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Aging and glycation promote erythrocyte phagocytosis by human endothelial cells: Potential impact in atherothrombosis under diabetic conditions

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HIGHLIGHTS

- The clearance of erythrocytes may be carried out by vascular cells in atherosclerotic conditions.
- Phagocytosis by endothelial cells is more pronounced with aged and glycated erythrocytes.
- Endothelial phagocytosis is enhanced with T2D erythrocytes.
- Interactions between erythrocytes and endothelial cells occur *in vivo,* in atherothrombotic/diabetic conditions.

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ABSTRACT

Background and aims: Atherothrombotic plaques of type 2 diabetic (T2D) patients are characterized by an increased neovascularization and intraplaque hemorrhage. The clearance of erythrocytes may be carried out by vascular cells. We explored the potential of human endothelial cells to bind and phagocyte *in vitro* aged and/or glycated erythrocytes as well as erythrocytes obtained from diabetic patients.

Methods: Fresh, aged and glycated-aged erythrocytes from healthy volunteers and T2D patients were tested for their binding and phagocytosis capacity as well as the potential functional consequences on endothelial cells (viability, proliferation and wound healing capacity). Immunohistochemistry was also performed in human carotid atherothrombotic samples (from patients with or without T2D).

Results: Aging and glycation of erythrocytes induced phosphatidylserine (PS) exposure and oxidative stress leading to enhanced endothelial cell binding and engulfment. Phagocytosis by endothelial cells was more pronounced with aged and glycated erythrocytes than with fresh ones. Phagocytosis was enhanced with T2D *versus* healthy erythrocytes. Furthermore, endothelial wound healing potential was significantly blunted after exposure to glycated-aged *versus* fresh erythrocytes. Finally, we show that interactions between erythrocytes and endothelial cells and their potential phagocytosis may occur *in vivo,* in atherothrombotic conditions, in neovessels and in the luminal endothelial lining.

Conclusions: Endothelial cells may play an important role in erythrocyte clearance in an atherothrombotic environment. Under diabetic conditions, erythrocyte glycation favors their engulfment by endothelial cells and may participate in endothelial dysfunction, thereby promoting vulnerable atherothrombotic plaques to rupture.

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1. Introduction

Atherothrombosis represents the first cause of mortality in diabetic patients [1]. Different risk factors such as hypertension, hypercholesterolemia or smoking are known to promote a more rapid plaque growth in diabetic conditions [2]. Increased platelet aggregation and activation of coagulation associated with a blunted fibrinolytic process lead to thrombus formation in case of both micro- and macrovascular diabetic vascular complications [3].

Chronic hyperglycemia induces protein glycation resulting in the formation of AGE (advanced glycated end-products) in the extracellular matrix and at the cell surface such as in red blood cells (RBC) that increase their adhesion to atherosclerotic plaques [4]. Increased thrombin and fibrinogen in diabetic conditions lead to the formation of a more compact clot, less prone to fibrinolysis, since plasmin glycation impairs its fibrinolytic activity [3]. In addition to a globally prothrombotic state, endothelial function is altered in diabetic patients, which favors atherosclerotic plaque formation. Chronic hyperglycemia induces glycation and oxidation of both lipids and proteins, which accumulate within the arterial wall, leading to a reduced NO production that affects vasodilation [5].

The presence of immature neovessels originating from adventitial *vasa vasorum* is associated with intraplaque hemorrhage that promotes plaque vulnerability to rupture [6,7]. Purushothaman et al. showed that both neovessel density and intraplaque hemorrhage were increased in diabetic *versus* non-diabetic patients [8]. Vessel leakage may cause extravasation of leukocytes and RBC, which represents an important source of cholesterol and oxidative stress [9], in particular following the release of hemoglobin into the extracellular compartment that will participate in plaque expansion [10]. In diabetic conditions, RBC protein glycation is observed, including that of hemoglobin (N-ter of the beta chain, HbA1c) [11]. Other proteins at the surface of RBC may also undergo glycation as shown by detection of AGE via the quantification of carboxymethyl-lysin and carboxyethyl-lysin residues [12]. In a pro-inflammatory and pro-oxidant environment, RBC undergo peroxidation as well as a loss of membrane lipid asymmetry, leading to apoptosis (also called eryptosis) [4]. The clearance of extravasated RBC by specialized phagocytes may be impaired in atherosclerotic conditions, in which macrophage foam cells display a reduced capacity to engulf apoptotic cells [13,14]. Other cell types such as smooth muscle cells (SMC) are able to phagocyte apoptotic bodies [15] via exposure of phosphatidylserine [16] and were shown to ingest aged RBC [17]. Endothelial cells represent the first barrier in contact with erythrocytes. In intraplaque neovessels, the reduced blood flow may favor interactions between RBC and endothelial cells, suggesting that a phagocytic process may occur *in vivo*, in particular for aged or modified RBC. Fens et al. have reported that endothelial cells are able to phagocyte RBC after PS exposure in both static and flow conditions [18]. Erythrocyte glycation in diabetic patients was shown to promote RBC/endothelial cells interactions via AGE and their receptors [19]. However, there are almost no data concerning the phagocytosis of aged and/or glycated RBC as well as the phagocytosis of T2D RBC by endothelial cells.

We consequently address in this study the impact of chronic hyperglycemia, as observed in diabetic patients, on RBC modifications, interactions with endothelial cells and potential vascular dysfunction. We tested the hypothesis that aged and glycated RBC may be more readily phagocytosed by endothelial cells than fresh, non-modified RBC. We have set up and characterized an *in vitro* model of RBC glycation and demonstrated that aged/glycated RBC engulfed by endothelial cells induced a decreased endothelial capacity for healing. This process may participate in the formation of intraplaque hemorrhage in diabetic patients with atherosclerosis.

2. Materials and methods

2.1. Erythrocytes preparation, aging and glycation

Erythrocytes were obtained from healthy (male and female) volunteers and type 2 diabetic patients with informed consents. Blood was collected on EDTA tubes (BD vacutainer®). Erythrocytes were washed 3 times with an isotonic solution (0.15 M NaCl, pH 7) and prepared in Phosphate Buffered Saline solution (1X PBS) – 5 mM glucose (PBS/0.1% glucose) at 20% hematocrit. Glycation and aging were induced by incubating RBC for 5 days at 37 °C with D-glucose (Sigma, Germany) at different concentrations (5, 17.5, 30, 55, 105 and 130 mM) [17]. Erythrocytes were either used fresh, aged with or without glycation on human endothelial cells.

2.2. Red blood cell hemolysis test (free radical induced hemolysis test)

The ability of erythrocytes to resist to lysis induced by free radicals was investigated [20]. Fresh, aged and glycated-aged erythrocytes from healthy subjects and T2D patients were washed and diluted at 50% in an isotonic solution (0.15 M NaCl, pH 7). Hemolysis was induced by addition of 40 µL of 0.5 M 2,2′-Azobis[2-methyl-propionamidin] dichloride (AAPH, Sigma-Aldrich, Germany) in each well containing 10^8 erythrocytes. Turbidimetry at 450 nm was recorded every 10 min using a 37°C- thermostated microplate reader (FLUOstar Omega, BMG Labtech, Germany). Results were expressed as 50% of maximal hemolysis time (HT50 in min).

2.3. HbA1c (%) determination

HbA1c measurements were performed on diluted aged and glycated-aged erythrocytes using a high-pressure liquid chromatography method performed on automate D-10 Hemoglobin analyser (Biorad).

2.4. Thiobarbituric assay

The thiobarbituric acid (TBA) assay was used for 5-hydroxymethylfurfural (HMF) determination as described by McFarland et al. [21]. 0.5 mL of diluted aged and glycated-aged erythrocytes (1:2 dilution in 0.15 M NaCl) from healthy and T2D patients were hydrolyzed for 1 h by addition of 0.5 mL of 0.3 N oxalic acid at 100 °C. After cooling to RT, 0.5 mL of 40% trichloroacetic acid was added and thoroughly mixed before centrifugation for 15 min at 3000 g. About 0.2 mL of 0.05 M TBA was added to 0.4 mL of supernatant and the mixture was incubated at 40 °C for 30 min. The absorbance was read at 443 nm. The 5-HMF concentration was determined by using a molar extinction coefficient of 4.10^8 and was expressed as mmol/mg of protein.

2.5. Endothelial cell culture

EA.hy926 endothelial cell line was obtained from the American Type Culture Collection (ATCC, USA, CRL-2922™) and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 25 mM glucose, 10% heat-inactivated Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 250 µg/mL amphotericin B and HAT (100 µmol/L hypoxanthine, 0.4 µmol/L aminopterin and 16 µmol/L thymidine). Cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

The human blood-brain barrier endothelial cell line HcMEC/D3, was obtained from Merck Millipore, France (SCC066). HcMEC/D3 cells were cultured in Endothelial Basal Medium 2 (EBM-2, Lonza) supplemented with 5.6 mM glucose, 5% heat-inactivated FBS, 100 units

penicillin/mL, 100 μg streptomycin/mL, 2.8 mM hydrocortisone, 1 mg/mL ascorbic acid, Chemically Defined Lipid Concentrate (1:100), 1 M HEPES buffer and 200 ng/mL bFGF. Cells were grown on coated tissue culture flasks with Cultrex® Rat Collagen I (50 μg/mL) at 37 °C with 5% CO_2 in a humidified atmosphere.

The human dermal microvascular endothelial cell line HMEC-1, obtained from ATCC (USA, CRL- 3243™) were cultured in MCDB131 medium supplemented with 5.6 mM glucose, 10 ng/mL epidermal growth Factor (EGF), 1 μg/mL hydrocortisone, 10 mM L-glutamine, 10% heat-inactivated FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

For all experiments, confluent endothelial cells (EA.hy926, HcMEC/D3 or HMEC-1) were stimulated with fresh, aged or glycated-aged erythrocytes from healthy volunteers or T2D patients at a density of 18.6×10^6 cell/cm^2 for 24 h at 37 °C.

