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Cross-talk between minimally primed HL-60 cells and resting HUVEC reveals a crucial role for adhesion over extracellularly released oxidants

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ABSTRACT

This study demonstrates that a long-lasting co-culture of neutrophil surrogates (HL-60 cells), minimally primed by platelet activating factor (PAF), and resting endothelial cells (EC) results in the elaboration of an hyper-adhesive endothelial surface, as measured by the increase in the expression of endothelial adhesion molecules E-Selectin, VCAM-1, and ICAM-1. This endothelial dysfunction is mediated by the activation of the redox-sensitive transcription factor NF-κB through an exclusive adhesion-driven mechanism active in the endothelial cell: reactive oxygen and nitrogen species, extracellularly released by minimally primed HL-60 cells, are not involved in the induction of the endothelial dysfunction.

By exploring for the first time the potential for minimally primed neutrophil surrogates to induce endothelial dysfunction, this study suggests a novel mechanism which may be operative in pathologies, mediated by minimally primed neutrophils, such as hyperdyslipidemia and cardiovascular complications.

1. Introduction

Given the impressive array of their harmful weapons, neutrophils (PMNs) have long been considered effective but indiscriminate attackers with a low control of their responses hence with a great potential for damaging the host tissue, should these weapons become uncontrolled and/or misdirected [1]. In recent years, however, it has become increasingly clear that the PMN response to pro-inflammatory stimuli is finely tuned to provide an appropriate level of reactivity. One of the regulatory mechanisms to control neutrophil responses is priming [1]. PMN activation, indeed, is not an all-or-none process; rather it can be modeled as a continuum from a nonresponsive to a fully activated phenotype. Within this continuum, PMN can exist in one of three functional states: quiescent, primed or activated [1].

Under non-infectious conditions PMNs are quiescent, exhibiting little or no release of oxidants; in this state, when first encountering a stimulus they reach a *primed status*. Upon encountering a second appropriate stimulus, PMNs would then proceed to full activation releasing oxidants, granule contents and inflammatory mediators in the surrounding milieu. Noteworthy, if the PMN has been primed, its functional response (ROS production, chemotaxis) to the second stimulus will be amplified, in comparison to that of unprimed (quiescent) cells. Importantly, the priming agent itself does not cause any noticeable functional response [1–3].

Interaction between PMNs and endothelial cells (EC) has been extensively investigated due to the crucial role these cells play in vascular biology and inflammatory processes. Indeed PMNs may trigger an endothelial injury seriously compromising the function of vasculature in several inflammatory pathologies as well as in relation to the inflammatory processes operative in atherosclerosis, diabetes and ischemia-reperfusion injury [4–6].

While it has extensively been demonstrated that fully activated PMNs do induce endothelial dysfunction [7], it is now becoming evident that also primed cells can be direct mediators of EC dysfunction. For instance, PMNs from patients with risk of developing atherosclerosis and cardiovascular complications (e.g. essential hypertension, diabetes mellitus type II or chronic renal failure) display a certain degree of priming that correlates positively with both the magnitude and type of the disease, leading to an oxidative imbalance within the endothelium [8–14]. This has led to propose that loose - but continuous - interaction between primed neutrophils and EC may induce a chronic threshold injury to the endothelium and a systemic oxidative stress able to initiate atherosclerosis and cardiovascular complications [8–10]. Conversely, EC-derived factors can also contribute to maintain and/or increment PMN activation.

Most of the studies have so far focused on the dysfunctioning effect produced in EC by fully activated or maximally primed PMNs

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[8,10,13]. While the evidence of an endothelial damage by maximally primed PMN can support a pathogenic role for these cells, investigating the interaction between minimally primed neutrophils and resting EC may help to understand the very early events promoting endothelial injury, possibly occurring before overt EC damage.

With this in mind, we have created a novel experimental model of resting EC/minimally primed PMN interaction to investigate whether, and to what extent, this cross-talk can lead to endothelial activation.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals were from Sigma–Aldrich (Poole, UK), and of the highest available purity grade.

