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Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death

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ABSTRACT

Neutrophil interaction with activated endothelial cells (EC) is required for transmigration. We examined consequences of this interaction on NETosis. Co-culture of activated EC with neutrophils induced neutrophil extracellular trap (NET) formation, which was partially dependent on production of IL-8 by activated EC. Extended neutophil/EC co-culture resulted in EC damage, which could be abrogated by inclusion of either diphenyleneiodonium to inhibit the NAPDH oxidase pathway required for NETosis, or DNAse to disrupt NETs. These findings offer new insight into mechanisms whereby NETs trigger damage to the endothelium in sepsis, small vessel vasculitis and possibly the villous trophoblast in preeclampsia.

1. Introduction

A unique aspect of polymorphonuclear neutrophil (PMN) antimicrobial defence is their ability to release chromatin and granular proteins to form an extracellular fibrillar matrix termed neutrophil extracellular traps (NETs) [1,2]. Several studies have determined that extracellular NETs ensnare and kill bacteria, fungi or even parasites [1,3–5]. While NETs contain anti-microbial proteins including elastase, cathepsin G, LL-37 and histones, an intact chromatin lattice appears to be required, as anti-microbial activity is diminished following exposure to DNases [2–4].

NETosis is triggered by a variety of stimuli, including microorganisms, activated platelets, placental micro-debris, pro-inflammatory cytokines (interleukin 8 (IL-8), tumor necrosis factor α (TNFα)) or phorbol 12-myristate 13-acetate (PMA) [1,6–8]. Reactive oxygen species play a central role in NETosis, as it is hindered by diphenyleneiodonium (DPI), a NADPH oxidase inhibitor [6]. Furthermore, patients with chronic granulomatosis, who have a genetically defective NADPH oxidase enzyme, do not produce NETs [9].

NETs have also been observed under inflammatory conditions and sepsis, implying a potential role in the pathogenesis of such disorders [2,7,8,10]. In preeclampsia, a severe pregnancy-related inflammatory disorder, large numbers of NETs have been observed in vivo in the intervillous space and are triggered in vitro by trophoblast micro-debris [8,11]. In experimental sepsis, platelet TLR4-neutrophil interaction induced NETs [7]. Of interest is that NETs were associated with endothelial injury [7]. Furthermore, in small vessel vasculitis (SVV) NETs were located in areas of tissue damage [12].

Although endothelial activation supports transmigration by neutrophils [13], the effect on NETosis is unclear. To examine this, we co-cultured human umbilical vein endothelial cell (EC) monolayers with isolated circulatory neutrophils.

2. Materials and methods

2.1. Isolation of neutrophils

Blood samples (20 ml each) were obtained from healthy donors at the Red Cross Blood Donation Center, Basel. PMNs were isolated

Abbreviations: DPI, diphenylene iodonium; EC, endothelial cells; IL-8, interleukin 8; NCM, neutrophil culture medium; NET, neutrophil extracellular trap; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear neutrophil; SVV, small vessel vasculitis; TNFα, tumor necrosis factor α

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using a Ficoll-Dextran method [8] and resuspended in neutrophil culture medium (NCM) comprising RPMI 1640 supplemented with 2% heat inactivated human serum, 2 mM L-glutamine, 100 U/ml penicillin 100 μg/ml and streptomycin (Invitrogen Life Technologies, Basel, Switzerland). Cell viability was 95–97% by trypan blue dye exclusion, and flow cytometry analysis for absence of CD11b ensured that PMN were not activated during isolation [8].

2.2. Endothelial cell culture

Human umbilical vein EC were purchased from PromoCell GmbH (Heidelberg, Germany) and cultured on 0.1% gelatine-coated culture plates in basal EC medium containing low serum (2% FCS) and EC growth supplement (PromoCell) [14]. NCM was used during co-culture experiments.

