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Phenazine methosulphate-treated red blood cells activate NF-κB and upregulate endothelial ICAM-1 expression

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ABSTRACT

Although enhanced Red Blood Cell (RBC) - Endothelial Cell (EC) interaction, as well as RBC induced EC activation, have been extensively studied in several RBC-linked pathologies, the specific individual effects of oxidatively modified RBC on EC activation has not yet been documented. However, increasing evidence in both experimental and clinical studies suggests that oxidatively modified RBC could be considered potential pathogenic determinants in several acute and chronic diseases displaying systemic oxidative stress. Therefore, the present study aimed to explore the specific effects of oxidized RBC interaction with endothelial cells on intracellular signaling *pathways* that promote EC activation. RBC were exposed to oxidative stress induced by phenazine methosulphate (PMS). It is shown that the interaction of oxidatively modified RBC with cultured human umbilical vein endothelial cells (HUVEC) results in: a) EC activation as indicated by the increased surface expression of intercellular adhesion molecule −1 (ICAM-1); b) the activation of transcription factor NF-κB, an indicator of cellular oxidant stress. These results emphasize the specific contribution of oxidatively modified RBC interaction to EC activation and their possible pathological role in vascular diseases and oxidative stress.

1. Introduction

Vascular endothelium serves as an anatomical and functional interface between blood and the underlying tissues, interacting with both to maintain homeostasis, vascular tone, red blood cell (RBC) and leukocyte adhesion and resistance to thrombosis. Normal red blood cells are readily deformable and are not adherent to quiescent endothelial cells (EC). However, RBC adhesion to the vasculature increases in several pathologic conditions. Enhanced adhesion of RBC to EC can be detrimental since it may impair blood flow, reduce oxygen delivery and cause vascular occlusion [1]. RBC obtained from patients with diabetes mellitus show altered redox status and increased propensity to adhere to cultured endothelial cells in vitro [2]. In sickle cell disease, the enhanced adhesion of sickle erythrocytes to endothelial cells generates oxidant stress causing EC activation [3–5]. In addition, abnormal RBC adhesive behavior has been reported in malaria, β-thalassemia, hereditary stomatocytosis, and chronic uremia; enhanced RBC–EC adhesion has been suggested to contribute to vascular damage and vaso-occlusive pathology in these diseases [6–9]. Recent publications have shown that enhanced RBC adhesion to vascular endothelial cells modulates EC gene expression and function, leading to EC activation [1].

EC activation promotes de novo expression of leukocyte adhesion molecules such as ICAM-1, VCAM-1 and *E*-selectin. [10–14]. The upregulated expression of cell adhesion molecules on endothelial cells alters the adhesive behavior of the vasculature and is one of the key events that leads to indiscriminate infiltration and movement of leukocytes across blood vessel walls and hence inflammation [15]. Numerous studies have shown that the transcription factor nuclear factor kB (NF-kB) is involved in the rapid induction of these adhesion molecules during immune and inflammatory responses [16–18]. Diverse agents such as inflammatory cytokines, oxidative stress and endotoxins activate NF-kB by distinct intracellular pathways that involve reactive oxygen species (ROS) as a common messenger [19,20]. The activation of NF-kB is capable of altering the expression of several genes associated with inflammation, including adhesion molecules, tissue factor, cytokines and acute phase proteins [21–23]. Nuclear factor-kB not only

Abbreviations: RBC, Red blood cell; EC, endothelial cell; PMS, phenazine methosulphate; HUVEC, human umbilical vein endothelial cells; NF-κB, nuclear factor kappa B; ICAM-1, intercellular adhesion molecule-1; ROS, reactive oxygen species

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triggers EC activation but also renders the endothelium more susceptible to apoptosis [24]. Thus, NF-kB is considered an important regulator of EC activation and EC apoptosis.