2.2. Cell cultures

Primary human umbilical vein ECs (HUVEC) were obtained from Lonza (Wokingham, UK) and cultured in endothelial growth medium (Lonza, UK) at 37 °C in a humidified atmosphere with 5% CO_2; cells were sub-cultured by trypsinization and used for the investigation up to passage four. HL-60 cells were obtained from European Type Culture Collection (ECACC, Sigma–Aldrich, Poole, UK) and maintained in RPMI-1640 with 10% heat-inactivated foetal bovine serum (Invitrogen, Paisley, UK), 1% L-glutamine, 1% non essential amino acids, 0.5% Gentamicin in a 5% CO_2 atmosphere at 37 °C; cellular density was maintained between 2 and 9×10^5 cells/ml. Cell number and viability were determined by Trypan blue exclusion. Differentiated HL-60 (dHL-60) cells were obtained by 5-day culturing in a medium containing 1% (v/v) dimethyl sulfoxide, as previously reported [15]. On day five, cells were harvested and re-suspended in fresh medium.

2.3. Assessment of HL-60 cell differentiation into neutrophil-like cells

CD11b expression was evaluated as marker of HL-60 cell differentiation into PMN-like cells [16]. Both HL-60 and dHL-60 cells were washed with a buffer containing PBS and 0.1% bovine serum albumin. Aliquots ($1–5 \times 10^4$ cells in 20 μl) were added to a 96-well plate and incubated at 4 °C with 3 μg/well of a mouse anti-human CD11b phycoerythrin-conjugated monoclonal antibody (Immunotech, Marseille, France). After 1 h at 4 °C, cells were washed and analyzed for fluorescence on a Becton Dickinson FACScan (NJ, USA) using a Cell Quest software. At least 10,000 events were analyzed for each sample. Data are expressed as median fluorescence intensity units measured in the FL2 (red) channel with a 488 nm (excitation) and 585 nm (emission) filter set.

2.4. Assessment of dHL-60 cell priming

Platelet activator factor (PAF) is a lipid mediator produced by enzymatic cleavage of membrane phospholipids [1]. In addition to its activating effects on neutrophils (at a concentration $>10^{-6}$ M), PAF may also exert many priming-type actions, evident at concentrations between 10^{-7} and 10^{-9} M, without stimulating PMN to produce significant amounts of oxidants [17]. Thus, differentiated HL-60 cells were pre-treated with PAF (10^{-10}–10^{-5} M) for 30 min at 37 °C and then activated with phorbol 12-myristate 13-acetate (PMA, 0.3×10^{-7} M at 37 °C for 60 min). Superoxide release was evaluated to assess the degree of priming. Measurements of the rate of superoxide release are based on SOD inhibitable reduction of 80 μM cytochrome *C* to its ferrous form. The rate of $O_2^{\bullet -}$ release was monitored from 10^6 either HL-60 or dHL-60 cells at 22 °C up to 90 min, applying a protocol previously described in detail [11].

2.5. HUVEC dysfunction

Given the minimal priming state of PMN, a long lasting co-culture was required to visualize changes in cell phenotype. Primary PMN are end-stage cells that cannot be cultured for long periods of time (>4 h) without losing responsiveness to various stimuli or becoming apoptotic [18]. HL-60 cells are widely reported to be similar to human neutrophils in morphology, expression of receptors, superoxide generation, chemotaxis and, unlike primary neutrophils, can be cultured for long periods of time [18].