2.6. MTT assay

Endothelial cell viability was evaluated by assessing the mitochondrial metabolic activity, using MTT (3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Cells were seeded in a 96-well plate at a density of 100 000 cells/cm^2 (32 000 cells/wells). After reaching confluence, cells were incubated or not with RBC for 24 h at 37 °C. Endothelial cells were then washed 3 times with sterile 1X PBS and incubated at 37 °C for 5 h with 200 μL of fresh medium supplemented with 20 μL of sterile filtered MTT solution (5 mg/mL in PBS). After removing the medium, the insoluble formazan crystals were dissolved in 200 μL/well dimethyl sulfoxide (DMSO). All samples were diluted in DMSO (1:2 dilution in 200 μL) and the absorbance was measured at 570 nm with a reference at 690 nm (FLUOstar Omega, BMG Labtech, Germany). Results are expressed as the percentage of control (untreated cells).

2.7. Cell division assay

The thymidine analogue 5-bromo-2′-deoxyuridine (BrdU, Sigma) was used to assess cell division kinetics of EA.hy926 cells. After 24 h of stimulation with erythrocytes in 24-well plates, EA.hy926 cells were incubated for 24 h at 37 °C with a 10 μM BrdU solution. Then, BrdU-labelled cells were washed with DMEM medium, collected by trypsinization and seeded out in DMEM/10% FBS (5000 cells/well) in 6 well plates. Cells were left in 5% CO_2 incubator for 10 days. After cells collection by trypsinization and three washes with cold PBS, EA.hy926 cells were successively fixed with ice cold ethanol (70% v/v) for 30 min at 4 °C, permeabilized with 2 N HCl/0.5% Triton X-100 to denature the DNA and neutralized with 0.1 M $Na_2B_4O_7$, pH 8.5 solutions. Then, cells were stained with anti-BrdU monoclonal antibody (Sigma, 1:100 dilution in PBS/0.5% Tween 20/1% BSA containing 10 μg/mL RNAse A) followed by an incubation with a secondary anti-mouse FITC-conjugated antibody.

(Molecular probes, 1:200 dilution). BrdU-labelled cells were washed and resuspended in PBS before analysis on FACScan (BD Biosciences) and CellQuest software.

2.8. Clonogenic assay

A clonogenic assay (or proliferation assay) was performed to assess the potential impact of aged and glycated-aged erythrocytes on endothelial function, according to the method previously described in Ref. [22] with slight modifications. After 24 h of stimulation with 1.8×10^8 of fresh, aged or glycated-aged erythrocytes, EA.hy926 cells were collected by trypsinization and seeded out in DMEM/10% FBS in 12-well plates (i.e 1500 cells/well). After 12 days of culture, cells were washed twice with PBS, were fixed with glutaraldehyde (6.0% v/v) and stained with crystal violet (0.5% w/v). The plates were carefully washed twice with tap water and the stain was solubilized by the addition of 100 μL of 1% sodium dodecyl sulphate. The absorbance was then read at 570 nm. Results were expressed as % proliferation relative to control untreated cells.

2.9. Flow cytometry assay

CD47 conformational changes analysis, phosphatidylserine exposure and ROS production by erythrocytes, apoptosis/necrosis of erythrocyte-stimulated EA.hy926 cells and bound erythrocytes to endothelial cells were assessed by flow cytometry, using a Becton Dickinson FACScan (BD Biosciences) and CellQuest software.

For conformational changes of CD47 on red blood cell membrane, washed erythrocyte samples were double stained with two distinct CD47 antibodies (10 μg/mL in HEPES) directed against two different epitopes: an APC-conjugated conformation-independent antibody (clone B6H12) and a FITC- conjugated conformation-dependent (clone 2D3) antibody.

For quantification of phosphatidylserine exposure, erythrocytes were labelled with 2 μg/mL annexin V-FITC in binding buffer (Biolegend) before flow cytometry analysis. Cells were fixed for 20 min in 4% paraformaldehyde solution and annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

For determination of ROS production, 50 μL of RBC (approximately 13×10^6 RBC) were incubated with 500 μL of a labeling solution (PBS 1X – DCFH-DA 1X or DHE 1X) for 30 min at 37 °C.

For apoptosis/necrosis study, EA.hy926 cells were collected by trypsinization, washed with PBS. Then, cells were incubated with 2 μg/mL Annexin V-FITC and 2 μg/mL PI (Sigma) in 100 μL binding buffer (BioLegend) for 15 min at RT before analysis.

For assessment of erythrocytes bound to endothelial cells, EA.hy926 were washed with PBS, trypsinized and fixed for 20 min in 4% paraformaldehyde solution. After fixation, cells were incubated with PBS/1% BSA for 30 min, in order to block non-specific sites. Cells were next incubated with rabbit anti-CD31 polyclonal antibody (for endothelial cells labeling, eBioscience, 1:100 dilution) for 30 min, followed by an incubation with anti-rabbit PE-conjugated secondary IgG (1:200 dilution). After that, cells were incubated with mouse anti-human glycophorin A antibody for erythrocytes labeling (Dako, 1.585 μg/mL) for 30 min followed by an incubation with a goat anti-mouse secondary antibody conjugated to Alexa 488 (Life technologies, 1:200 dilution). FACS analysis was performed on gated endothelial CD31-positive cells.

2.10. DAF colorimetric assay

Detection of erythrocytes associated with EA.hy926, HMEC-1 and HcMEC/D3 cells (either bound or internalized) was performed by using 2,7-diaminofluorene (DAF) reagent which quantifies the pseudo-peroxidase activity of RBC hemoglobin. DAF working solution was prepared by dissolving 10 mg of DAF in 10 mL of 0.2 M Tris-HCl pH 7.2/9% glacial acetic acid with vigorous vortexing and by adding 20 μL of 30% hydrogen peroxide just before use.

EA.hy926, HcMEC/D3 or HMEC-1 cells plated in 12-well plate and incubated for 24 h with PBS/0.1% glucose or RBC were washed three times with PBS. Hemoglobin contained in non-phagocytosed erythrocytes bound to endothelial cells was recovered by an osmotic shock with 100 μL of water for 3 min. Endothelial cells were then lysed to release hemoglobin contained in phagocytosed erythrocytes by adding 100 μL of PBS/1% Triton X100 for 3 min. Next, 100 μL of the DAF working solution were added to 40 μL of samples which were previously transferred to a 96-well plate. The reaction mixture was incubated for 20 min and the optical density (OD) was measured at 620 nm using a spectrophotometer microplate reader. Bound and internalized RBC number was calculated by using a calibration curve of RBC (10^2-10^5 cells/μL). The results were expressed as RBC number/μL of sample.

2.11. Visualization of erythrocyte binding and internalization by immunofluorescence

Ea.hy926 cells were seeded on coverslips and incubated for 24 h with PBS/0.1% glucose, healthy fresh, aged or glycated-aged RBC, washed three times with PBS and fixed for 20 min in 4% paraformaldehyde solution. Cells were then permeabilized with PBS/0.05% Triton X100 for 10 min. For immunofluorescence including confocal microscopy, RBC were stained with a mouse anti-human glycophorin A antibody (Dako, 1.585 µg/mL), endothelial cell cytoplasm was stained with a rabbit anti-human alpha tubulin antibody (Sigma, 2 µg/mL). The secondary antibodies used were respectively a goat anti-mouse antibody conjugated to Alexa 488 (Life technologies, 1:200 dilution), a goat anti-rabbit antibody conjugated to Alexa 594 (Invitrogen, 1:200 dilution). Endothelial cells nuclei were stained with DAPI (Sigma, 0.1 µg/mL). Controls using a non-relevant IgG (Dako) were included in each set of experiments.

2.12. Wound healing assay

To study endothelial cell ability to repair a lesion, a scratch assay or wound healing assay was performed on EA.hy926 endothelial cells according to the method described previously [23]. EA.hy926 cells were plated in 60 mm petri dishes and incubated for 24 h with PBS/0.1% glucose, fresh, aged or glycated-aged RBC before three washes with PBS and incubation with complete medium. Four scratches were performed on the cell monolayer with a 200 µL tip. The debris were removed and cells were incubated with fresh complete medium. Cells were maintained at 37 °C with 5% CO_2 in a humidified atmosphere for 24 h. Microscopic bright field pictures were taken at 0 h, 2 h, 4 h and 10 h after the scratch induction on an inverted microscope Nikon Ti–U eclipse. The scratch area was measured with imageJ software (imageJ 1.43 software, USA). Results were expressed as the reduction of wound area in AU of five independently performed experiments.

2.13. Carotid endarterectomy sample - histological analysis

Hemorrhagic carotid atherosclerotic plaque samples from diabetic (n = 3) and non-diabetic patients (n = 3) were obtained from the MADi study (Marqueurs d'Athérothrombose chez le Diabétique, CHU de La Réunion, France). All patients gave their informed consent to participate to the study (CPP N°2013/69, A01202-43). After surgery, a representative endarterectomy sample was fixed in 4% paraformaldehyde and then embedded in paraffin using standard histological protocols. Six-micron paraffin sections were rehydrated before immunostaining. Antigen retrieval was performed by incubation in pH6 10 mM citrate buffer at 95 °C for 5 min. Dako Envision procedure was then applied according to the manufacturer's instruction. Briefly, endogenous peroxidase activity was inhibited by 0.3% H_2O_2, non-specific sites were blocked by 1% bovine serum albumin in TBS-T for 30 min (50 mM TRIS-HCl pH7.6, 150 mM NaCl containing 0.05% Tween 20). The primary antibodies were incubated at room temperature for 60 min: anti-glycophorin A at 2.6 µg/mL (M0819, Dako), anti-von Willebrand factor at 3.17 µg/mL (M0616, Dako). A non-immune mouse IgG1 was applied as control in the same conditions (X0931, Dako). The secondary anti-mouse antibodies conjugated to horseradish peroxidase and linked to a polymer were incubated for 30 min at RT and the reaction was performed using diamino-benzidine. All steps were separated by 3 washes with TBS-T. The nuclei were counterstained with Harris hematoxylin for 1 min. After a final wash with tap water, the sections were dehydrated and the slides were mounted in Eukitt resin before analysis with NanoZoomer S60 digital slide scanner (Hamamatsu).