Oxidative stress is now known to be a major factor in the development of most pathological events associated with neurological disorders, cancer, cardiovascular disorders, influenza, Down syndrome, hepatitis, rheumatoid arthritis, ulcers, pneumonia, cataract, glaucoma and human aging [25–27]. RBC are particularly vulnerable to oxidative stress, and oxidatively modified RBC and their abnormal interactions with vascular EC are considered as crucial factors in several pathologic conditions characterized by systemic oxidative stress. Although abnormal RBC-EC interactions have been extensively studied in several RBC linked pathologies, it is often difficult to determine the specific effects of oxidatively modified RBC on EC activation inasmuch as linked pathologies can affect multiple RBC properties.

The present study was designed to explore the specific effects of oxidized RBC on EC activation and to further evaluate intracellular signaling events that are involved. In order to mimic RBC damage in vivo, RBC were treated with phenazine methosulphate (PMS), an agent that causes oxidative damage. EC activation was examined by measuring the surface expression of the adhesion molecule ICAM-1 and NF-kB activity in cultured human umbilical vein endothelial cells (HUVEC). We hypothesized that oxidized RBC activate the NF-kB signaling pathway and enhance the expression of ICAM-1 leading to EC activation.

2. Materials and methods

2.1. Reagents

Mouse antihuman ICAM-1, peroxidase conjugated rabbit anti-mouse IgG and rabbit polyclonal IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-conjugated anti-rabbit IgG was obtained from Invitrogen (Singapore). Oligonucleotides recognizing NF-κB DNA consensus sequences were purchased from 1st Base, Singapore. The Dig Gel shift assay system was obtained from Roche Applied Science, and all other chemicals were purchased from local sources at the highest purity available.

2.2. RBC preparation and induction of oxidative stress by PMS

Blood was obtained by sterile venipuncture from the antecubital vein of healthy adult donors into EDTA (1.5 mg/mL) and used within four hours; all donors provided verbal informed.

consent. RBC were separated from whole blood by gentle centrifugation (2000 ×*g*, 10 min), then washed three times in isotonic phosphate buffered saline (PBS, 10 mM phosphate, 285 mOsm/kg, pH = 7.4). To stimulate intracellular generation of superoxide [28], normal RBC were treated with various concentrations of PMS (25 μM, 50 μM, 100 μM, 200 μM, 400 μM, 800 μM and 1000 μM) in PBS for 60 min at 37 °C under constant mixing, after which RBC were thoroughly washed with PBS.

2.3. Endothelial cell culture and exposure to PMS-treated RBC

HUVEC were obtained from American Type Culture Collection (ATCC, No. CRL-1730™) and cultured in 0.1% gelatin coated flasks using F-12K medium supplemented with 10% fetal bovine serum, 0.2% bovine brain extract, 0.1 mg/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin; culturing was done in a 5% CO_2 humidified water jacketed incubator. At 80% confluence, the cells were sub-cultured using 0.25% trypsin-EDTA and cells from passage 4–7 were used for the experiments. For dose dependent experiments, HUVEC were incubated with RBC pretreated with various PMS concentrations (i.e., 25, 50, 100, 200, 400, 800 and 1000 μM PMS) for 24 h at 37 °C in serum free medium. For time course experiments, endothelial cells were incubated with RBC pretreated with 800 μM PMS for different time periods ranging from 0.5 h to 24 h (i.e., 0.5, 1, 2, 3, 6, 9, 12 and 24 h). Untreated HUVEC and HUVEC treated with normal RBC under identical conditions served as controls.

2.4. ELISA for measurement of ICAM-1 expression

The cell surface expression of ICAM-1 on the endothelial monolayers was quantified by ELISA using a modification of the methods described previously [29]. HUVEC were seeded at a concentration of 2×10^4 cells/well in 96-well gelatin-coated plates, cultured to confluence and exposed to PMS- treated RBC as described earlier. At the end of the indicated incubation period, the cells were washed with PBS and fixed with acetone-methanol (1:1, v/v) at 4 °C for 10 min. Non-specific binding was blocked by using bovine serum albumin (1% in PBS). Cells were incubated with anti-ICAM-1 monoclonal antibodies overnight at 4 °C (dilution 1:500), washed with PBS, followed by incubation with horse peroxidase conjugated rabbit anti-mouse secondary antibody (dilution 1:200) for 120 min at room temperature. The cells were then washed with PBS and exposed to the peroxidase substrate *p*-nitrophenyl phosphate (1 mg/ml) in 0.1 M glycine buffer, pH 10.4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. The absorbance was measured at 405 nm using a microplate reader (Beckman Coulter). The surface expression of ICAM-1 is shown as mean ± standard deviation (SD) of the optical density (OD) after subtracting the blank value.