HUVEC were grown on 6-well plates and when confluent incubated with or without 1×10^6 dHL-60 cells for 16 h, with or without 1×10^{-7} M PAF. When required, superoxide dismutase (SOD) and catalase (CAT) were added in combination (200 U/ml each enzyme) to the culture medium. In the experiments employing cell-permeable antioxidant, HUVEC were pretreated for 2 h with N-acetyl-cysteine (NAC, 30 mM), washed with fresh medium and then co-cultured for 16 h with dHL-60 cells as above described. In the experiments designed to prevent dHL-60 cell adhesion to HUVEC, Transwell™ polycarbonate membranes inserts (24 mm diameter, 0.4 μm pore size, Corning B.V. Life Sciences (Amsterdam, The Netherlands) were employed: confluent HUVEC were co-cultured for 16 h with PAF-primed dHL-60 cells added in the Transwell™ insert.

In all cases, at the end of the incubation time HUVEC were washed with PBS, harvested with cell dissociation medium and diluted in a washing buffer containing PBS, 0.1% bovine serum albumin and 1 mM $CaCl_2$. Aliquots ($0.1–0.5 \times 10^5$ cells in 20 μl) were added to a 96-well plate and incubated with 2 μg/well of a mouse anti-human ICAM-1 (CD54), PECAM-1 (CD31) or E-selectin (CD62E) monoclonal antibody (clones 15.2, 13.5 and18.6, respectively; AbD Serotec, Kidlington, UK). After 1 h at 4 °C, cells were washed and incubated with 20 μl of a rabbit anti-mouse $F(ab')_2$ polyclonal FITC-conjugated secondary antibody (Serotec, Kidlington, UK) for a further 1 h. Finally, samples were washed and analyzed for fluorescence on a Becton Dickinson FACScan using a Cell Quest software. HUVEC and dHL-60 cells were clearly distinguished for their forward and side scatter characteristics. At least 20,000 events were analyzed for each sample. Data are expressed as median fluorescence intensity units, measured in the FL1 (green) channel with a 488 nm (excitation) and 530 nm (emission) filter set.

2.6. Detection of intracellular reactive oxygen and nitrogen species

Intracellular levels of ROS and RNS were assessed using the non-fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCDHF-DA) as previously reported [19]. In brief, either EC or dHL-60 cells were cultured as described above. At selected time points, cells were washed with warm PBS, 100 μM DCDHF-DA (Molecular Probes, OR, USA) was added to the culture system and cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. After 45 min cells were washed with PBS, harvested, diluted in a FACS buffer containing PBS and 0.1% bovine serum albumin, and analyzed for fluorescence on a Becton Dickinson FACScan using a Cell Quest software. At least 20,000 events were analyzed for each sample. Data are expressed as median fluorescence intensity units as measured in the FL1 channel.

2.7. NF-κB luciferase assay

NF-κB activity was examined by transfecting HUVEC with pNF-κB-Luc luciferase construct (Stratagene, CA, USA). Transfections were carried out using Amaxa Nucleofector Technology (Lonza,

UK). Briefly, HUVEC were passaged two days before nucleofection and, on the day of the experiment, 100 μl of a cell suspension at 0.5×10^6 cell/ml was combined with 3 μg of pNF-κB-Luc luciferase construct, transfected according to the manufacturer protocol and incubated for 12 h in a 5% CO_2 atmosphere at 37 °C. Transfected HUVEC were thereafter co-cultured with PAF-primed dHL-60 cells and the specific treatments applied as detailed above. After 6 h, cells were washed with PBS and lysed for 5 min at 4 °C using a lysis buffer according to the manufacturer's instructions (Promega, WI, USA). Luciferase activity is expressed as relative luminescence units (RLU) $\times 10^3$ following reading in a TD/2020 luminometer (Turner Biosystems, CA, USA).

2.8. Statistical analysis

Data are reported as mean ± SEM of *n* experiments performed in triplicate or quadruplicate. Differences among experimental groups were assessed using one-way analysis of variance and, once significant, applying the Dunnett test to selected experimental groups. A probability value less than 0.05 was considered significant.

