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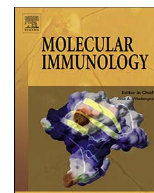

Morphometric characteristics of neutrophils stimulated by adhesion and hypochlorite

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ABSTRACT

The aim of this work was to compare cell form, size and volume as well as the locomotor activity of polymorphonuclear leukocytes (PMNLs) stimulated by adhesion to glass and exposed to hypochlorous acid at non-toxic dose. After 20 min of adhesion to a glass surface, volume, cell surface area and projection area of PMNLs were equaled to $143.1 \pm 21.4\ \mu m^3$, $288.8 \pm 28.8\ \mu m^2$ and $248.3 \pm 32.3\ \mu m^2$, respectively. Projection area of PMNLs exposed to NaOCl was noticeably enlarged as compared with control samples. The cell volume of 20 min adherent cells exposed to NaOCl was enlarged in comparison with both control cells and 5 min adhered exposed to NaOCl cells. NaOCl exposure induced a degranulation of PMNLs as measured by lysozyme release. Granules could be found both above the cell surface and on the substratum near the cell. The S/V ratio for PMNLs increased (from 1.52 to $2.02\ \mu m^{-1}$) with the increasing of cell activation time. But at NaOCl addition the reverse tendency was observed (from 2.10 to $1.87\ \mu m^{-1}$). In cells exposed to NaOCl the redistribution and decrease of concentration of F-actin took place. This observation supports the hypothesis that the priming of PMNLs with hypochlorous acid modifies cell motility and morphology and reflects also on other functions.

1. Introduction

Polymorphonuclear leukocytes (PMNLs or neutrophils) are rapidly recruited from peripheral blood to inflammatory sites, where they are able to recognize, phagocytose and destroy unwanted microorganisms (Edwards, 1994). During interaction with pathogens, these cells release numerous antimicrobial proteins and proteases pre-stored in granules into the phagolysosomes, and generate reactive oxidants (Babior et al., 2002; Nauseef, 2014; Robinson, 2008; Winterbourn and Kettle, 2013). These agents altogether contribute to inactivation of microbes. A further defense mechanism is the formation of extracellular traps (Brinkmann et al., 2004; Metzler et al., 2011).

The heme-containing protein myeloperoxidase (MPO) is only found in azurophilic granules of PMNLs and to a lesser extent in monocytes (Halliwell and Gutteridge, 1999). Upon activation of neutrophils, MPO is secreted into phagolysosomes and also released from the cells (Arnhold and Flemmig, 2010; Babior et al., 2002; Halliwell and Gutteridge, 1999; Metzler et al., 2011; Nauseef, 2014). Due to its high pK_a value, it easily associates with negatively charged surface epitopes, serum proteins and trap components (Arnhold and Flemmig, 2010). Among the chemical reactions catalyzed by MPO is the generation of the potent oxidant hypochlorous acid (HOCl) (Winterbourn and Kettle, 2013). This agent interacts with DNA, proteins, lipids, carbohydrates and other molecules (Arnhold and Flemmig, 2010; Hawkins and Davies, 2002; Hawkins et al., 2003; Pirillo et al., 2010). Cysteine and methionine residues of proteins are rapidly oxidized by HOCl to modify proteins function (Cook et al., 2012; Ronsein et al., 2014). With amines HOCl forms chloramines (Hawkins and Davies, 2002; Winterbourn and Kettle, 2000).

MPO and its product HOCl are also involved in regulatory processes at inflammatory sites. This protein binds to CD11β/CD18 integrins on PMNLs, leading to the induction of intracellular signaling cascades and as a result to up-regulated degranulation, integrin expression, and NADPH-oxidase activation in an autocrine manner (Lau et al., 2005). Low doses of HOCl are known to enhance the oxidative activity of PMNLs and augment cell aggregation (Zholnerevich and Semenkova, 2010a, 2010b). These mechanisms might play a role in the stepwise activation of novel recruited PMNLs to already existing inflammatory

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loci. These data indicate a priming effect of HOCl on neutrophil functions. However, it remains unclear to what extent HOCl affects the adhesive and morphological properties of PMNLs.

We addressed here the question which effects exhibit the incubation of PMNLs with HOCl on morphological cell properties and adhesion of cells to glass surfaces. To reveal and evaluate these alterations intravital light, fluorescent and atomic-force microscopy (AFM) and spectrophotometry have been applied with the aim of estimation of cell form, size and volume, locomotor activity, degranulation as well as F-actin distribution.

2. Material and methods

2.1. Chemicals

Earl's balanced salt solution (EBSS) containing 0.12 M NaCl, 5.4 mM KCl, 0.9 mM $NaH_2PO_4{\cdot}2H_2O$, 0.8 mM $MgSO_4{\cdot}7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2$, 26.2 mM $NaHCO_3$ (pH 7.4), phosphate buffer solution (PBS) containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 15 mM KH_2PO_4, (pH 7.4) and 0.15 M NaCl were freshly prepared. EBSS and PBS components, Wright-Giemsa stain were obtained from Analysis X Company, Minsk, Belarus.

Dextran, ficoll, verografin, NaOCl, glutaraldehyde, Triton X-100, paraformaldehyde, *Micrococcus lysodeikticus* were obtained from Sigma, St. Louis, USA. Alexa Fluor 532 Phalloidin was obtained from Thermo Fisher Scientific (Life Technologies), Oregon, USA.