2.5. Immunofluorescent staining for NF-KB translocation

For immunofluorescent staining, HUVEC were seeded onto Lab-Tek tissue culture chamber slides (Nunc, Inc., Naperville, IL). After treatment with PMS-treated RBC, cells were fixed with freshly prepared 4% paraformaldehyde in PBS for 20 min at room temperature and then permeabilized with 0.5% Triton X-100 for 15 min. After a 1 h blocking with 10% normal goat serum at room temperature, cells were incubated with rabbit polyclonal IgG (dilution 1:50 in PBS) against the p65 subunit of NF-kB for 2 h, washed twice with PBS and then incubated with Alexa fluor 488 goat anti-rabbit IgG (dilution 1:100) for 1 h. Images were captured using a Zeiss confocal laser scan microscope. Excitation wavelength/detection filter settings were as follows: 585/665 nm for Alexa Fluor 488, 495/519 nm for NF-κB p65 visualization.

2.6. NF-KB activation assay

To determine NF-kB activation, an electrophoretic mobility shift assay (EMSA) was performed on endothelial cell nuclear extracts using a modified protocol of Schreiber et al [30]. Cells were washed with PBS and harvested by a trypsin-EDTA method. After centrifugation, the cells were collected in 100 μl ice-cold cell lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 1 mM Na_2MoO_4 with complete protease inhibitor cocktail). Cells were allowed to swell on ice for 10 min before 20 μl of 20% NP-40 (nonyl phenoxypolyethoxylethanol) was added. The tube was then mixed thoroughly using a vortex mixer for 10 s, after which it was centrifuged at 10,000 *g* at 4 °C for 3 min. The nuclear pellets thus obtained were resuspended in 50 μL of ice-cold nuclear extraction buffer (10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 1 mM Na2MoO4, 400 mM Nacl, 1% NP-40 with complete protease inhibitor cocktail) and kept on ice for 15 min under intermittent agitation. Finally, the samples were subjected to centrifugation for 2 min at 4 °C at 14000 g and the supernatant was stored at −80 °C until use.

EMSA was carried out using a digoxigenin (DIG) gel shift kit (Roche Applied Science), according to the manufacturer's instructions. Briefly, the oligonucleotides, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, and 3′-TCA ACT CCC CTG AAA GGG TCC G-5′ were annealed and labeled at the 3′ end with digoxigenin. This probe (0.4 ng/μl) was incubated at room temperature for 10 min with 10 μl of nuclear extract in a binding

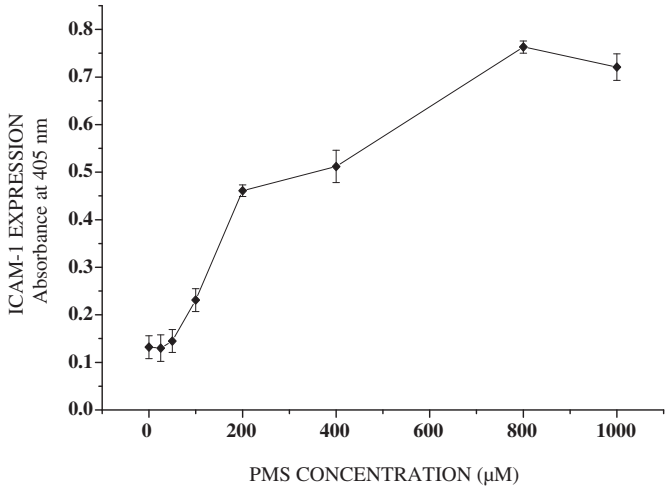

Fig. 1. ELISA showing surface expression of ICAM-1 in HUVECs exposed to PMS-treated RBC. HUVECs were exposed to normal and PMS treated (25 μM to 1000 μM PMS)) RBC for 24 h at 37 °C for ICAM-1 expression and was measured by ELISA as described in the Materials and Methods section. The data presented are representative of three independent experiments. Results are expressed as mean ± SD of OD at 405 nm.

