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Lipid alterations in human blood-derived neutrophils lead to formation of neutrophil extracellular traps

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ABSTRACT

The formation of neutrophil extracellular traps (NETs) as a host innate immune defence mechanism has been shown to be the result of a novel cell death process called NETosis. The objective of this study was to investigate the role of cholesterol in the formation of NETs. To this end, primary human neutrophils were treated with different concentrations of methy-β-cyclodetxrin (MβCD) to reduce cholesterol level in the cell. The formation of NETs was studied using immunofluorescence microscopy and Picogreen-quantification of released dsDNA. Neutrophils treated with MβCD showed a significant release of NETs in a process that is independent of NADPH-oxidase. The effect of MβCD on the lipid composition of the cells was determined using high performance thin layer chromatography (HPTLC). The identities of lipids separated by HPTLC were confirmed by mass spectrometry. Treatment of neutrophils with MβCD revealed distinct changes in the lipid composition: The percentage of cholesterol in the cell was significantly reduced; other lipids as sphingomyelin were only slightly affected. Interestingly, neutrophils treated with sphingomyelin-degrading sphingomyelinase also showed significant release of NETs. In conclusion, this study shows that lipid alterations facilitate formation of NETs.

Introduction

Neutrophil extracellular traps (NETs) have recently been described by Brinkmann et al. (2004) as a novel phagocytosis-independent host innate immune defence mechanism against microbes. Interestingly, Fuchs et al. (2007) reported that NET-releasing neutrophils have been shown to enter a cell death programme called NETosis which clearly differs from classical cell death pathways such as apoptosis or necrosis. During NETosis the nuclear and cellular membrane dissolve, and the components, such as chromatin decorated with granular proteins are released into the extracellular space as shown by Papayannopoulos and Zychlinsky (2009). Those extracellular NETs can then be used by the dying cell to entrap and kill various microbial pathogens as reviewed by von Köckritz-Blickwede and Nizet (2009).

Mechanisms leading to the release of NETs are still not completely understood. Importantly, Chow et al. (2010) found that statin treatment of primary blood-derived neutrophils induced the formation of NETs. Statins block the rate-limiting enzymes in the cholesterol biosynthesis through the inhibition of the HMG-CoA reductase. However, it remained unclear if this statin-mediated NET-formation is a result of direct cholesterol depletion in the cell or eventually only a secondary effect caused by statin-mediated transcriptional changes as reported for simvastatin-mediated changes in transcript expression of various cytokines in monocytes by Rezaie-Majd et al. (2002). The objective of the study was to investigate the effect of cholesterol depletion on the formation of NETs.

Materials and methods

Isolation of neutrophils

The blood of healthy human donors was used for the isolation of polymorphnuclear cells (PMNs). Therefore blood was layered onto Polymorphprep™ (Progen) without mixing. A centrifugation step with 30 min at 370 × *g* without brake was performed. The plasma and mononuclear cells were removed and the PMNs including neutrophils were washed with 1× PBS (PAA); contaminating erythrocytes were lysed using water (Roth, pure water, sterile,

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pyrogen free). Finally, the pellet was resuspended in 1 ml RPMI 1640 (PAA, without Phenol red, with L-glutamin).

Lipid extraction and preparation

To gain an insight into the effect of MβCD on the lipid composition of neutrophils, high performance thin layer chromatography (HPTLC) was performed. Therefore $1 * 10^5$/ml blood-derived human neutrophils were treated with three concentrations of MβCD (1 mM, 5 mM and 10 mM) or with 0.1 U/ml sphingomyelinase (SMase) for 2 h at 37 °C with 5% CO_2. Subsequently the cells were lysed using 1% SDS and a 26 G cannula, and prepared for TLC as described by Bligh and Dyer (1959). Briefly, 2 ml methanol and 1 ml chloroform were added to each sample. Then, the samples were rotated at room temperature for 30 min followed by centrifugation at 7 °C for 5 min at 1952 × *g*. The supernatant was transferred to a new glass tube and 1 ml chloroform and 1 ml water was added, mixed briefly and centrifuged again at 7 °C for 10 min at 17,000 × *g*. After centrifugation, the upper aqueous layer was removed and the remaining organic phase was vacuum dried and stored at −20 °C for further analysis.

Thin layer chromatography

Thin layer chromatography was performed as previously described by Brogden et al. (2014) with minor alterations. Lipids were resuspended in 150 μl chloroform/methanol (1:1). Polar and neutral lipids (Fig. 1B) were analysed using the following protocol: Five μl of the lipid extract was loaded onto 20 × 10 cm high performance thin layer chromatography (HPTLC) silica gel 60 plates from Merck (Germany). The HPTLC plates were developed using three solvent systems consecutively. Firstly, acetic acid ethyl ester/1-propanol/chloroform/methanol/0.25% potassium chloride (27:27:11:10), followed by n-hexane/diethyl ether/acetic acid (75:23:2), and finally n-hexane. The plates were then stained with phosphoric acid/copper sulphate solution (10:7.5) in water. Finally plates were heated at 170 °C for 10 min. The lipid bands were identified by comparison to authentic standard substances and analysed using the CP ATLAS software (Lazarsoftware). Lipid identification was confirmed by Electrospray Ionisation-Tandem Mass Spectrometry (ESI-MS/MS) using a TLC-MS Interface (CAMAG) coupled to the pump of a 1200 Series Binary LC System (Agilent) and to the Turbo V ESI source of a 4000 QTrap mass spectrometer (AB SCIEX). Lipid bands were extracted using methanol with 5 mM ammonium acetate at a flow rate of 0.1 ml/min. Fatty acyl subspecies were determined in the positive (SM, PC, PS, PE, DAG, cholesterol, CE) or the negative (PI, fatty acids) ion mode by specific precursor ion or neutral loss scans. Precursor ions of m/z 184 were monitored for the determination SM and PC subspecies, precursors of m/z 369 for cholesterol and CE subspecies, and precursors of m/z 241 for the analysis of PI subspecies. A neutral loss of 185 was selected for the determination of PS subspecies, a loss of 141 for PE subspecies, and a loss of 35 for the analysis of DAG subspecies. Fatty acids were analysed in the full scan mode. The instrument settings for nebuliser gas (Gas 1), turbogas (Gas 2), curtain gas, and collision gas were 50 psi, 55 psi, 20 psi, and medium, respectively. The interface heater was on, the ESI source temperature was 350 °C, and the ionspray voltage was 5.5 kV. For all scans the values for declustering potential, entrance potential, and cell exit potential were 80 V, 10 V, and 10 V, respectively. The collision energies ranged from 35 to 55 V.