2.3. Analysis of NETs

NETs were evaluated using the membrane-impermeable DNA binding dye SYTOX green (Molecular Probes, Invitrogen Life Technologies) to detect extracellular DNA as detailed previously [1,8]. Experiments were performed in 96-well culture plates. In a first set of experiments freshly isolated PMN (1.5×10^5 cells/well) were directly stimulated with PMA (25 nM), thapsigargin (500 nM) or rTNFα (20 ng/ml) (all from Sigma Chemicals, St. Louis, MO, USA). In a second set, EC (1×10^4 cells/well) were treated with the same agents for 18 h, rinsed repeatedly with PBS to remove all traces of agents, and then co-cultured with unstimulated PMN (1.5×10^5 cells/well). SYTOX green (5 μM) was added to the cultures after selected periods of incubation (30–180 min), and fluorescence measured 5 min later using arbitrary fluorescence units. To visualize NETs, live-cell cultures were imaged with an Olympus IX-50 inverted fluorescence microscope. Here the co-cultured EC were loaded with PKH26 red fluorescent cell linker (Sigma) prior to the treatment with different agents.

2.4. Scanning electron microscopy

PMN (1×10^5 cells) seeded onto 12 mm 0.001% polylysine-coated coverslips were incubated in the absence or presence of PMA (25 nM), thapsigargin (500 nM) or rTNFα (20 ng/ml) for 3 h, fixed with 2.5% glutaraldehyde, postfixed using repeated incubations with 1% osmium tetraoxide/1% tannic acid, and dehydrated with a graded ethanol series (30%, 50%, 70%, 100%). After dehydration and critical-point drying, the specimens were coated with 2 nm platinum and analyzed by a Philips XL-30 ESEM scanning electron microscope at ZMB, Biozentrum, University of Basel.

2.5. Analysis of EC damage

A cellular DNA fragmentation ELISA (Roche Diagnostics GmbH, Mannheim, Germany) was used to quantitatively determine EC damage. BrdU-labelled EC [15] (1×10^4 cells/well in 96-well plates) were cultured for 18 h in the absence of presence of PMA (25 nM), thapsigargin (500 nM) or rTNFα (20 ng/ml). EC were rinsed to remove all traces of activators and cultured for a further 18 h in the absence or presence of unstimulated PMN (1.5×10^5 cells/well) and without or with inclusion of 500 U/ml DNase (Sigma) or 25 μM diphenylene iodonium (DPI; Sigma) or 100 μg/ml IL-8 neutralizing antibodies (anti-CXCL8/IL-8 (clone 6217), R&D Systems, Abingdon, UK) or 100 μg/ml isotype control IgG (Sigma). The plates were centrifuged (10 min, 250×*g*) and supernatants collected for analysis of BrdU-labelled DNA fragments. EC damage is expressed as the fold-increase in the released DNA fragments over that in untreated monolayer EC cultures. EC cultured for 18 h under conditions of complete serum deprivation (i.e. 0.1% BSA replacing 2% FCS), which results in ≈90% cell detachment/death [14], served as a positive assay control; release of BrdU-labelled DNA fragments into culture supernatants was in the order of 20-fold greater than control EC.

2.6. IL-8 ELISA

EC (1×10^5 cells) were seeded into 6-well plates, allowed to attach and then cultured for 18 h with or without PMA (50 nM), thapsigargin (500 nM) or rTNFα (200 ng/ml). EC were washed with PBS to remove all traces of activators and further cultured for 18 h in NCM. IL-8 in supernatants was measured using a human IL-8 ELISA kit (R&D Systems, Abingdon, UK).

2.7. Statistics

Experiments were performed in triplicate on at least three separate occasions. Data, given as mean ± S.D., were analyzed using GraphPad Prism software. Where appropriate either two-tailed Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison test were used to calculate the statistical significance of the differences between experimental groups. *P* values of less than 0.05 were considered significant.