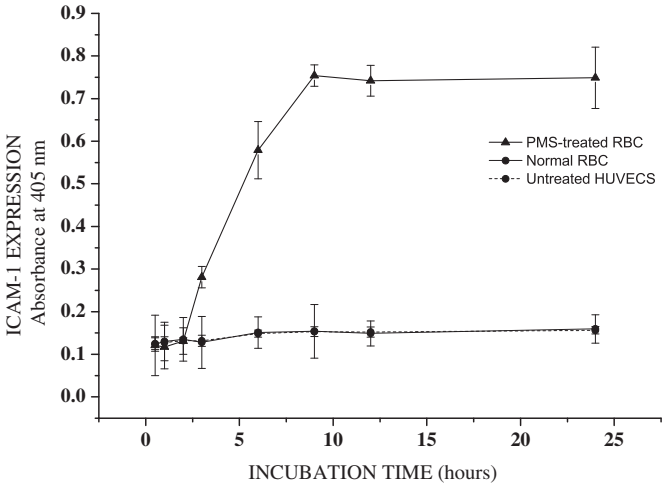

Fig. 2. ELISA showing time course of ICAM-1 expression in HUVECs exposed to PMS- treated RBC. HUVECs were incubated with RBC treated with 800 μM PMS for different time periods ranging from 0.5 h to 24 h (0.5 h, 1.0 h, 2.0 h, 3.0 h, 6.0 h, 9.0 h, 12.0 h and 24 h) at 37 °C for ICAM-1 expression and measured by ELISA as described in the Materials and methods section. Untreated HUVECs and HUVECs treated with normal RBC under identical conditions served as controls. The data presented are representative of three independent experiments. Results are expressed as mean ± SD of OD at 405 nm.

buffer containing 20 mM HEPES, pH 7·6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0·2% Tween20, 30 mM KCl, 1 μg poly d(I−C), and 0·1 μg poly L-lysine. The protein-DNA complexes were then separated on a 6% native polyacrylamide gel: DNA probes bound to NF-kB were retarded, whereas unbound DNA probes were not. After blotting to a nylon membrane, labeled oligonucleotides were detected by alkaline phosphatase antidigoxigenin (Gab)2 fragments. The specificity of binding was examined by competition experiments, where a 100-fold excess of unlabeled oligonucleotide with the same sequence was added to the reaction mixture prior to the addition of the DIG-labeled oligonucleotide.

3. Results

3.1. ICAM-1 expression of HUVEC

Initial efforts were directed towards identifying the effect of oxidatively-modified RBC on ICAM-1 expression by endothelial cells (EC). As shown in Fig. 1, incubation of EC with PMS- treated RBC resulted in an increased expression of ICAM-1 that correlated with the PMS concentration. This stimulatory effect can be observed starting from 50 μM PMS onwards and gradually increases by six-fold at 800 μM then plateaus. Apparently, saturation is reached at about this level, though it should be noted that concentrations above 1000 μM were not employed in this study.

The time dependence of ICAM-1 expression is shown in Fig. 2, where it is clear that incubation of PMS-treated RBC with HUVEC results in a time dependent increase of the surface expression of ICAM-1. An increase of ICAM-1 expression was first noted at 3 h of incubation and continued to increase up to an incubation time of 9 h; thereafter it remained unchanged for up to 24 h. To determine whether the effect observed was specific for oxidatively modified RBC, HUVEC were also incubated with normal RBC and without RBC under identical conditions, then assayed for ICAM-1 expression. As shown in Fig. 2, there was no difference of ICAM-1 expression compared to untreated EC (dashed line).