2.14. Statistical analysis

Data were expressed as mean ± standard deviation (SD). All assays were performed at least in three independent experiments, with triplicate for each of them. Statistical analysis was achieved using Prism software (Prism software, USA). Significant differences ($p < 0.05$) between the means were determined by analysis of variance one-way ANOVA procedures followed by Tukey's multiple comparison test, respectively.

Table 1
HbA1c levels in diabetic patients.

Patients ID	HbA1c %
1	10.4
2	16.7
3	9.3
4	13.6
5	10.6
6	10.8
8	9.7
9	10.3
10	10.7
11	11.1
12	13.2
Mean	11.5
SD	2.2

Quantification of glycated hemoglobin (% HbA1c) in diabetic RBC was performed on an automate D-10 Hemoglobin analyser (Biorad).

3. Results

3.1. Effects of glycation and aging on erythrocytes

Red blood cells were aged and/or glycated *in vitro* by incubation at 37 °C for 5 days with or without D-glucose at different concentrations, ranging from 5 to 130 mM. In order to evaluate *in vitro* RBC glycation, the percentage of glycated hemoglobin (HbA1c) was measured by routine automated HPLC (Bio-Rad). As expected, RBC incubated with high D-glucose concentrations showed significantly increased percentage of HbA1c compared to non-glycated RBC (11.4 ± % *vs.* 5 ± %, $p < 0.05$) at 130 mM of D-glucose, Supplemental Fig. 1A). This value is equivalent to that of patients with a poorly controlled T2D (Table 1). 5-Hydroxymethylfurfural (5-HMF), a marker of glucose degradation formed during Maillard reaction, was then quantified. High levels of D-glucose (105 and 130 mM) induced a significant increase in RBC 5-HMF production during the glycation process as compared to that of non-glycated RBC (Supplemental Fig. 1B).

Phosphatidylserine (PS) exposure at the cell surface is often recognized as an initial step for binding and subsequent phagocytosis (“eat-me” signal). This parameter was assessed in aged RBC ± glycation by evaluating the percentage of FITC-annexin V-positive erythrocytes using flow cytometry. Aging ± glycation significantly increased PS exposure (ranging from 76.08% to 95.8% annexin V-positive cells) compared to fresh RBC from healthy volunteers (4.29% annexin V-positive cells). Glycation significantly enhanced PS externalization by RBC relative to aging alone (Fig. 1A).

In parallel, the expression of CD47, an RBC membrane protein, has been reported to be modulated under aging condition and its conformational change could represent another “eat-me” signal involved in erythrophagocytosis signalization [24]. We have used two different antibodies against CD47 detecting respectively a constant conformational epitope (clone B6H12) and a conformational variable epitope (clone 2D6) (Fig. 1B). No significant change in membrane CD47 expression (B6H12) was observed for fresh, aged and aged/glycated RBC whereas a significant increase in CD47-FITC fluorescence was observed for aged/glycated erythrocytes using the 2D3 clone antibody, reflecting a conformational change. This result suggests that albeit an unchanged global expression of membrane RBC CD47, a conformational change is

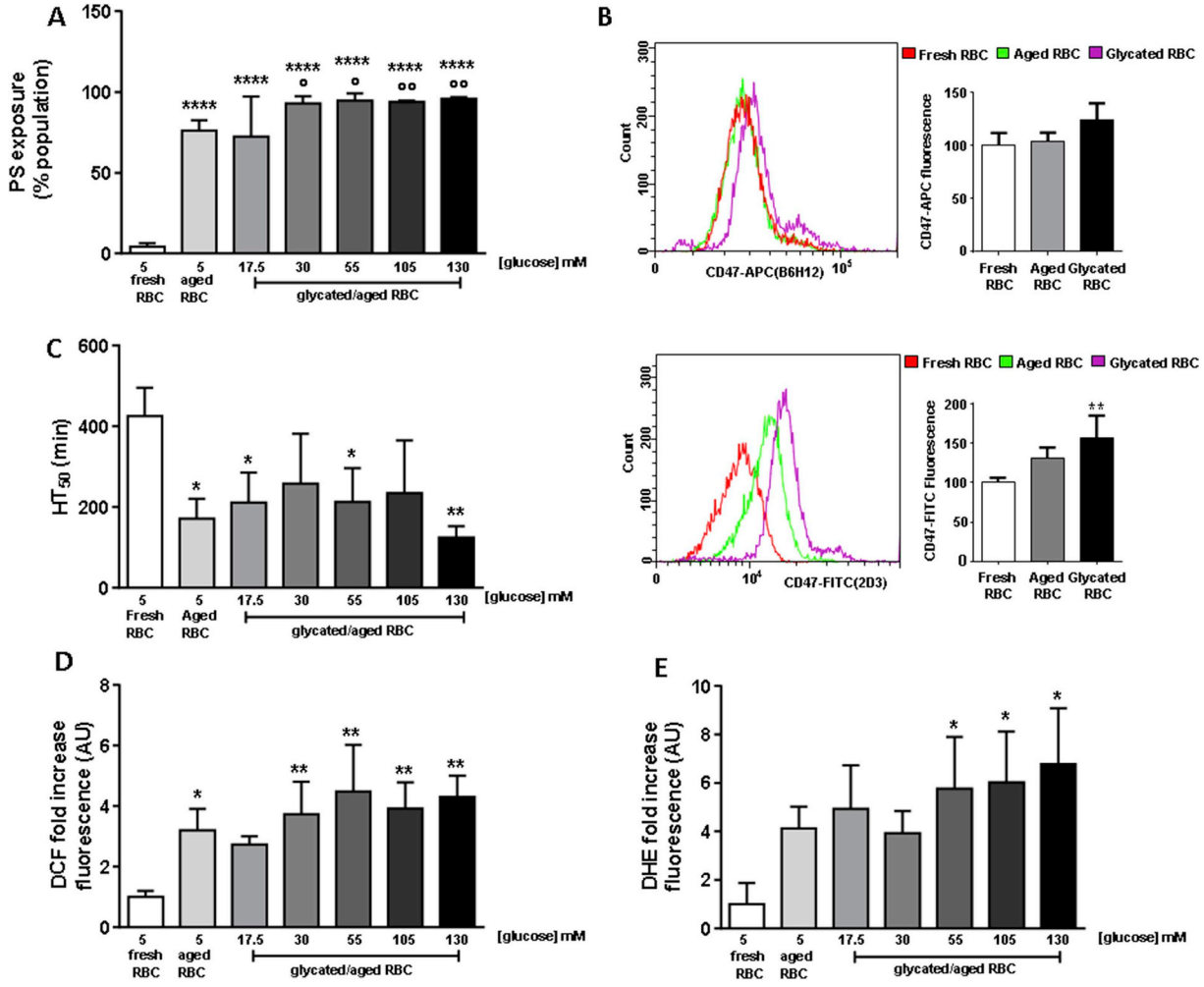

Fig. 1. Characterization of a red blood cell (RBC) model subjected to aging and glycation for investigation of endothelial erythrophagocytosis.
RBC were either incubated in PBS/0.1% glucose at 20% hematocrit for 5 days at 37 °C (aged RBC) or supplemented with different amounts of glucose (5–130 mM). Alternatively, erythrocytes were used freshly isolated from the same healthy volunteers. (A) Representative histograms of APC-labelled CD47 (clone B6H12) and FITC-labelled CD47 (clone 2D3) for fresh, aged and glycated RBC (with 130 mM glucose) obtained by flow cytometry. Anti-CD47 antibodies detect conformational independent (clone B6H12) and conformational dependent (clone 2D3) epitopes of CD47, respectively. Fluorescence quantification data of CD47-APC and CD47-FITC are mean ± SD of four independent experiments. (**$p < 0.01$ compared to healthy fresh RBC). (B) Phosphatidylserine exposure of healthy fresh, *in vitro* aged and glycated/aged RBC was assessed by flow cytometry. Histograms represent the percentage of PS-exposing RBC, results are the mean ± SD of three independently-performed experiments. RBC aging increased significantly PS exposure compared to healthy fresh RBC. RBC glycation increased significantly PS exposure compared to aged RBC. (****$p < 0.0001$ compared to healthy fresh RBC. *$p < 0.05$ and **$p < 0.01$ compared to aged RBC). (C) Hemolysis test was used to assess healthy fresh and *in vitro* modified RBC ability to resist to an oxidative insult. Hemolysis was triggered by adding 2,2′-Azobis[2-methyl-propionamidin] dichloride (AAPH) and the absorbance at 450 nm was monitored every 10 min at 37 °C. Results are expressed as 50% of maximal hemolysis time (HT_{50} min) as mean ± SD (n = 6; *$p < 0.05$ and **$p < 0.01$ compared to healthy fresh RBC). (D) Intracellular ROS production by healthy fresh and *in vitro*-modified RBC was measured by flow cytometry. (E) RBC were incubated with DCFH-DA and DHE in PBS for 30 min at 37 °C for determination of H_2O_2 and superoxide anion production, respectively. Histograms expressed the fold increase in fluorescence as mean ± SD (n = 3; * $p < 0.05$ and **$p < 0.01$ compared to healthy fresh RBC for DCF and DHE production).

observed in aged/glycated erythrocytes, suggesting a potential supplementary "eat-me" signal presented by these cells (in addition to PS exposure) to potential phagocytes.

We then evaluated the effect of aging and/or glycation on RBC ability to resist free radical-induced hemolysis. In this test, healthy fresh RBC exhibited a higher hemolysis half-time (HT50) than that of aged/glycated RBC (Fig. 1C). RBC aging ± glycation reduced HT50 compared to healthy fresh RBC, respectively by 60 and 71% (130 mM of D-glucose). Both aging and glycation induced RBC fragility in response to an oxidative insult.