3. Results and discussion

3.1. Endothelial cell activation elicits NETs formation

The expected release of DNA and visible NET formation by PMNs following activation by PMA [1,6,8] or TNFα [16] is shown in Fig. 1A and C. Thapsigargin, which mobilizes Ca^{2+} from intracellular pools, also effectively induced NETs in PMNs (Fig. 1A and C). Since EC are activated by PMA, TNFα and thapsigargin [14] we examined whether EC treated with them might induce NETosis. Fluorescence in co-cultures with untreated EC and PMN was negligible (Fig. 1B). In contrast an increase in SYTOX green fluorescence in co-cultures of activated EC and unprimed PMN suggested that NETosis had been triggered (Fig. 1B). This was most pronounced for EC pre-treated with PMA, followed by TNFα and thapsigargin (Fig. 1B). The kinetics was similar, albeit quantitatively less than by direct of PMN (c.f. Fig. 1A and B). Induction of NETs was confirmed by live-cell fluorescence microscopy, which showed the presence of typical extracellular DNA lattices in co-cultures of PMN and activated EC, but not with untreated EC (Fig. 1C). From these photomicrographs, recorded at the end of the 3 h culture period, it is evident that in both neutrophil monoculture and EC–PMN co-cultures the incidence of SYTOX green stained cells was considerably higher than that of NET-like structures (Fig. 1C). Thus increases in SYTOX green fluorescence readings (Fig. 1A and B) can be assumed to reflect both the process of NETosis *per se* as well as cell death following NETosis. To unequivocally demonstrate the presence of NETs we performed high resolution scanning electron microscopy on control and activated PMN. In activated PMN we observed structures characteristic of NETs [1]; they consisted of smooth fibers with a 15–17 nm-diameter and globular domains of 25–28 nm-diameter (Fig. 1D). A major structural component of these extracellular lattices was DNA as demonstrated by high magnification fluorescence microscopy after live-cell SYTOX green staining of activated PMN on a parallel set of cover slips (Fig. 1D).

3.2. NETs can induce EC injury

After prolonged (18 h) co-culture of PMNs and activated EC, we noted that a large proportion of EC stained for both PKH26 and SYTOX green (Fig. 2A) indicating the occurrence of EC death during