3.2. Nuclear translocation of transcription factor NF-kB

To monitor NF-kB activity at a cellular level, the NF-kB p65 antibody was used to track the nuclear translocation signal of p65 via confocal laser scanning microscopy. As shown in Fig. 3A and B, the untreated control cells and HUVEC treated for 24 h with normal RBC showed a strong fluorescence of the cytoplasm. The cells exposed to RBC that were PMS-treated at lower concentrations (25 μM PMS) clearly showed a NF-kB fluorescence signal in the cytoplasm but no signal in the nucleus (Fig. 3C). Cells exposed to 50 μM PMS-treated RBC for 24 h showed a slightly larger nuclear signal (Fig. 3D), but from 100 μM upwards the cells showed a strong nuclear fluorescence pattern (Figs. 3E, 100 μM; 3F, 200 μM; 3G, 400 μM; 3H, 800 μM and 3I, 1000 μM), thus clearly indicating the NF-kB translocation in response to the increasing PMS concentration. Time course experiments revealed that during the first 2 h following incubation with PMS-treated RBC, the fluorescence signal can be detected in the cytoplasm (Fig. 4A–C), whereas after 3 h, the fluorescence signal becomes visible in the nucleus (Fig. 4D). It should be noted that the intensity of the fluorescence further increases between 6 and 24 h (Fig. 4E–H). These results therefore indicate that oxidatively modified RBC interfere with the translocation of the p65 subunit from the cytoplasm to the nucleus.

3.3. Oxidatively modified RBC- induce NF-kB activity

To assess the signal transduction pathway activated by oxidatively modified RBC, activation of the transcription factor NF-kB was quantified via gel shift assays. For this purpose, the DNA binding activity to NF-kB-specific oligonucleotides was analyzed in nuclear extracts of HUVEC. Compared to NF-kB activity in untreated HUVEC (Fig. 5, lane 1), endothelial cells exposed to PMS-treated RBC (50 μM to 1000 μM) showed an increase in NF-kB activity correlating with the increase of PMS concentration (Fig. 5, lanes 4 to 9). Incubation of normal red blood cells with HUVEC did not lead to an increase of the NF-kB activity compared to untreated HUVEC. Furthermore, incubation of HUVEC with 25 μM PMS-treated RBC did not affect the basal NF-kB activity (Fig. 5, lanes 2 and 3). As shown in Fig. 6, the gel shift assay for the time course experiments showed that there was a minimal activation of NF-kB activity when HUVEC were incubated with PMS-treated RBC for up to 3 h (lane 4). However, after 3 h, there was a distinct increase in the intensity of the DNA binding signal (lane 5, 6, 7 and 8). These results thus indicate that the interaction of oxidatively modified RBC with HUVEC causes a greater activation of NF-kB compared to that observed