To quantify oxidative stress induced either by aging or glycation on RBC, different types of radical oxygen species (ROS) were measured by flow cytometry (Fig. 1D and E). Hydrogen peroxide production was increased in both aged and aged/glycated RBC (DCFH-DA test). With the measurement of DHE fluorescence, only glycation at 55, 105 and 130 mM of D-glucose induced an increase in superoxide anion production when compared to aged RBC (Fig. 1E).

3.2. Erythrocytes binding and phagocytosis by endothelial cells

To investigate whether RBC may bind and be phagocytosed by endothelial cells, fresh, aged and aged/glycated erythrocytes were incubated with human endothelial EA.hy926 cells for 24 h at 37 °C. Epifluorescence and confocal microscopy were used to show the interaction between RBC and endothelial cells (Fig. 2A, RBC glycophorin A in green, endothelial cell alpha tubulin in red and nuclei in blue). Aging increased RBC interactions with endothelial cells relative to healthy fresh RBC, and glycation amplified this phenomenon (left-

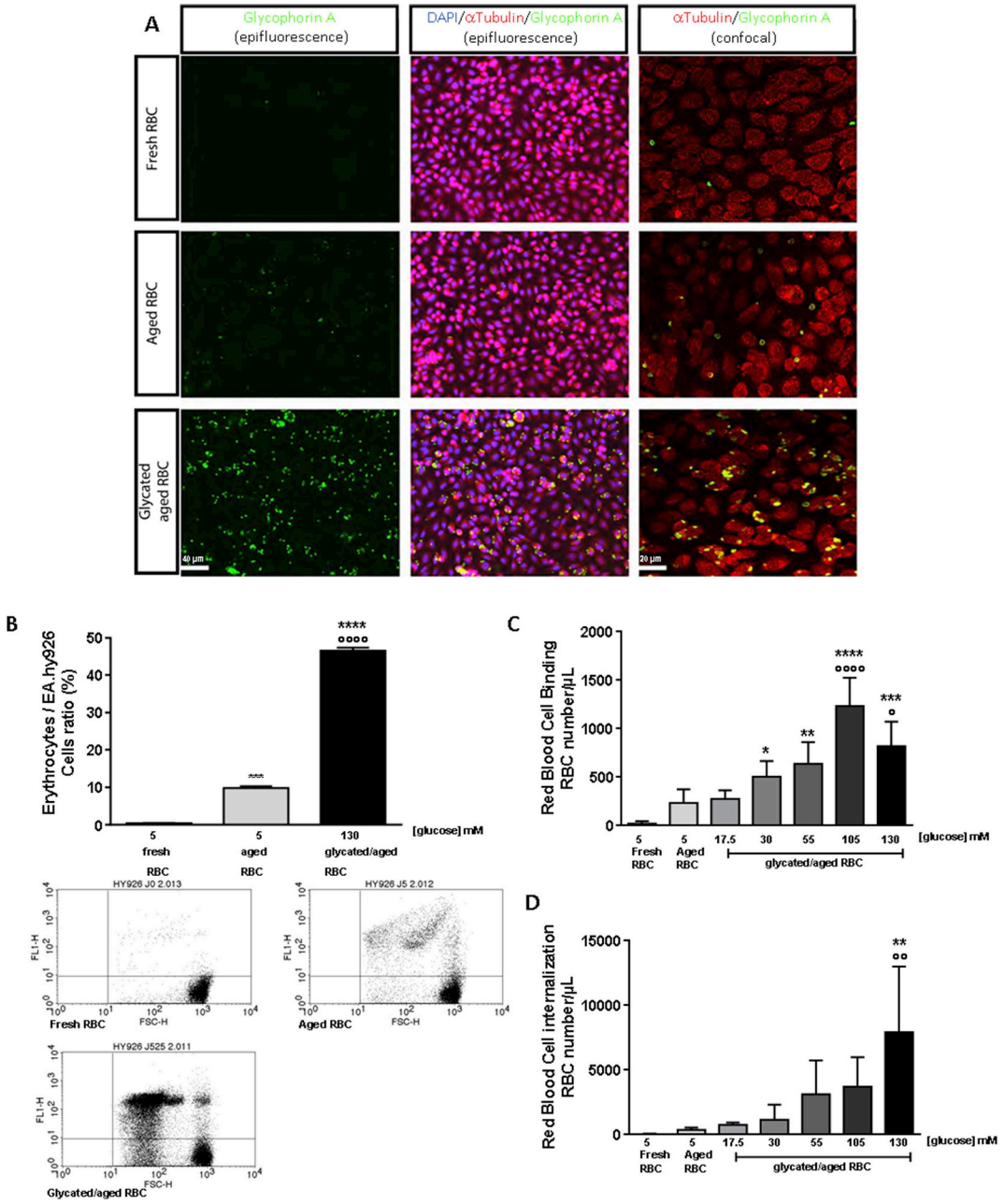

Fig. 2. Human endothelial cells EA.hy926 are able to bind to and internalize glycated/aged RBC *in vitro*.

Human endothelial cells EA.hy926 were incubated with fresh, aged or glycated/aged (with 130 mM glucose) RBC for 24 h at 37 °C. After incubation, cells were washed three times with PBS 1X to discard unbound RBC. (A) Immunostaining of EA.hy926 (α tubulin in red) and RBC (glycophorin A in green) after co-incubation. α Tubulin antibody was used for labeling of the cytoplasm of EA.hy926 endothelial cells and glycophorin A for RBC membrane labeling. Both aged and glycated/aged RBC exhibit an enhanced interaction with EA.hy926 compared to fresh RBC (left panels, epifluorescence). Confocal imaging analysis (right panels) was performed, showing an enhanced phagocytosis of aged and glycated/aged compared to fresh RBC. Cell nuclei were stained with DAPI (blue, except for confocal analysis). Scale bar = 40 µm (left and middle panels) and scale bar = 20 µm (right panels). (B) RBC interaction with EA.hy926 was assessed by flow cytometry. The analysis was performed after RBC and endothelial cell labeling respectively with glycophorin A and CD31. CD31 positive cells were selected as endothelial cells, population of interest. Aging and glycation enhanced RBC interaction with EA.hy926. Histograms represent RBC/EA.hy926 cells ratio (%), results are the means ± SD of three independent experiments. (***$p < 0.001$ and ****$p < 0.0001$ compared to fresh RBC and ****, $p < 0.0001$ compared to aged RBC). (C and D) RBC indirect quantification was performed by DAF colorimetric assay to evaluate the number of bound (C) or internalized (D) RBC by EA.hy926. RBC amount was evaluated on bound (in H_2O) and internalized cells (in PBS/1% Triton X100). Histograms represent bound or internalized RBC number/µL by EA.hy926. Results are the means ± SD of six independently performed experiments. Aging and glycation enhanced RBC binding and phagocytosis/internalization by EA.hy926. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ and ****$p < 0.0001$ compared to fresh RBC and ° $p < 0.5$; ** $p < 0.01$ and **** $p < 0.0001$ compared to aged RBC). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

middle panels). Furthermore, confocal microscopy analysis shows that aging and even more glycation increased RBC interaction with endothelial cells and their subsequent internalization (Fig. 2A, right panels). To support these data, and in order to quantify this phenomenon, flow cytometry analysis was performed after labeling RBC and endothelial cells, respectively by glycophorin A and CD31 (Fig. 2B). Erythrocyte aging increased RBC interactions with endothelial cells *versus* fresh RBC, whereas glycation markedly amplified this phenomenon (9.8% *vs.* 46.5% erythrocytes/EA.hy926 cell ratio respectively for 5 and 130 mM glucose). To reinforce these results, hemoglobin was quantified by DAF colorimetric assay to evaluate the number of RBC bound to endothelial cells and those that were internalized (Fig. 2C and D). Only glycation significantly increased RBC binding to endothelial cells compared to aged or fresh RBC, in a dose-dependent manner (from 30 to 130 mM of D-glucose). Only glycated RBC with 130 mM of D-glucose allowed a significant internalization by endothelial cells compared to the other conditions.

These results were confirmed using two other types of human endothelial cells: HcMEC/D3, a model of blood-brain barrier [25] and HMEC-1, a model of microvascular endothelial cells (Supplemental Fig. 2).

3.3. Erythrocyte phagocytosis by endothelial cells did not alter cell viability

After RBC incubation with endothelial EA.hy926 cells, viability was investigated using two types of experiments. First, mitochondrial activity was explored, showing that neither RBC aging nor glycation modified endothelial cell viability (Supplemental Fig. 3A). Then, apoptosis and necrosis of endothelial cells were assessed by flow cytometry (Supplemental Figs. 3B–D). Neither RBC aging nor glycation increased significantly the percentage of annexin V and propidium iodide positive cells.

3.4. Effect of RBC aging and glycation on endothelial cell proliferation and wound repair capacity

To evaluate human endothelial cell ability to proliferate after exposure to RBC, EA.hy926 cells were incubated with BrdU, a thymidine analogue. EA.hy926 cells were grown for 12 days and then analyzed by flow cytometry to detect BrdU by immunofluorescence, the proliferation being proportional to BrdU dilution. Fig. 3A upper panel shows control cell proliferation (without RBC treatment). Both aged RBC and glycated-aged RBC decrease EA.hy926 cells proliferation, attested by a reduced dilution of BrdU. In order to confirm this result, a clonogenic assay was used (Fig. 3B). Aged and aged/glycated RBC significantly reduced endothelial cell proliferation compared to healthy fresh RBC.