3. Results

3.1. Priming effect of PAF on dHL-60 cells

Primed PMN are pre-activated cells such that exposure to a subsequent inflammatory stimulus generates an enhanced response with respect to resting cells. Preliminary assays were carried out to establish the concentration of PAF required to yield minimally primed dHL-60 cells. As shown in Fig. 1, stimulation with PAF $< 10^{-7}$ M did not prime cells, as they did not release any significant amount of $O_2^{\bullet-}$ above the levels of control cells exposed to PMA. In contrast, PAF concentrations between 10^{-7} and 10^{-5} M primed dHL-60 cells in a concentration-dependent fashion, as judged by the increment of PMA-stimulated superoxide release (Fig. 1). No release of $O_2^{\bullet-}$ was measured when cells were not treated with PMA, irrespective of the PAF concentration used for priming (not shown). Thus, 10^{-7} M PAF was regarded to be the minimal concentration required to significantly prime, without activating, dHL-60 cells.

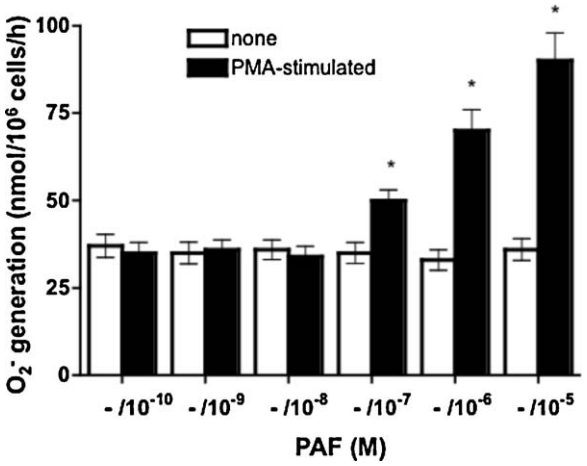

Fig. 1. Effect of PAF on dHL-60 cell-released $O_2^{\bullet-}$. Cell were exposed to the indicated concentration of PAF immediately prior to addition of PMA (as indicated in Section 2); the rate of $O_2^{\bullet-}$ release is measured as nmol/10^6 cells/h. Data are mean ± SE of 3 distinct experiments with different cell preparations. $^*P < 0.05$ vs. control.

3.2. Co-culture of minimally primed dHL-60 cells with resting HUVEC causes EC dysfunction

Next we investigated the effects of a long-lasting co-culture of minimally primed dHL-60 cells with resting HUVEC on the expression of the major EC adhesion molecules. Flow cytometry allowed endothelial cells and adherent leukocytes to be distinguished in view of their different scatter profiles: HUVEC showed a spread distribution with high FSC and SSC values (Fig. 2A and B)