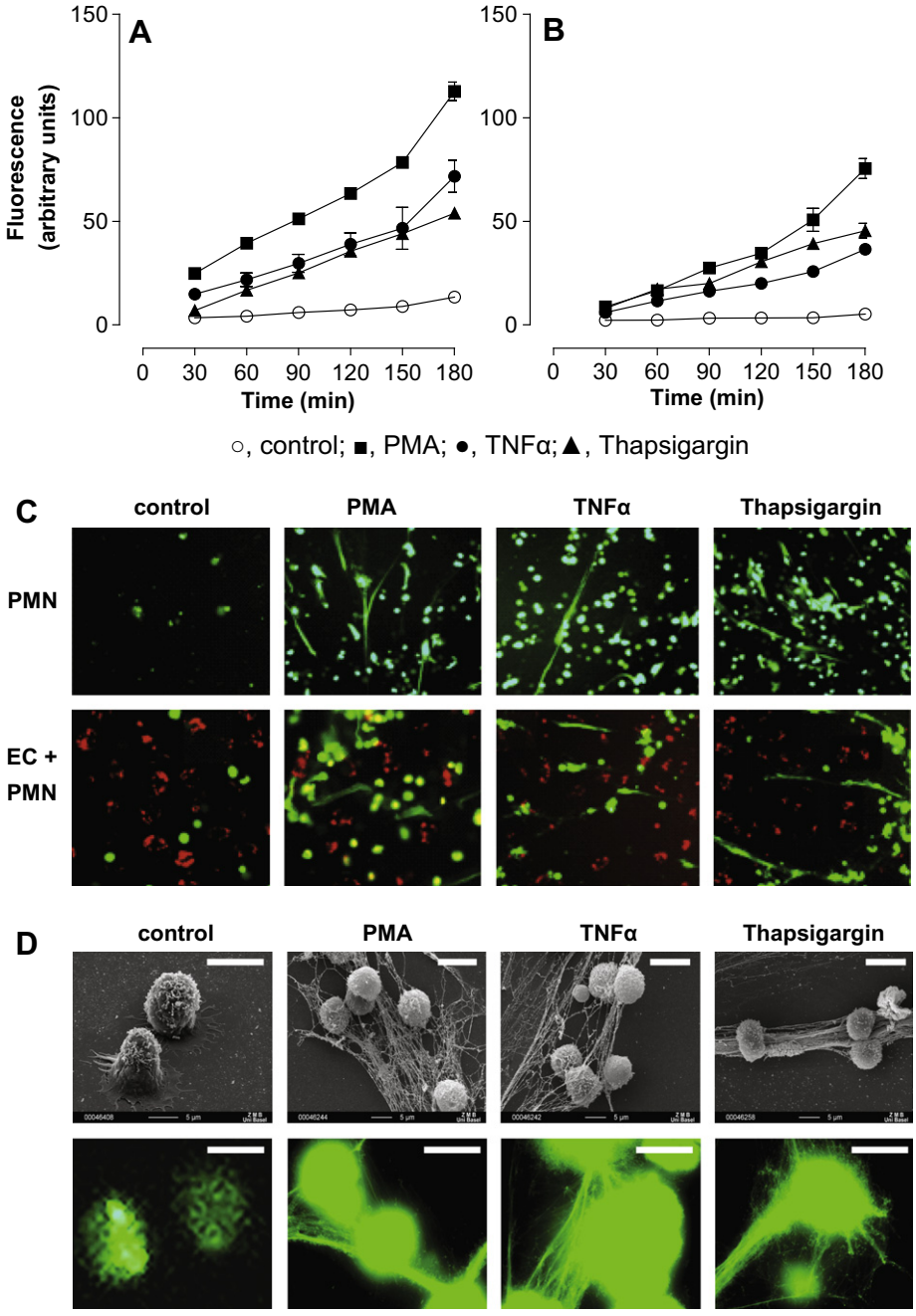

Fig. 1. Activated EC induce NETs. (A) PMN were incubated for up to 3 h in the absence (control) or presence of PMA (25 nM), TNFα (20 ng/ml) or thapsigargin (500 nM) during which induction of NETs was monitored by SYTOX green fluorimetry. (B) EC were incubated for 18 h in the absence (control) or presence of PMA, TNFα or thapsigargin and rinsed to remove all traces of activators. Then untreated PMN were added to EC ($t = 0$) and SYTOX green fluorescence in co-cultures was monitored for up to 3 h. (C) Fluorescence microscopy of NETs induced in PMN after 3 h of direct stimulation (top panel) or co-culture with activated EC (lower panel). NETs were visualized by SYTOX green staining (green). EC were pre-loaded with PKH26 (red). (D) High-resolution scanning electron microscopy of NETs induced by PMN following a 3 h period of stimulation (bars, 10 μm). SYTOX green staining shows that DNA is a major component of the structural lattice (bars, 15 μm).

extended co-culture. Such "co-staining" was negligible when activated EC and PMN were co-cultured for only 3 h (Fig. 1C). Interestingly, SYTOX green staining of EC during extended co-culture with PMN was prevented by inclusion of either DNAse, which destroys the DNA backbone of NETs [1]), or the NADPH oxidase inhibitor DPI, which hinders NETosis [6] (Fig. 2A). Thus, intact NETs in contact with EC for extended time periods evidently damage EC. A similar observation was made in sepsis and SVV [7,12].

In order to quantitate EC damage after co-culture with PMN we used BrdU-labelled EC and measured release of BrdU-labelled DNA fragments into co-culture supernatants by cellular DNA fragmentation ELISA. Release of BrdU-labelled DNA under short-term (3 h) culture conditions was negligible for both EC monolayer and EC–PMN co-cultures (data not shown). Under extended (18 h) culture conditions we detected significant damage to EC when PMNs were co-cultured with activated EC, but not with non-activated EC (Fig. 2B). The release of BrdU-labelled DNA fragments from EC co-cultured with PMN was almost entirely abolished by the inclusion of DNase and also largely reduced by the addition of DPI (Fig. 2B). Taken together these data support a PMN/NET-mediated mechanism of EC injury subsequent to priming of neutrophils by activated EC.