NET induction

To examine the implication of membrane lipids in NET formation, cholesterol or sphingomyelin were depleted by using different concentrations of MβCD (Sigma, final 1 mM, 5 mM, and 10 mM) or with 0.1 U/ml SMase, respectively. As a positive control for NET release, PMA was used at a final concentration of 25 nM (Sigma). $2 * 10^5$/100 μl neutrophils were seeded into a 48-well-plate (Nunc), on poly-L-lysine (Sigma, 0.01%) coated glass slides. The plate was centrifuged for 5 min at 370 × *g* at room temperature, then incubated for 2 h at 37 °C with 5% CO_2 and fixed with 4% PFA. For the time kinetic experiment, the cells were treated with 10 mM of MβCD for 10, 30, 60, 90 and 120 min. Untreated neutrophils were used as a negative control. To test the role of NADPH-oxidases in NET-formation, neutrophils were incubated with 10 mM of MβCD or 25 nM PMA in the presence and absence of 10 μg/ml diphenylene iodonium (DPI, Sigma) for 2 h at 37 °C with 5% CO_2 to block NADPH-oxidase-dependent ROS-formation. Finally the cells were fixed with 4% PFA.

For live imaging of cell death, $5 * 10^5$/250 μl neutrophils were seeded into a 24-well glass bottom well plate and stimulated with 10 mM MβCD or with 0.1 U/ml SMase. RPMI was used as negative control. Plates were incubated at 37 °C and 5% CO_2. After 30 min of incubation, cells were centrifuged for 5 min at 370 × *g* and supernatant was removed. 150 μl of LIVE/DEAD® Viability/Cytotoxicity dye (Invitrogen) was added according to the manufacturer's recommendation for 15 min. Staining of mitochondrial DNA was done as previously described (Yousefi et al., 2009): Briefly, samples were treated with 0.5 μM Syto13 (Invitrogen) to label nucleic acids and 5 μM MitoSOX Red (Invitrogen) to stain mitochondrial DNA. Finally, samples were analysed using the Leica TCS SP5 confocal inverted-base fluorescence microscope with a HCX PL APO 63× 0.75–1.25 oil immersion objective.

Immunofluorescence microscopy

For visualisation of the NETs, glass slides were washed three times with PBS. Then cells were permeabilised and blocked using 2% BSA, 0.2% Triton X-100 in PBS for 45 min at room temperature. Next, neutrophils were incubated with a mouse monoclonal antibody against H2A–H2B–DNA complex (PL2-6, Losman et al., 1992, 2.65 mg/ml, diluted 1:2000 in PBS containing 2% BSA, 0.2% Triton X-100), a polyclonal antibody against MPO (rabbit anti-MPO, Dako, diluted 1:300 in PBS containing 2% BSA, 0.2% Triton X-100) or the respective isotype controls (mouse IgG2a or rabbit IgG in PBS containing 2% BSA, 0.2% Triton X-100) over night at 4 °C. Finally, an Alexa 488-conjugated goat-anti-mouse antibody (Dy Light 488 conjugated highly cross-absorbed, Thermo; diluted 1:1000 in PBS containing 2% BSA, 0.2% Triton X-100) or Alexa 633-conjugated goat-anti-rabbit antibody (Invitrogen, IgG (H+L), highly cross-adsorbed; diluted 1:1000 in PBS containing 2% BSA, 0.2% Triton X-100) were used as secondary antibody, and after washing the glass slides were embedded in ProLong® Gold antifade reagent with Dapi (Invitrogen). Samples were visualised using a Leica TCS SP5 confocal inverted-base fluorescence microscope with a HCX PL APO 40× 0.75–1.25 oil immersion objective. Settings were adjusted with control preparations using an isotype control antibody. For each sample, a minimum of 6 randomly selected images per independent experiment were acquired and used for quantification of NET-producing cells. Data were expressed as percentages of NET-forming cells in relation to the total number of cells.

Quantitative cell death measurement by LDH release

For the quantification of LDH release, supernatant of the cells from time kinetic experiments were collected prior to fixation. The supernatants were stored at −20 °C until usage. For the total cell lysate, 25 μl of 1% Triton X-100 was added to the well. Finally, 100 μl of cells and 100 μl CytoTox-ONE™ reagent (Promega) were incubated for 10 min at 22 °C on a 96-well plate. After incubation, 50 μl of stop solution was added and the fluorescence

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