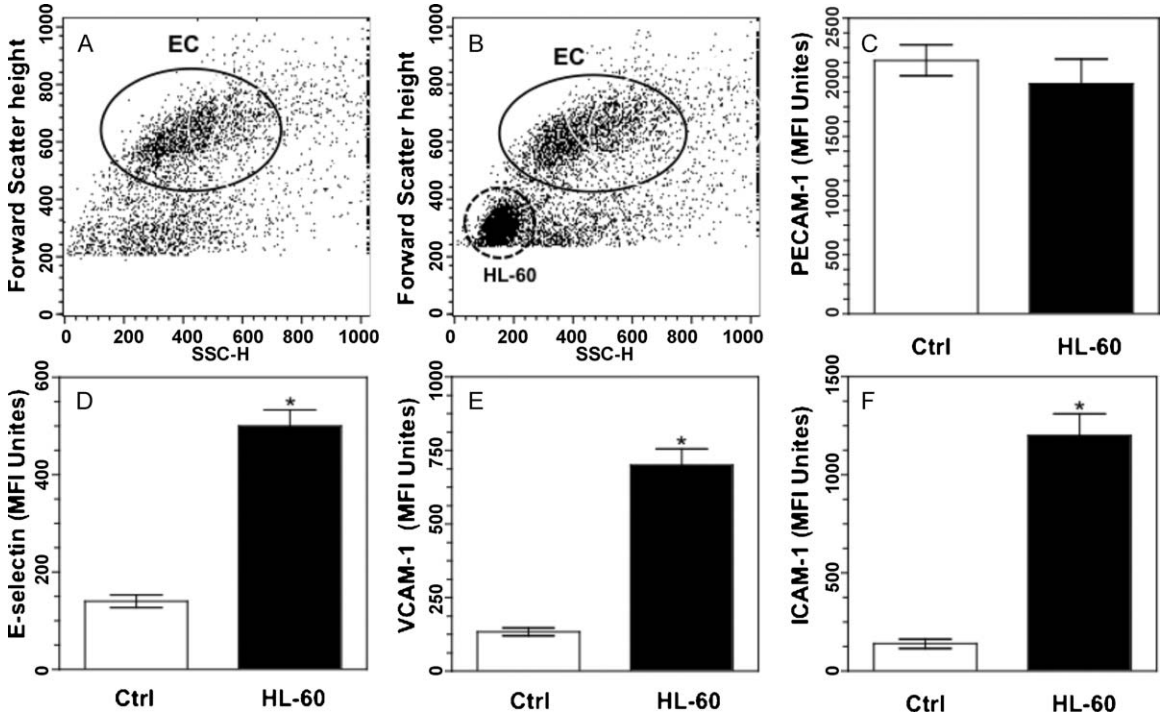

Fig. 2. Flow cytometry analysis of endothelial PECAM-1, E-selectin, VCAM-1 and ICAM-1 expression induced by PAF-primed dHL-60 cells. (A and B) Dot plots of the scatter characteristic of HUVEC alone (panel A) and co-cultured with PAF-primed dHL-60 cells (panel B), representative of five separate experiments. Solid lines represent gating on HUVEC. (C–F) Endothelial PECAM-1, E-selectin, VCAM-1 and ICAM-1 expression induced by PAF-primed dHL-60 cells. HUVEC were cultured either in the absence (CTRL) or in the presence of PAF-primed dHL-60 cells (HL-60). Data are mean ± SEM of 5 separate experiments. $^*P < 0.05$ *vs.* control.

whereas dHL-60 cells appeared as a lower and more compact population (Fig. 2B). Co-incubation of minimally primed dHL60 cells with HUVEC for 16 h induced a significant increase of E-Selectin, VCAM-1 and ICAM-1 levels ($P < 0.01$) (Fig. 1D–F, respectively) whereas the expression of PECAM-1 did not show any significant change (Fig. 1C), demonstrating that the HUVEC dysfunction was not consequent to non-specific loss of cell viability or phenotype.

3.3. Minimally primed dHL-60 cell-induced endothelial dysfunction is mediated by a redox-dependent mechanism

We then investigated the mechanisms by which minimally primed dHL-60 cells might cause the HUVEC activation. Since changes in endothelial adhesion molecule expression can be induced by changes of the endocellular redox milieu [20], oxidative stress inside EC was then evaluated. Co-incubation of minimally primed dHL-60 cells with resting HUVEC induced a time-dependent increase of reactive species (ROS/RNS) inside EC that peaked at 4 h, returning to basal levels >12 h (Fig. 3A).

Closer monitoring of the time-course of E-Selectin, VCAM-1 and ICAM-1 expression in the same conditions, provided evidence that the increase of the adhesion molecule expression started only after a critical ROS/RNS level was achieved (i.e. 4 h), with a maximum expression at 16 h (Fig. 3A, inset). Consistent with this, pretreatment of HUVEC with N-acetyl-cysteine (NAC), a cell-permeable thiol radical scavenger widely used to assess the role of reactive species in gene expression [21], abrogated the expression of ICAM-1, VCAM-1 and E-selectin induced by minimally primed dHL60 (Fig. 3B).