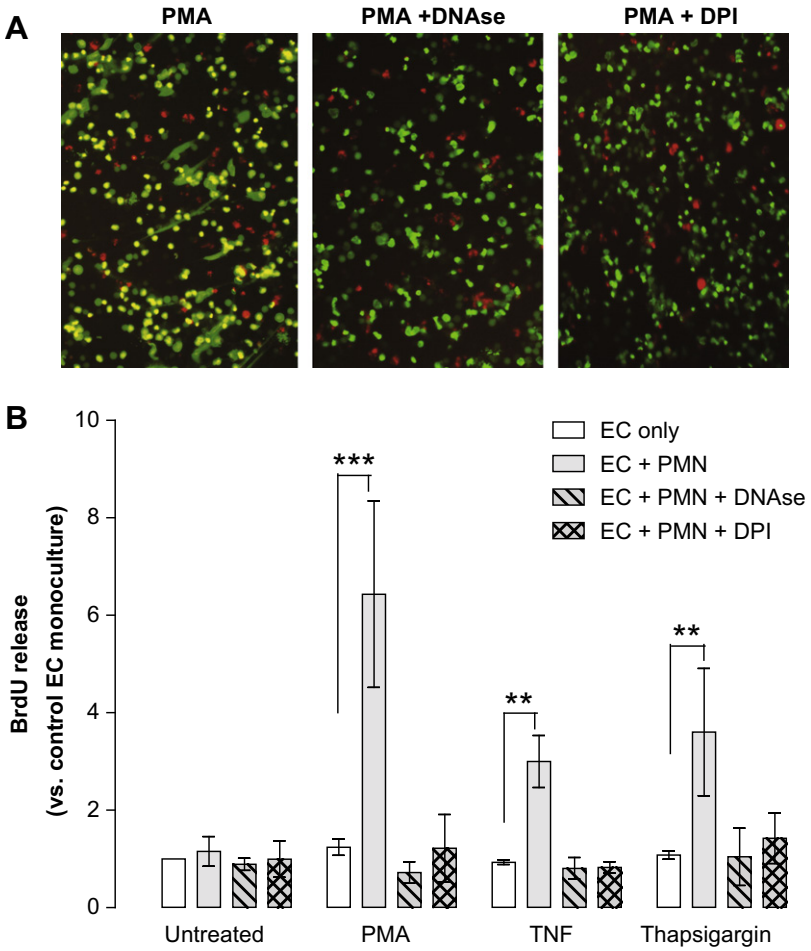

Fig. 2. NET-induced EC injury. (A) Fluorescence microscopy of NET-induced EC death and its hindrance by DNAse or DPI. PKH26-loaded EC (red) were treated with PMA for 18 h, rinsed well to remove all traces of PMA, and then co-cultured with PMN in the absence or presence of DNAse or DPI for 18 h. (B) NET-induced EC death. BrdU-labelled EC were treated with PMA, TNFα or thapsigargin for 18 h. After rinsing to remove all traces of activators EC were cultured without or with inclusion of PMN for a further 18 h, after which the release of BrdU-labelled DNA fragments from EC was analysed. Significant differences between EC monoculture and EC–PMN co-culture are indicated (**$P < 0.01$, ***$P < 0.001$).

NET-induced EC death appears to be akin to what has been observed for the anti-microbial activity of NETs, in as much as an intact DNA lattice structure is required [2,3,17]. The proposed containment of toxic PMN granular contents by NETs may not be sufficient to prevent cell damage by direct contact of NETs [18]. On the other hand, disruption of the DNA lattice could lead to a more widespread effect of released granular contents. This aspect awaits further examination.

3.3. Neutrophil/NETs-mediated EC injury is partially dependent on IL-8 produced by activated EC

Activated EC produce cytokines and adhesion molecules enabling adherence and transmigration of neutrophils. Since IL-8 is both produced by activated EC [19,20] and a potent inducer of NETs [1,6,8], we asked whether it plays a role in NETosis-mediated by activated EC. We determined whether EC continue to produce IL-8 during the 18 h period of co-culture following treatment with PMA, TNFα and thapsigargin [19,20]. A considerable production of IL-8 occurred in cultures of EC exposed to TNFα or thapsigargin (Fig. 3A), while production by EC exposed to PMA was low (Fig. 3A). Inclusion of DPI during the post-activation culture period abrogated the production of IL-8 by EC activated with TNFα or thapsigargin, but had little effect in PMA activated EC (Fig. 3A), indicating a role for NAPDH oxidase in the former cases but not the latter.