Finally, human endothelial cell ability to recover after a lesion was investigated using the wound healing/scratch assay (Fig. 3C). After incubation with healthy fresh, aged or aged/glycated RBC, scratches were generated on a human endothelial cell monolayer. Cells were maintained at 37 °C and pictures were taken every 2 h for 10 h. Only glycation decreased significantly endothelial cell wound repair relative to healthy fresh RBC. In conclusion, RBC aging/glycation decreased endothelial cell proliferation whereas glycation is required to drastically blunt endothelial cell wound repair capacity, integrative of both proliferation and migration processes.

3.5. Erythrocytes from T2D patients are eagerly phagocytosed by endothelial cells

In order to investigate the effects of diabetes on RBC interaction with human endothelial cells, fresh or aged RBC from healthy or T2D patients (Supplemental Fig. 4) were incubated with human endothelial cells EA.hy926 for 24 h at 37 °C (microscopy analysis and biochemical quantification of hemoglobin were performed**).** Before incubation with endothelial cells, we first characterized RBC modifications under diabetic conditions. Erythrocytes from diabetic patients presented more externalised PS than those from non-diabetics (Fig. 4A), associated with higher levels of intracellular ROS detected by the DCFH-DA (Fig. 4B–C). However, whereas a trend towards a decreased resistance to hemolysis was observed in diabetic RBC (lower HT50), it did not reach statistical significance ($p = 0.13$) (Fig. 4D). We then tested the capacity of endothelial cells to phagocyte erythrocytes from diabetic *vs.* non-diabetic patients. As expected, aging enhanced the interaction of T2D RBC with endothelial cells (Fig. 5). More importantly, fresh T2D RBC interacted more with endothelial cells than healthy fresh RBC, suggesting that *in vivo* RBC glycation is sufficient to promote binding/internalization and potentially impact on endothelial function.

3.6. Intraplaque hemorrhage and interactions between endothelial cells and RBC in human carotid endarterectomy samples

We analyzed the presence of neovessels and potential interactions between endothelial cells and RBC in human carotid atherosclerotic plaques (Fig. 6). We selected samples of both non-diabetic (A-D) and diabetic (E-H) patients, with intraplaque hemorrhage as attested by the accumulation of glycophorin A-positive RBC (A, C, E, G). In the sample from non-DT2 patients, only few neovessels could be observed (vWF immunostaining), suggesting that blood may come from a massive plaque rupture, rather than from a neovessel leakage. In the diabetic sample, many neovessels are visible surrounded by erythrocytes (G and H). Some figures of discontinuous endothelial layer and extravasating erythrocytes are shown by black arrowheads. In the non-diabetic sample, in spite of a massive intraplaque hemorrhage, the neovessels observed appeared thick and non-leaky (black arrowheads, C and D). The presence of a luminal thrombus can also promote interactions between endothelial cells and RBC (glycophorin A immunostaining, Figs. 6I-L). This may be observed in both non-diabetic (I and J) and DT2 patients (K and L). At higher magnification (J and L), interactions between erythrocytes and endothelial cells may be observed. Endothelial positivity for glycophorin suggests that these cells have taken up RBC, which is particularly visible in the carotid section from the diabetic subject.

4. Discussion

In this study, we have set up an *in vitro* model of glycated RBC that may mimic diabetic conditions in order to investigate the role of chronic glucose exposure on erythrocytes and its effect on endothelial function. We report that aged/glycated RBC are characterized by an intracellular oxidative stress, the loss of lipid membrane asymmetry via phosphatidylserine (PS) exposure and an increased susceptibility to hemolysis. Glycated RBC were able to massively bind to endothelial cells and to be internalized by these cells as compared to healthy fresh RBC. In these conditions, cell viability was not altered, but both cell proliferation and migration were blunted in endothelial cells exposed to glycated RBC.

In our RBC treatment model, we observe an increased exposure of PS due to aging similar to that observed by Kolb et al. [17]. This model consisting in an incubation at 37 °C for 5 days was chosen as similar conditions may be observed during blood stasis in neovessels of atherothrombotic plaques. Incubation with glucose further increased PS exposure independently of the concentration used (from 30 to 130 mM). This may be due to a plateau that is reached at 95% of PS exposure in high-glucose conditions. Several groups have used RBC models of glycation [11,26] with different glucose concentrations and incubation times. Here, we report that intracellular concentrations of superoxide anion and hydrogen peroxide are increased in aged and glycated RBC. Glycation tends to further increase oxidative stress in aged erythrocytes (DHE and DCFH-DA tests), as reported in other models in which lipid peroxidation was increased by incubation with high-glucose concentration [11,26]. In order to characterize the level of

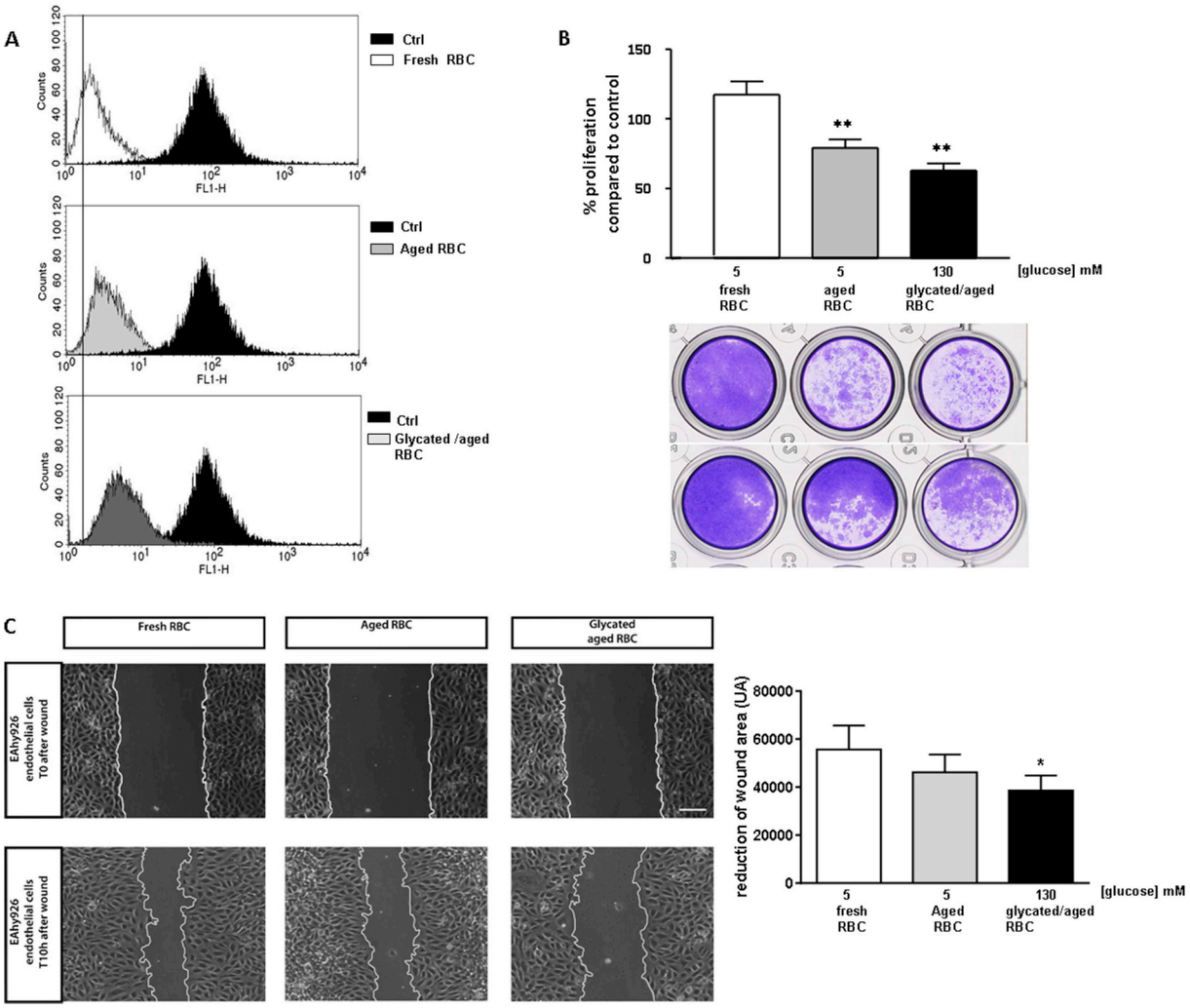

Fig. 3. RBC phagocytosis impaired EA.hy926 cell proliferation.
Human endothelial cells EA.
hy926 were incubated with fresh, aged or glycated/aged RBC for 24 h at
37 °C. After incubation, cells were washed three times with 1X PBS to discard unbound RBC. EA.hy926 were then incubated with or without BrdU. After BrdU incorporation, cells were seeded at a known density and grown for 12 days at 37 °C. (A) Nuclear BrdU incorporation was detected by flow cytometry. Phagocytosis of aged and glycated/aged RBC delayed EA.hy926 proliferation relative to that of fresh RBC. (B) EA.hy926 ability to form a colony was evaluated using a clonogenic assay. Cells were stained with crystal violet and the absorbance was read at 570 nm. Histograms represent the percentage of proliferation compared to the control (upper panel), results are the mean ± SD of three independent experiments. Representative pictures of the clonogenic survival assay after 12 days of growth (lower panels) (**$p < 0.01$ compared to fresh RBC). (C) Wound healing assay. The ability of EA.hy926 to migrate and/or proliferate after a lesion was evaluated. Representative images of the scratch area after the lesion (T0 and T12 h) (left panels, magnification ×10 scale bar = 40 μm). Histograms represent the quantification of wound area (AU, right panel), results are the mean ± SD of five independently performed experiments. Only glycated/aged RBC phagocytosis by EA.hy926 altered their wound healing capacity (*$p < 0.05$ compared to healthy fresh RBC).

glycation, we used two different assays: HMF (hydroxymethylfurfural, which results from the dehydration of the glucose moiety of glycated proteins) and determination of % HbA1C. A dose-dependent increase in HMF and % HbA1C was observed in our model, reaching statistical significance for the highest glucose concentrations used (100 and 130 mM). These results are in accordance with those reported by other groups, respectively for HMF [11] and glycated hemoglobin [26]. In our study, we used supra-physiological glucose concentrations to reach significant.