3.4. Activation of NF-κB in HUVEC by minimally primed dHL-60 cells

It is well established that endothelial adhesion molecule expression is under control of several redox-regulated transcription factors, including the master minder NF-κB [22]. To determine whether this possible cascade of events was indeed occurring in our experimental settings, we transfected HUVEC were with a NF-κB reporter construct prior to co-culture with minimally primed dHL-60 cells. Luciferase activity in HUVEC co-cultured with control un-primed dHL-60 cells was not significantly different than that observed in HUVEC alone (not shown). Conversely, co-culture of HUVEC with minimally primed dHL-60 cells caused a significant increase (~3-fold) in the activation of this transcription factor within the EC (Fig. 4A).

3.5. HUVEC dysfunction is mediated by cell–cell interaction and not by extracellularly released oxidants

It has been reported that PMN-induced EC activation can be triggered by oxidants released by adherent PMN, adhesion molecule ligation, or both [7]. In order to investigate which event was to be considered crucial for the EC activation measured in our system we determined the effect of superoxide dismutase and catalase on NF-κB activation and ICAM-1 upregulation in HUVEC. No differences in both NF-κB activation and ICAM-1 up-regulation were observed between HUVEC stimulated in the absence or in the presence of these two antioxidant enzymes (Fig. 4B), suggesting that released oxidants, whenever released by adherent PMN, were ineffective on the modulation of redox-regulated signaling pathways.

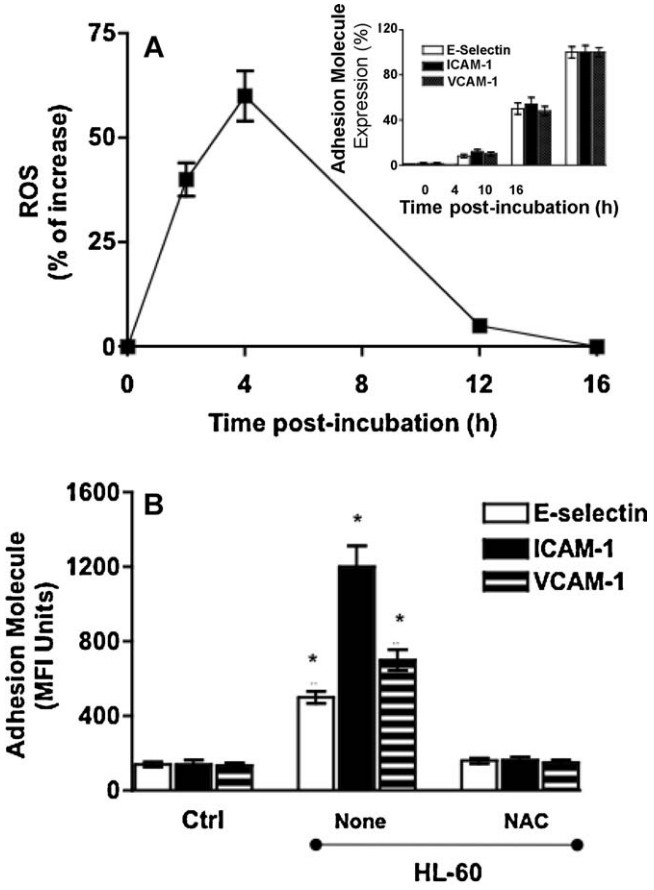

Fig. 3. Time-course of EC dysfunction. (A) Kinetics of endothelial ROS/RNS increase and E-selectin, VCAM-1 and ICAM-1 expression (inset) induced by PAF-primed dHL-60 cells. (B) Effect of NAC on E-selectin, VCAM-1 and ICAM-1 expression induced by PAF-primed dHL-60 cells. HUVEC were either left untreated (CTRL) or co-cultured with PAF-primed dHL-60 cells (HL-60/NONE) or pretreated with NAC and then co-cultured with PAF-activated dHL-60 cells (HL-60/NAC). Data are mean ± SE of 3 separate experiments. $^*P < 0.05$ vs. control.