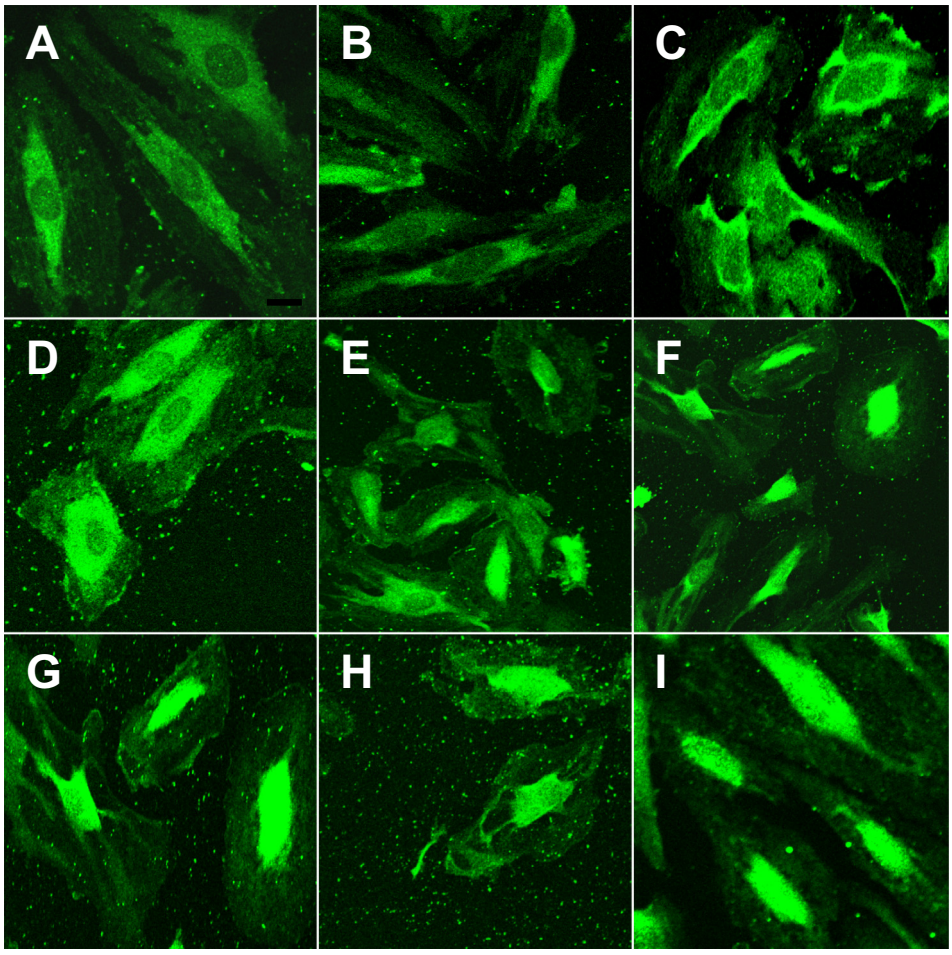

Fig. 3. Immunofluorescence staining detecting NF-κB nuclear translocation in HUVECs. HUVECs exposed to normal and PMS-treated (25 μM to 1000 μM PMS) RBCs were fixed, stained with an antibody specific for NF-κB and visualized under a confocal microscope. The results showed that the signal of NF-κB was diffusely distributed in the cytoplasm in untreated HUVEC (A) and cells exposed to normal RBC (B) and 25 μM PMS-treated RBCs (C). Cells exposed to 50 μM PMS treated RBC showed slight nuclear signal (D). A strong nuclear signal was observed in cells exposed to high concentration PMS-treated RBC indicating the translocation of NF-κB from cytoplasm to nucleus (E-100 μM PMS treated, F-200 μM PMS treated, G- 400 μM PMS treated, H-800 μM PMS treated and I-1000 μM PMS treated).

with normal RBC or untreated EC.

4. Discussion

It is now recognized that oxidatively modified or damaged RBC can play an important role in the pathogenesis of various pathological conditions. Although much effort has been focused on determining how the interaction of RBC with EC induces EC activation in different pathological states, the molecular responses of EC differ according to the pathological background of the interacting cells. Unfortunately, there appear to be no published studies detailing the exact effects of oxidatively modified RBC on EC intracellular signaling events. Our present study thus presents new information directly relevant to the specific contribution of oxidized RBC to EC activation. In addition, while PMS is often employed to study RBC oxidative stress responses at cellular and subcellular levels, this study reports the unique use of PMS to study heterotypic (i.e., RBC-EC) interactions at the molecular level.