To determine the functional role of IL-8 in the EC–PMN interaction, an IL-8 neutralizing antibody or isotype control IgG was included throughout the period of co-culture of activated EC and PMNs. Anti-IL-8 antibodies reduced NET-induced EC death following activation with TNFα and thapsigargin (Fig. 3B), whereas only a minimal reduction was observed after activation with PMA (Fig. 3B). These data demonstrate an IL-8 mediated mechanism in PMN-mediated injury of EC after they were treated with thapsigargin or TNFα. The factors mediating NETosis by PMA activated EC still needs to be determined, but may involve cell-cell interaction *via* members of the ICAM family [21].

4. Conclusion

In conclusion, activated EC not only interact with PMN during transmigration [13,20–22], but may also induce NETosis and ensuing EC death. Since activated EC are present under conditions of inflammation [13,20], this interaction could escalate the inflammatory response. This could have severe consequences in conditions where large numbers of NETs have been found in vivo, such as in the intervillous space of preeclamptic placentae [8], or glomeruli in SVV affected patients [12]. To address these questions, it will be necessary to determine whether NETosis induced by activated EC occurs in vivo, whether it is *per se* associated with tissue damage, and what role scavenger cells such as macrophages play in

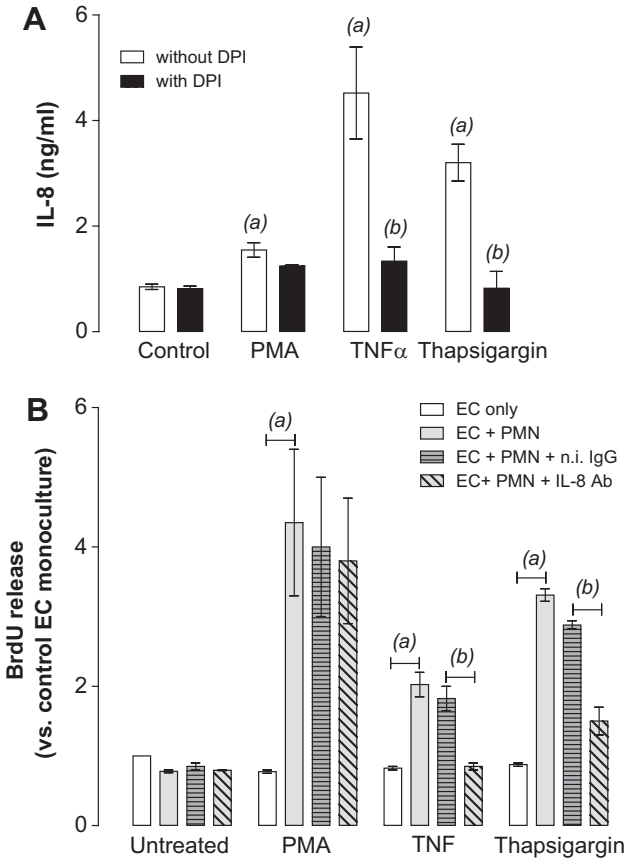

Fig. 3. IL-8 produced by activated EC and NET-induced EC death. (A) Production of IL-8 by activated EC. EC were activated with PMA, TNF or thapsigargin for 18 h in the absence or presence of DPI, rinsed extensively and cultured for a further 18 h, following which IL-8 levels in the supernatant were determined. *(a)* = Significantly increased accumulation compared with control, untreated EC (P at least < 0.05). *(b)* = Significant inhibitory effect of DPI (P at least <0.01). (B) BrdU-labelled EC were activated with PMA, TNF or thapsigargin for 18 h, rinsed extensively and cultured for a further 18 h without or with PMN and in the absence or presence of 100 μg/ml neutralizing anti-IL-8 antibodies or isotype non-immune (n.i.) IgG. Release of BrdU-labelled DNA fragments from EC was measured by ELISA. *(a)* = significant difference between EC monoculture and EC–PMN co-culture (P at least <0.001). *(b)* = significant inhibitory effect of anti-IL8 antibodies (P at least <0.001).

rapidly removing NETs to prevent damage to the underlying/surrounding tissue.

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