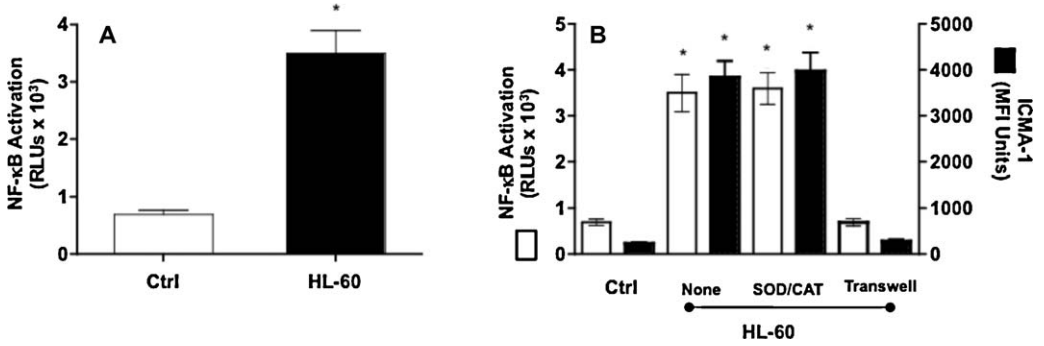

Fig. 4. NF-κB activation in EC. (A) Effect of PAF-primed dHL-60 cells on endothelial NF-κB activation. HUVEC were either left untreated (CTRL) or co-cultured with PAF-primed dHL-60 cells (HL-60) for 6 h. (B) Effect of cell-impermeable antioxidants or Transwell® inserts on NF-κB activation and ICAM-1 expression induced by PAF-primed dHL-60 cells. HUVEC were co-cultured with PAF-primed dHL-60 cells either in the absence (NONE) or presence of superoxide dismutase and catalase (SOD/CAT; 200 U/ml) or employing Transwell® inserts (TW). Untreated HUVEC were also tested (CTRL). Data are mean ± SE of 3 separate experiments. $^*P < 0.05$ vs. control.

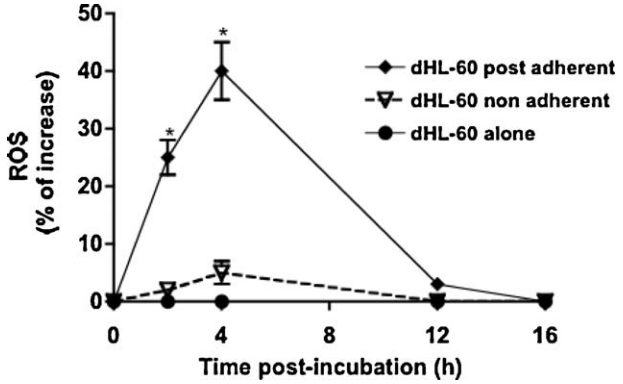

Fig. 5. Time course of intracellular ROS/RNS generation in dHL-60 cells. Differentiated HL-60 cells were primed with 10^{-7} M PAF and either left alone (●), or co-cultured with HUVEC in the absence (▽) or in the presence (◆) of Transwell® inserts. At selected time points, ROS/RNS generation inside dHL-60 was evaluated. Data are mean ± SE of 3 separate experiments. *$P < 0.05$ vs. dHL-60 alone.

To investigate the role of physical adhesion between HUVEC and minimally primed dHL-60 cells on the process leading to endothelial dysfunction, HUVEC were co-cultured with minimally primed dHL-60 cells, either in the absence or in the presence of a Transwell™ insert, a device that allows EC and dHL-60 cells to exchange soluble molecules, but prevents the physical contact between the two cell types. As shown in Fig. 4B, physical separation between cells abolished both NF-κB activation and ICAM-1 up-regulation, indicating that cell adhesion is necessary and sufficient to trigger the signaling events conducive to EC dysfunction.