RBC modifications, but it allowed us to obtain glycation levels similar to those observed in diabetic patients: at 130 mM glucose, hemoglobin glycation reached 11.4% *vs.* 11.5% in some uncontrolled diabetic patients, suggesting that our model is pathophysiologically relevant.

In diabetic patients, RBC were reported to display an increased oxidative stress and reduced antioxidant activity, associated with accelerated senescence and susceptibility to hemolysis [27]. Accordingly, in our model, both aging and glycation induced an increased susceptibility to hemolysis induced by an oxidative insult (AAPH). Babu et al. demonstrated that erythrocyte aggregation was enhanced and deformability was reduced in diabetic RBC [4]. An inverse correlation between RBC life span and HbA1c percentage has been reported by Virtue et al. (6.9 days for each 1% increase in glycated hemoglobin) [28]. The short lifespan of RBC in diabetic conditions [29,30] may be due to these biochemical and morphological changes leading to an increased clearance by the reticulo-endothelial system. Other studies report that increased glycation of erythrocyte membrane proteins and hemoglobin may be due to the much longer lifespan of RBC [31]. The spleen may be less effective in the removal of senescent RBC leading an increased number of erythrocytes exposing PS [32].

In our *in vitro* conditions, we report that endothelial cells were able to bind and phagocyte both aged and aged/glycated RBC in a glycation

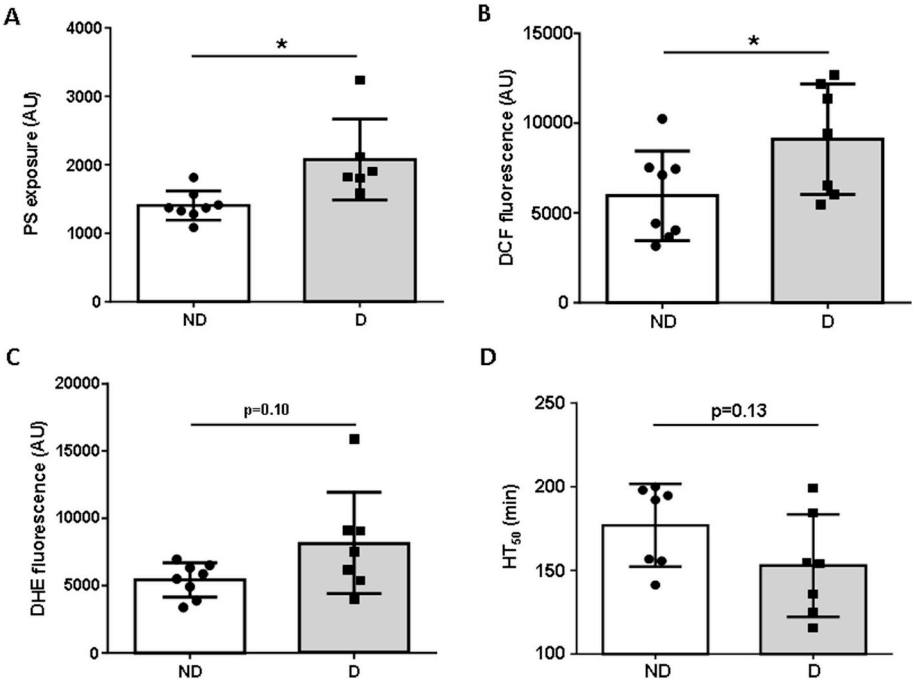

Fig. 4. Characterization of fresh RBC from T2D or healthy subjects model for investigation of endothelial erythrophagocytosis.
(A) Phosphatidylserine exposure of healthy and diabetic RBC was assessed by flow cytometry. Histograms represent the average PS exposure level (AU) for erythrocytes membranes of non diabetic (N = 8) and diabetics (N = 7). Results are mean ± SD (n = 8 ND and 7 D). (B) Hemolysis test was used to assess healthy and diabetic RBC ability to resist to an oxidative insult. Results are expressed as 50% of maximal hemolysis time (HT_{50} min) as mean ± SD (n = 8 ND and 7 D). (C) Intracellular ROS production by healthy RBC was measured by flow cytometry. RBC were incubated with DCFH-DA and DHE (D). Histograms expressed the fold increase in fluorescence as mean ± SD (n = 8 ND and 7 D). (*$p < 0.05$ compared to healthy ND RBC).

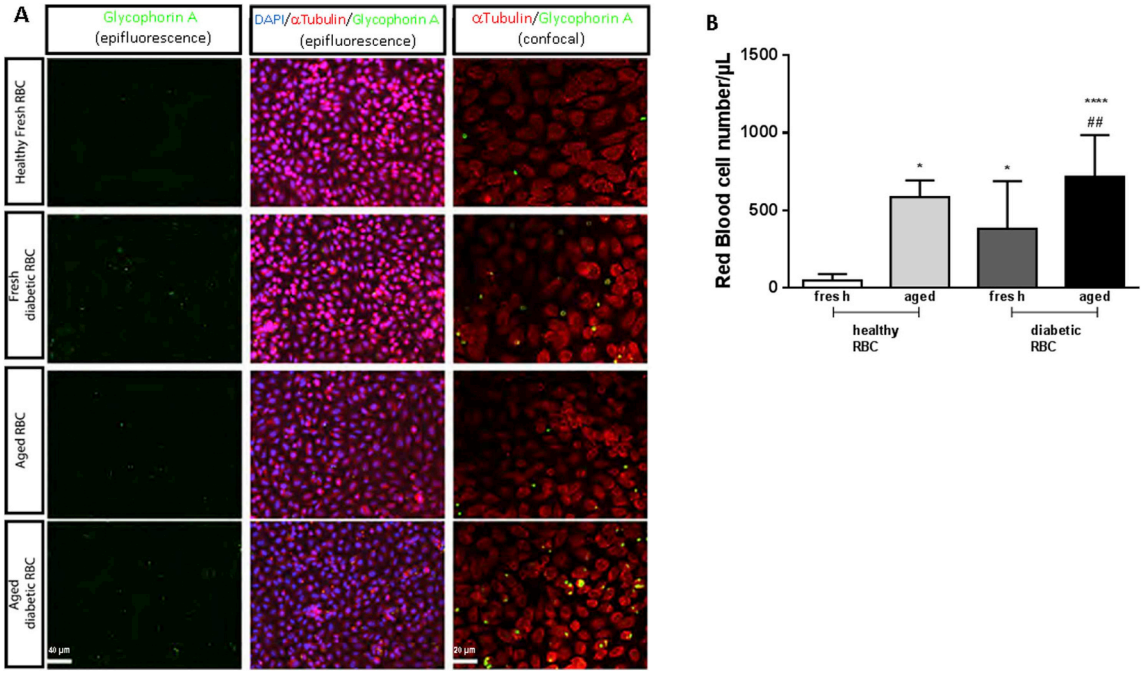

Fig. 5. Diabetes enhances binding/internalization of RBC by human endothelial cells.
Human endothelial cells EA. hy926 were incubated with either fresh or aged RBC from T2D or healthy subjects for 24 h at 37 °C. After incubation, cells were washed three times with PBS 1X to discard unbound RBC. (A) Immunostaining of EA.hy926 (α tubulin in red) and RBC (glycophorin A in green) after co-incubation. Both fresh and aged RBC from T2D patients displayed an enhanced interaction with EA.hy926 relative to healthy fresh or aged RBC (left panels). Confocal imaging analysis was performed showing an enhanced phagocytosis of T2D RBC by EA.hy926 compared to that of healthy volunteers (right panels). Cell nuclei were stained with DAPI (blue, except for confocal analysis). Scale bar = 40 µm (left and middle panels) scale bar = 20 µm (right panels). (B) RBC indirect quantification was performed to evaluate the number of RBC associated with human endothelial cells EA.hy926 (either bound or internalized). Histograms represent RBC number/µL interacting with EA.hy926, results are the means ± SD of 6 independent experiments. (*$p < 0.05$ and ****$p < 0.0001$ compared to healthy fresh RBC and $^{\#\#}p < 0.01$ compared to fresh T2D RBC). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