Several studies have provided indirect evidence that EC activation,

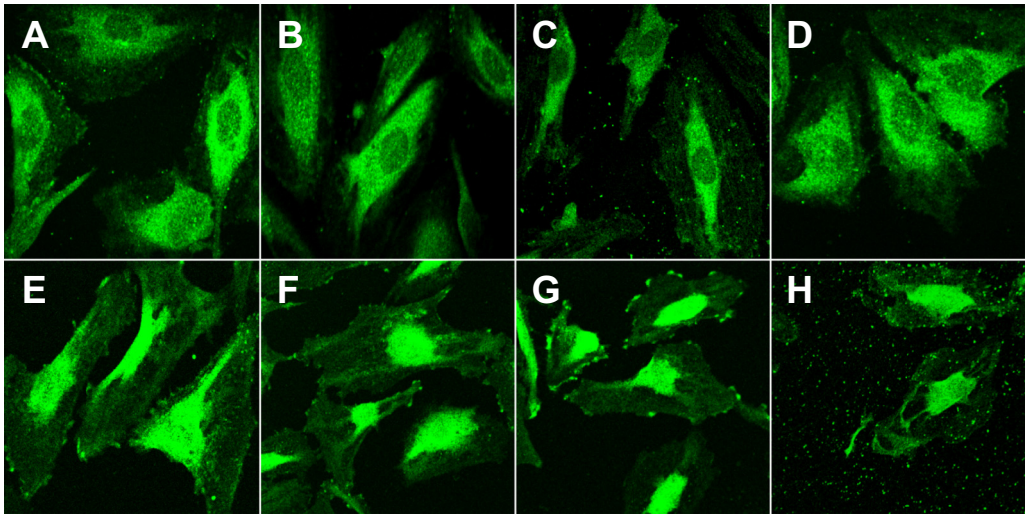

Fig. 4. Immunofluorescence staining detecting NF-κB nuclear translocation in HUVECs exposed to 800 μM PMS-treated RBC at different time points from 0.5 h to 24 h. Cytoplasmic p65 was observed as green fluorescence signal at 0.5 h, 1.0 h and 2.0 h (A–C). At 3 h, the fluorescence signal became visible in the nucleus (D) and the intensity of the fluorescence further increased between 6 and 24 h (E–H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

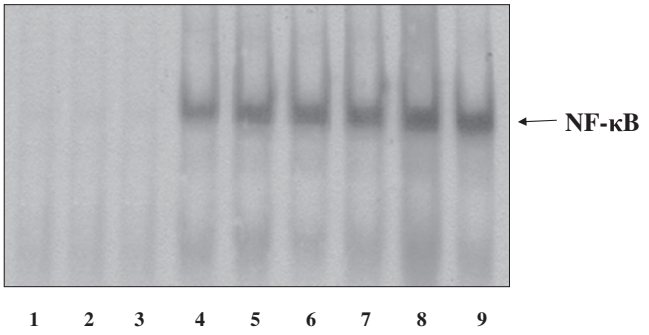

Fig. 5. EMSA showing NF-κB binding activity in nuclear extracts of endothelial cells exposed to PMS-treated RBC for 24 h. Nuclear extracts were prepared and NF-κB activity was analyzed as described in Materials and Methods. The arrowhead indicates DNA-protein complex. Untreated cells (Lane 1), cells treated with normal RBC (Lane 2) and 25 μM PMS-treated RBC (Lane 3) showed no NF-κB binding activity whereas nuclear extracts of cells exposed to high concentration PMS-treated RBC showed NF-κB binding activity corresponding to increase in PMS concentration. (Lane 4, 50 μM PMS-treated RBC; Lane 5, 100 μM PMS-treated RBC; Lane 6, 200 μM PMS-treated RBC; Lane 7, 400 μM PMS-treated RBC; Lane 8, 800 μM PMS-treated RBC and Lane 9, 1000 μM-PMS treated RBC).

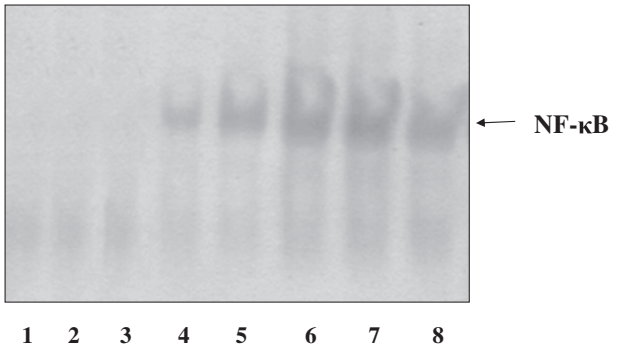

Fig. 6. EMSA showing NF-κB binding activity in nuclear extracts of endothelial cells exposed to 800 μM PMS-treated RBC at different time points. Labeled oligonulceotides moved freely in the gel without showing any bands for cells incubated with 800 μM PMS-treated RBC upto 2 h. (Lane 1, cells incubated with PMS treated RBC for 0.5 h; Lane 2, incubation for 1.0 h; Lane 3, incubation for 2 h). Cells incubated for 3 h showed minimal NF-κB binding activity (Lane 4) and a distinct increase in NF-κB binding activity was observed between 6 and 24 h (Lane 5, Incubation for 6 h; Lane 6, Incubation for 9 h; Lane 7: Incubation for 12 h; Lane 8, Incubation for 24 h).