3.6. Cross-talk between minimally primed dHL-60 cells and resting HUVEC

Though priming pre-disposes neutrophils to a prompt activation, primed PMN by themselves are not capable of producing reactive species. When co-cultured with resting HUVEC for 16 h, minimally primed dHL-60 cells showed a significantly increase in the endocellular levels of ROS/RNS, peaking at 4 h. On the other hand, when they were prevented to adhere to EC, by being cultured inside a Transwell™ inserts, no intracellular production of ROS/RNS was detected (Fig. 5). This provides evidence that adhesion-driven signals establish a real cross-talk between EC and dHL-60 cells, yielding EC activation. In our experimental settings cell adhesion represents the "second stimulus" that leads primed-neutrophils to full activation.

4. Discussion

This study investigates endothelial dysfunction mediated by primed PMN, and explores mechanistic aspects of minimally primed neutrophil surrogates to potentially induce EC activation. Our results support previous studies on the pathogenic role of primed neutrophils [1,8–14] where it has been shown that minimally primed neutrophils are able to induce over-expression of E-Selectin, VCAM-1 and ICAM-1, thus promoting an activating phenotype of EC. However, several novel observations were made here.

First, minimally primed neutrophil surrogates can activate EC as long as they are co-cultured for a time period considerably higher (16 h) than that reported in studies employing highly primed neutrophils from hemodialysis, diabetic or hyperlipidemic patients (15 min) [10,13,14]. Secondly, minimally primed dHL-60 cells neither alter EC viability nor induce irreversible lethal events that would result in cell detachment and apoptosis, outcomes reported when highly primed PMN are used [10,13,14]. This is coherent with the threshold degree of priming employed here, at variance from that used in previously published studies. At a mechanistic level, our data demonstrate for the first time that minimally primed HL-60 generate endothelial intracellular signals, namely variation in the endothelial redox mileu and downstream activation of NF-κB, resulting in EC dysfunction.

In endothelial cells, the activation of NF-κB by oxygen radicals is a cardinal event leading to up-regulation of several adhesion molecules including ICAM-1 and VCAM-1. Furthermore, treatment of TNF-α-stimulated cells with antioxidants such NAC and pyrolidine dithiocarbamate reduces Ser32 phosphorylation leading to a reduction in NF-κB activity in endothelial cells. Our results are consistent with these findings and show a strong reduction of E-selectin, ICAM-1 and VCAM-1 upregulation in HUVEC co-cultured with minimally primed dHL-60 in presence of NAC providing a direct link between activation of NF-κB and the increased expression of these adhesion molecules.

More importantly, if integrated in the wider frame of knowledge related to the pathogenic role of primed neutrophils, our results also suggest that EC dysfunction can be the result of a continuous interaction of minimally primed PMN with endothelium, finally leading to a clear injury. Moreover, we suggest that the extent of neutrophil priming is crucial to influence the way the EC response can be triggered. Indeed, we show that when neutrophils are minimally primed, EC activation is achieved through adhesion-driven signals whereas, as elsewhere reported [10], soluble factors released by these cells overcome direct adhesive effects when highly primed PMN are used.

Finally, a pivotal function for physical adhesion between EC and minimally primed dHL60 was found. Adhesion to the monolayers led to production of reactive species in the neutrophil-like cells too, stimulating a real cross-talk between these two cell types instead of a mere "one-way" activation of EC.

In conclusion, the present study reveals a function for intracellular, but not extracellularly released, oxidants and, more importantly, describes how a minimal degree of leukocyte activation might be sufficient to elicit these intracellular responses which would, then, impact on the cell phenotype. Based on these results we propose a new mechanism of EC dysfunction that maybe relevant to clinical states where a persistent threshold level of neutrophil activation occurs, without overt cell activation or neutrophil-dependent cell injury. Indeed, since initial endothelial dysfunction is a fundamental event in hyperdyslipidemia-associated and inflammation-based diseases such as atherosclerosis and cardiometabolic complications, our findings may possibly suggest new pharmacological targets helpful to the development of preventive strategies.

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