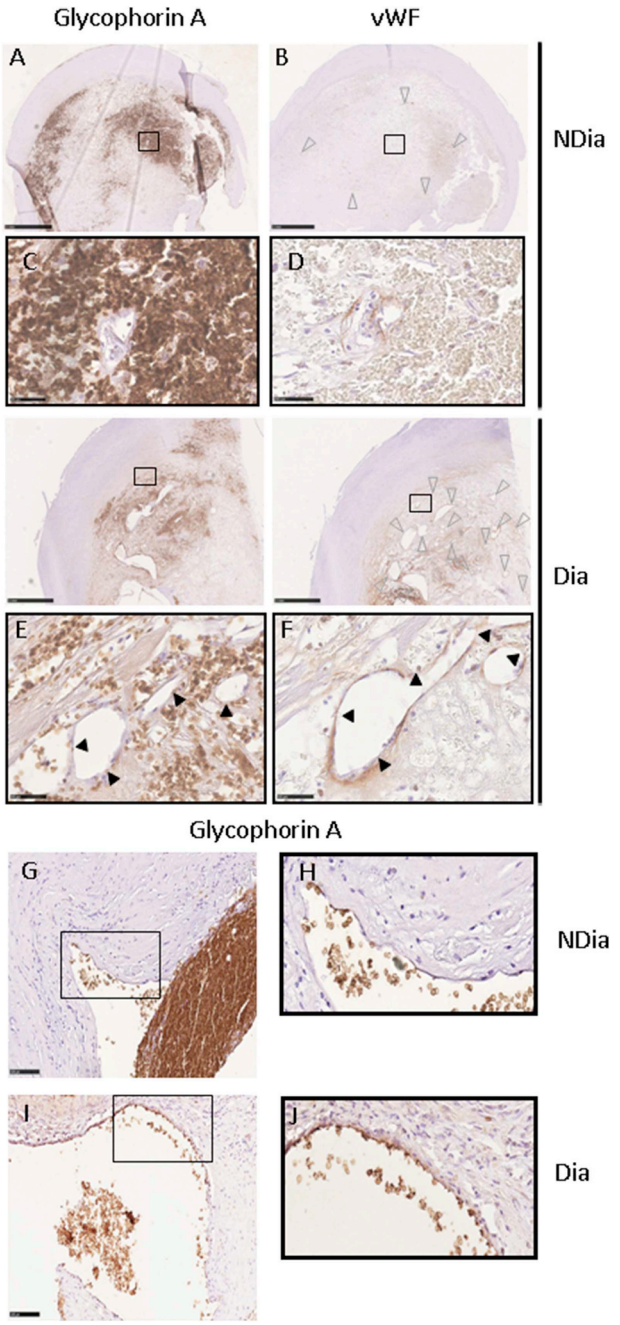

Fig. 6. Glycophorin A and von Willebrand factor (vWF) immunostaining in hemorrhagic human carotid sections from DT2 and non-DT2 patients.
Non-diabetic (NDia) and diabetic (Dia) patients carotid plaque with intraplaque hemorrhage were analyzed for the presence of glycophorin A (A, C, E, G-J)) and vWF (B, D, F). The presence of vWF- positive neovessels is pointed by empty grey arrowheads (B–D). Interactions between luminal endothelial cells and RBC are observed in both NDia and Dia samples, with a marked glycophorin A immunostaining in endothelial lining (J). Black arrowheads point at discontinuous endothelial layer areas and extravasating erythrocytes. High magnification images of the areas delimited by squares are presented in E, F and H, J. Black bars represent 1 mm (A-D, G, I) and 50 μm (E and F).

level-dependent manner. Wautier et al. first demonstrated that AGE (advanced glycated end-products) were formed *in vivo* under diabetic conditions at the surface of RBC [30] and then reported that glycated RBC binding to HUVEC was mediated by interactions with RAGE (receptor for AGE) [19]. CD36 scavenger receptor is also expressed by both endothelial cells and macrophages, and able to bind various ligands including oxidized LDL, plasmodium falciparum-infected RBC as well as AGE [33].

In diabetic patients, glycation induces AGE formation at the surface of RBC [12]. Binding and subsequent phagocytosis of glycated RBC could thus occur via CD36 expressed by endothelial cells. Another receptor for PS and oxidized LDL was shown to mediate apoptotic RBC binding to HUVEC[34].

In a different model of oxidatively modified RBC (by tert-butylhydroperoxide, tBHP), Fens et al. reported a PS exposure and a lactadherin-dependent phagocytosis by HUVEC. The same results were obtained under static and flow conditions. They also showed that in the absence of lactadherin (an opsonin known to bind PS, αvβ3 and αvβ5 integrins), less binding and uptake of erythrocytes by HUVEC were observed whereas in the presence of lactadherin many intracellular erythrocytes could be detected [18,35].

Phosphatidylserine exposure at the surface of senescent cells is a well-established “eat-me” for recognition and subsequent clearance of apoptotic cells by phagocytes. Closse et al. provided strong evidence that human erythrocytes-endothelial cell interactions are mediated by phosphatidylserine recognition by HUVEC (human venous endothelial cells) [36]. They report that annexin V was able to inhibit RBC-HUVEC adhesion in a dose-dependent manner. This first interaction could be a pre-requisite for other ligand-receptor interactions; after erythrocyte tethering to endothelial cells, other molecules may initiate phagocytosis pathways, such as CD47-TSP-SIRPα or RAGE-AGE [24]. In our model, the role of different scavenger receptors such as CD36, as well as the involvement of phosphatidylserine and lactadherin in the process of binding and engulfment, should be investigated in more details.

We then investigated potential expression and conformational changes of CD47 in our experimental conditions. CD47 is present at the RBC membrane and may be involved in erythrophagocytosis as an “eat-me” or “do not eat-me” signal, depending on its conformation [24]. We show that CD47 underwent a conformational change in aging/glycation conditions, whereas its global expression remained unchanged. This conformational change could represent an “eat me” signal, as previously reporter by Burger et al. [24]. In the context of hemorrhagic stroke, it was shown that mice knocked down for CD47 on erythrocytes displayed an enhanced hematoma clearance and a reduced brain injury [37]. The same group recently reported similar beneficial effects using CD47 blocking antibodies [38]. Theses results suggest that modulation of CD47 expression or function may impact on RBC clearance. In our *in vitro* model, the enhanced phagocytosis of aged/glycated RBC by non-professional phagocytes such as endothelial cells, may induce deleterious effects in the context of atherosclerosis, via the destabilization of the endothelial layer.

In the study by Fens et al., they report a cytotoxic effect of RBC treated by 3 mM tBHP after a 24 h-incubation period [18]. In our experimental conditions, we demonstrated that endothelial cells did not undergo apoptosis or necrosis after 24 h of incubation with aged/glycated RBC. These differences in terms of mortality may be due to the cell type used (cell line *vs.* primary culture of HUVEC) or to the RBC treatment (glycation *vs.* oxidative modification).

From a functional point of view, incubation of RBC with endothelial cells did not impact their capacity to bind monocytes (THP1 cell line, data not shown). However, we demonstrate for the first time that endothelial cell ability to migrate and proliferate was reduced following incubation with aged/glycated RBC. We have also observed that endothelial cells placed in hyperglycemic conditions were more prone to bind and engulf fresh aged erythrocytes (data not shown), suggesting that the global uptake of RBC by endothelial cells in diabetics may be increased. In pathological conditions, the endothelial layer must face mechanic, oxidative and inflammatory aggressions, resulting in the necessity for healing. This capacity is markedly decreased after aged/glycated RBC phagocytosis. *In vivo*, in atherosclerotic conditions, the presence of immature microvessel in hemorrhagic plaque has been reported [39], suggesting that a blunted healing capacity for the

endothelium may promote RBC leakage into the necrotic core, thereby worsening intraplaque lipid accumulation and heme-associated oxidative stress. Other vascular cell types have been reported to be able to phagocyte aged RBC such as smooth muscle cells [17,40], leading to the formation of foam cells [40]. The results from Delbosc et al. suggest that endothelial cells may phagocyte RBC *in vivo* as hemoglobin was focally detected in the endothelium of human fatty streaks [40]. Macrophages are also able to phagocyte erythrocytes leading to iron secretion and subsequent LDL oxidation [41].

To our knowledge, there is no *in vivo* study on the impact of RBC glycation in atherosclerotic conditions. Wautier et al. reported that the clearance of RBC from diabetic rats injected into normal rats was higher than that of non-diabetic rats. This accelerated clearance was reduced by pre-treatment with anti-RAGE immunoglobulins, suggesting that glycated RBC removal was dependent on endothelial RAGE [19]. We demonstrate that RBC from diabetic patients were more prone to bind to and be internalized by endothelial cells relative to those from healthy non-diabetic subjects. These results suggest that our *in vitro* model of RBC glycation is relevant and may mimic diabetic conditions; they are in accordance with a study that tested, in flow conditions, the binding of RBC from diabetic patients to endothelial cells [42]. Finally, we have shown that interactions between endothelial cells and RBC occur *in vivo* in atherothrombotic conditions, potentially promoting intraplaque hemorrhage. A specific study should be designed in order to compare diabetic *vs.* non-diabetic plaques with intraplaque hemorrhage and to quantify potential differences according to the diabetic status. Our aim was to illustrate the relevance of endothelial interactions with resident RBC and their possible uptake by these cells, *in vivo*, in human samples. Iron metabolism within atherothrombotic plaque is of major importance since erythrocytes may undergo hemolysis if they are not eliminated, and the released hemoglobin may subsequently be oxidized to ferri- and ferrylhemoglobin. The resulting free heme and iron may promote further oxidation and glycoxidation of lipids, particularly under diabetic conditions [43]. Oxidized low-density lipoproteins may in turn induce a switch in macrophage phenotype in which iron export is compromised [44]. Altogether, defects in erythrocyte clearance and iron metabolism within atherothrombotic plaques represent additional factors of instability.

In conclusion, we have set up a well-characterized patho-physiologically relevant model of RBC glycation that induces endothelial dysfunction (reduced capacity to migrate and proliferate) after phagocytosis. These deleterious effects of glycated RBC on the endothelium may account for the increased vulnerability of atherosclerotic plaques to rupture in diabetic patients.

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Author contributions

AC, CT, JP, PR, OM, ND, NAY carried out experiments. AC, PR, ND, EB and OM conceived experiments, analyzed data and wrote the manuscript.

XD, NLM, MB, RV, AGD participated in study design and data collection.

All authors were involved in writing the paper and had final approval of the submitted version.

Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.10.015.