i.e. increased surface expression of VCAM-1, ICAM-1 and E-selectin, can be linked to the elevation of circulating levels of soluble adhesion molecules, thrombomodulin and NO [31–39]. Activation of cultured endothelial cells with pro-inflammatory cytokines such as TNF-α and IL-1β increases ICAM-1 expression [40]. Our results clearly demonstrate that RBC oxidized by PMS are capable of inducing endothelial expression of ICAM-1, thus suggesting a direct effect of oxidatively modified RBC on the activation of EC. In contrast, no alteration of endothelial ICAM-1 expression was found using untreated EC or EC treated with normal RBC (Fig. 2), thereby supporting the concept that normal RBC do not activate quiescent EC.

It has been proposed that the diverse effects of endothelial cell activation share a common intracellular control mechanism through the activation of the transcription factor NF-kB [41]. Several studies have shown that NF-kB primarily responds to oxidative stress and that it enhances the transcription of a variety of genes [20,41]. In particular, reactive oxygen species (ROS) act as a second messenger, responsible for signal transduction from extracellular signaling molecules and their membrane receptors to intracellular regulatory systems such as NF-kB [42]. A stimulating agent acting at the endothelial cell surface causes the activation of cytoplasmic NF-kB; once activated, the NF-kB is transported into the nucleus and turns on the transcription of a large number of genes associated with inflammation including adhesion molecules [22]. Here, in this study, the interaction of PMS-treated RBC with endothelial cells resulted in the translocation of NF-kB from cytoplasm to nucleus and increased NF-kB activity in the nuclear extracts (Figs. 3 and 5), indicating the direct activation of the transcription factor NF-kB by means of oxidatively modified RBC. It should be noted that our results also indicate a correlation between NF-kB activation and ICAM-1 expression and that previous studies have also suggested a similar connection between NF-kB in ICAM-1 expression in cultured endothelial cells [3,43,44].

Treatment of human RBC with PMS results in a sustained intracellular production of superoxide anions (O_2^-) and other oxygen free radicals, thereby modifying the ionic equilibrium of the erythrocytes [28,45,46]. The adherence/contact of oxidatively modified RBC to endothelial cells initiates cellular signaling. Consequently, activation of transcription factor NF-kB occurs in HUVEC, leading to the binding of activated NF-kB to the consensus sites in the regulatory regions of DNA for several genes including a subset of CAMs, thus bringing about the cell-surface expression of ICAM-1. Garg et al., reported that TNF-α induced free radical generation in a manner similar to H_2O_2 and that it activated inflammatory signaling pathways including NF-kB in vascular cells [47]. It is interesting to note that the interaction of sickle erythrocytes with endothelial cells can also induce the surface expression of ICAM-1 and activation of transcription factor NF-kB [3].

The expression of many other adhesion molecules has also been reported in sickle cell disease stimulated EC, including P-selectin and *E*-selectin [48–50]. The mechanisms of increased P-selectin expression on EC are often related with increasing ROS and EC reticulum stress triggered by sickle red blood cells and/or RBC-released agents such as histamine and high mobility group 1B protein (HMGB1) [48,49]. For present study, however, it is still unclear as to whether increased ICAM-1 and NF-κB are also related to increasing ROS and EC reticulum stress.

In overview, this study presents specific individual effects of oxidatively modified RBC on *intracellular signaling pathways* that promote *endothelial cell* activation. The interaction of oxidatively modified RBC with endothelial cells resulted in the up-regulation of ICAM-1 expression and the activation of NF-kB, thereby demonstrating the potency of oxidatively modified RBC to contribute to EC activation. These observations provide a molecular framework to gain more insight into the pathophysiology of variety of acute and chronic illnesses characterized by systemic oxidative stress.

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