References

[1] T.F. Luscher, M.A. Creager, J.A. Beckman, F. Cosentino, Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: Part II, Circulation 108 (2003) 1655–1661.
[2] J. Plutzky, The vascular biology of atherosclerosis, Am. J. Med. 115 (Suppl) (2003) 55S–61S.
[3] F. Picard, J. Adjedj, O. Varenne, [Diabetes Mellitus, a prothrombotic disease], Ann. Cardiol. Angeiol 66 (2007) 385–392.
[4] N. Babu, M. Singh, Influence of hyperglycemia on aggregation, deformability and shape parameters of erythrocytes, Clin. Hemorheol. Microcirc. 31 (2004) 273–280.
[5] M.A. Creager, T.F. Luscher, F. Cosentino, J.A. Beckman, Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: Part I, Circulation 108 (2003) 1527–1532.
[6] M.R. Hayden, S.C. Tyagi, Vasa vasorum in plaque angiogenesis, metabolic syndrome, type 2 diabetes mellitus, and atherosclerosis: a malignant transformation, Cardiovasc. Diabetol. 3 (2004) 1.
[7] G.K. Kolluru, S.C. Bir, C.G. Kevil, Endothelial dysfunction and diabetes: effects on angiogenesis, vascular remodeling, and wound healing, Int. J. Vasc. Med. 2012 (2012) 918267.
[8] K.R. Purushothaman, M. Purushothaman, P. Muntner, P.A. Lento, W.N. O'Connor, S.K. Sharma, V. Fuster, P.R. Moreno, Inflammation, neovascularization and intraplaque hemorrhage are associated with increased reparative collagen content: implication for plaque progression in diabetic atherosclerosis, Vasc. Med. 16 (2011) 103–108.
[9] D.N. Tziakas, G.K. Chalikias, D. Stakos, H. Boudoulas, The role of red blood cells in the progression and instability of atherosclerotic plaque, Int. J. Cardiol. 142 (2010) 2–7.
[10] F.D. Kolodgie, H.K. Gold, A.P. Burke, D.R. Fowler, H.S. Kruth, D.K. Weber, A. Farb, L.J. Guerrero, M. Hayase, R. Kutys, J. Narula, A.V. Finn, R. Virmani, Intraplaque hemorrhage and progression of coronary atheroma, N. Engl. J. Med. 349 (2003) 2316–2325.
[11] J. Viskupicova, D. Blaskovic, S. Galiniak, M. Soszynski, G. Bartosz, L. Horakova, I. Sadowska-Bartosz, Effect of high glucose concentrations on human erythrocytes in vitro, Redox Biol. 5 (2015) 381–387.
[12] R. Nagai, E.K. Deemer, J.W. Brock, S.R. Thorpe, J.W. Baynes, Effect of glucose concentration on formation of AGEs in erythrocytes in vitro, Ann. N. Y. Acad. Sci. 1043 (2005) 146–150.
[13] B. Buttari, E. Profumo, R. Rigano, Crosstalk between red blood cells and the immune system and its impact on atherosclerosis, BioMed Res. Int. 2015 (2015) 616834.
[14] D.M. Schrijvers, G.R. De Meyer, M.M. Kockx, A.G. Herman, W. Martinet, Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 1256–1261.
[15] R.E. Garfield, S. Chacko, S. Blose, Phagocytosis by muscle cells, Lab. Investig. 33 (1975) 418–427.
[16] M.R. Bennett, D.F. Gibson, S.M. Schwartz, J.F. Tait, Binding and phagocytosis of apoptotic vascular smooth muscle cells is mediated in part by exposure of phosphatidylserine, Circ. Res. 77 (1995) 1136–1142.
[17] S. Kolb, R. Vranckx, M.G. Huisse, J.B. Michel, O. Meilhac, The phosphatidylserine receptor mediates phagocytosis by vascular smooth muscle cells, J. Pathol. 212 (2007) 249–259.
[18] M.H. Fens, R. van Wijk, G. Andringa, K.L. van Rooijen, H.M. Dijstelbloem, J.T. Rasmussen, K.M. de Vooght, R.M. Schiffelers, C.A. Gaillard, W.W. van Solinge, A role for activated endothelial cells in red blood cell clearance: implications for vasopathology, Haematologica 97 (2012) 500–508.
[19] J.L. Wautier, M.P. Wautier, A.M. Schmidt, G.M. Anderson, O. Hori, C. Zoukourian, L. Capron, O. Chappey, S.D. Yan, J. Brett, et al., Advanced glycation end products (AGEs) on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface- associated AGEs and diabetic complications, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 7742–7746.
[20] Prost, M., Process for the determination by means of free radicals of the antioxidant properties of a living organism or a potentially aggressive agents, In, U.S. Patent 5 135-850 USA 1992.
[21] K.F. McFarland, E.W. Catalano, J.F. Day, S.R. Thorpe, J.W. Baynes, Nonenzymatic glucosylation of serum proteins in diabetes mellitus, Diabetes 28 (1979) 1011–1014.
[22] N.A. Franken, H.M. Rodermond, J. Stap, J. Haveman, C. van Bree, Clonogenic assay of cells in vitro, Nat. Protoc. 1 (2006) 2315–2319.
[23] C.C. Liang, A.Y. Park, J.L. Guan, In vitro scratch assay: a convenient and

inexpensive method for analysis of cell migration in vitro, Nat. Protoc. 2 (2007) 329–333.
[24] P. Burger, P. Hilarius-Stokman, D. de Korte, T.K. van den Berg, R. van Bruggen, CD47 functions as a molecular switch for erythrocyte phagocytosis, Blood 119 (2012) 5512–5521.
[25] B.B. Weksler, E.A. Subileau, N. Perriere, P. Charneau, K. Holloway, M. Leveque, H. Tricoire-Leignel, A. Nicotra, S. Bourdoulous, P. Turowski, D.K. Male, F. Roux, J. Greenwood, I.A. Romero, P.O. Couraud, Blood-brain barrier-specific properties of a human adult brain endothelial cell line, FASEB J. 19 (2005) 1872–1874.
[26] W. Sompong, H. Cheng, S. Adisakwattana, Protective effects of ferulic acid on high glucose-induced protein glycation, lipid peroxidation, and membrane ion pump activity in human erythrocytes, PLoS One 10 (2015) e0129495.
[27] Y.R. Sailaja, R. Baskar, D. Saralakumari, The antioxidant status during maturation of reticulocytes to erythrocytes in type 2 diabetics, Free Radic. Biol. Med. 35 (2003) 133–139.
[28] M.A. Virtue, J.K. Furne, F.Q. Nuttall, M.D. Levitt, Relationship between GHb concentration and erythrocyte survival determined from breath carbon monoxide concentration, Diabetes Care 27 (2004) 931–935.
[29] R.D. Brunning, H.S. Jacob, W.D. Brenckman, F. Jimenez-Pasquau, F.C. Goetz, Fragmentation haemolysis in patients with severe diabetic angiopathy, Br. J. Haematol. 34 (1976) 283–289.
[30] J.L. Wautier, R.C. Paton, M.P. Wautier, D. Pintigny, E. Abadie, P. Passa, J.P. Caen, Increased adhesion of erythrocytes to endothelial cells in diabetes mellitus and its relation to vascular complications, N. Engl. J. Med. 305 (1981) 237–242.
[31] Q. Zhang, M.E. Monroe, A.A. Schepmoes, T.R. Clauss, M.A. Gritsenko, D. Meng, V.A. Petyuk, R.D. Smith, T.O. Metz, Comprehensive identification of glycated peptides and their glycation motifs in plasma and erythrocytes of control and diabetic subjects, J. Proteome Res. 10 (2011) 3076–3088.
[32] A.B. Manodori, F.A. Kuypers, Altered red cell turnover in diabetic mice, J. Lab. Clin. Med. 140 (2002) 161–165.
[33] Y.M. Park, CD36, a scavenger receptor implicated in atherosclerosis, Exp. Mol. Med. 46 (2014) e99.
[34] O. Borst, M. Abed, I. Alesutan, S.T. Towhid, S.M. Qadri, M. Foller, M. Gawaz, F. Lang, Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX, Am. J. Physiol. Cell Physiol. 302 (2012) C644–C651.
[35] M.H. Fens, E. Mastrobattista, A.M. de Graaff, F.M. Flesch, A. Ultee, J.T. Rasmussen, G. Molema, G. Storm, R.M. Schiffelers, Angiogenic endothelium shows lactadherin-dependent phagocytosis of aged erythrocytes and apoptotic cells, Blood 111 (2008) 4542–4550.
[36] C. Closse, J. Dachary-Prigent, M.R. Boisseau, Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium, Br. J. Haematol. 107 (1999) 300–302.
[37] W. Ni, S. Mao, G. Xi, R.F. Keep, Y. Hua, Role of erythrocyte CD47 in intracerebral hematoma clearance, Stroke 47 (2016) 505–511.
[38] C. Jing, L. Bian, M. Wang, R.F. Keep, G. Xi, Y. Hua, Enhancement of hematoma clearance with CD47 blocking antibody in experimental intracerebral hemorrhage, Stroke 50 (2019) 1539–1547.
[39] J. Le Dall, B. Ho-Tin-Noe, L. Louedec, O. Meilhac, C. Roncal, P. Carmeliet, S. Germain, J.B. Michel, X. Houard, Immaturity of microvessels in haemorrhagic plaques is associated with proteolytic degradation of angiogenic factors, Cardiovasc. Res. 85 (2010) 184–193.
[40] S. Delbosc, R.G. Bayles, J. Laschet, V. Ollivier, B. Ho-Tin-Noe, Z. Touat, C. Deschildre, M. Morvan, L. Louedec, L. Gouya, K. Guedj, A. Nicoletti, J.B. Michel, Erythrocyte efferocytosis by the arterial wall promotes oxidation in early-stage atheroma in humans, Front. Cardiovasc. Med. 4 (2017) 43.
[41] X.M. Yuan, W.L. Anders, A.G. Olsson, U.T. Brunk, Iron in human atheroma and LDL oxidation by macrophages following erythrophagocytosis, Atherosclerosis 124 (1996) 61–73.
[42] M.P. Wautier, T. Khodabandehlou, C. Le Devehat, J.L. Wautier, Modulation of RAGE expression influences the adhesion of red blood cells from diabetic patients, Clin. Hemorheol. Microcirc. 35 (2006) 379–386.
[43] E. Nagy, J.W. Eaton, V. Jeney, M.P. Soares, Z. Varga, Z. Galajda, J. Szentmiklosi, G. Mehes, T. Csonka, A. Smith, G.M. Vercellotti, G. Balla, J. Balla, Red cells, hemoglobin, heme, iron, and atherogenesis, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 1347–1353.
[44] L. Marques, A. Negre-Salvayre, L. Costa, F. Canonne-Hergaux, Iron gene expression profile in atherogenic Mox macrophages, Biochim. Biophys. Acta 1862 (2016) 1137–1146.