2.2. PMNLs isolation and treatment

Neutrophils were isolated from heparinized peripheral blood of healthy donors (23 persons) after written consent by centrifugation (25 min, 400*g*) in a ficoll-verografin density gradient (Böyum, 1976). Remaining erythrocytes were eliminated by hemolytic shock during 20 s. Cells were twice washed with EBSS, suspended in it and counted in the Goryaev's chamber. 500 000 cells per ml were routinely applied for further investigations. The neutrophil number in the suspension was not less than 90%.

Four groups of samples were investigated: neutrophils adherent for 5 and 20 min to glass were in two control groups, neutrophils exposed to sodium hypochlorite simultaneously with adhesion (5 and 20 min) were in the other two groups.

A working solution of NaOCl in a concentration of 1.5 mM was prepared from commercial 5% NaOCl solution by diluting with 0.15 M NaCl and used immediately. 15 μl of working NaOCl solution was added to a 1.5 ml sample containing a neutrophil suspension (15 μl of 0.15 M NaCl was added to the control samples). The concentration of NaOCl to be added was calculated by a standard procedure from absorbance values measured at 292 nm ($\varepsilon_{292}(OCl^-) = 350\ M^{-1}cm^{-1}$) for NaOCl/NaOH solutions (100 mM NaOH, pH 12) (Morris, 1966).

The final non-toxic concentration of NaOCl was 15 μM. We showed earlier that NaOCl addition at 15 μM to PMNLs did not lead to LDH release from cells during 30 min (Zholnerevich and Semenkova, 2010a, 2010b).

2.3. Light microscopy

Intravital survey was carried out in 35 mm round dishes with freshly isolated neutrophils from five donors for 20 min using an Olympus VX-51WI light microscope (Olympus, Japan) with a water-immersion objective. During the survey neutrophils adhere to the surface, were activated and migrated along the dish bottom. Two groups of samples were investigated: neutrophils adherent 20 min to glass were in control group, neutrophils exposed to NaOCl in a final concentration of 15 μM simultaneously with adhesion (20 min) were in another group.

Freshly isolated neutrophils from five donors (500 000 cells per ml) in EBSS (with or without NaOCl) were allowed to adhere to glass surface for 20 min at 37 °C. Non-adherent cells were discarded by washing with PBS. Adhered cells were fixed in iced ethanol for 15 min, dried and stained by Wright-Giemsa stain for 10 min. Samples were rinsed three times with distilled water and analyzed using an Olympus VX-51WI light microscope (Olympus, Japan) with a water-immersion objective. The number of adhered neutrophils in samples were counted in 6 randomly selected fields of 20 Giemsa-stained samples at 40× magnification.

2.4. Atomic force microscopy

For atomic force microscopy sample preparation droplet of freshly isolated neutrophils (500 000 cells per ml) was placed on glass slide. The cells were allowed to adhere to glass surface for 5 or 20 min at 37 °C. Non-adherent cells were discarded by washing with EBSS. Adherent cells were fixed with 1% glutaraldehyde for 15 min, rinsed with EBSS and twice with distilled water, air-dried at room temperature thereafter. Twelve different neutrophil preparations from three donors were applied to get the data.

AFM-examination was made using atomic force microscope NT-206 (MicroTestMachines Co., Minsk, Belarus). Standard AFM tips CSC38 (MikroMasch, St.-Peterburg, Russia) were used. The microscope had a maximum horizontal scanning range of 50 × 50 μm and a vertical range of 3 μm. The imaging was carried out in air in contact scanning mode. The size of single neutrophils image was about 900 μm^2. Each image was collected in 250 × 250 pixels resolution. AFM height images of entire leukocytes were analyzed. Typical image consists of a three-dimensional map of the apical cell surface with a limited number of points, representing the cell height. Assuming that the basal cell membrane has a close contact with glass substrate a cell volume was estimated. The program for visualization, processing and analysis of scanning probe microscopy data SurfaceXplore 1.3.11 (MicroTestMachines Co., Minsk, Belarus) was used to calculate cell projection area, cell surface area and cell volume.

2.5. Fluorescent microscopy of neutrophils actin cytoskeleton

Freshly isolated neutrophils were allowed to adhere to glass surface for 5 or 20 min at 37 °C. Adherent cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature. Then glass coverslips with fixed cells were washed twice with PBS. For permeabilization cells were treated with 0.2% Triton X-100 in PBS for 10 min. Samples were washed twice with PBS and incubated with Alexa Fluor 532 Phalloidin at a final concentration of 6.6 μM for 30 min at room temperature in humidified atmosphere. After washing with PBS coverslips with fixed cells were rinsed with distilled water for 10 min. Confocal fluorescence analysis was performed using a Raman 3D-scanning confocal microscope NanoFinder High End (Tokyo Instruments, Japan – Lotis TII, Minsk, Belarus). Confocal images were processed with NanoFinder Data Viewer system software (Tokyo Instruments, Japan – Lotis TII, Minsk, Belarus) to calculate the integral fluorescence intensity of Alexa Fluor 532 in cells which corresponds the concentration of F-actin in cells. Twelve different neutrophil samples from three donors were applied to get the data.

2.6. PMNLs degranulation assay

Freshly isolated neutrophils were divided into two groups and allowed to adhere to glass surface for 20 min at 37 °C. In second group samples NaOCl was added in a final concentration of 15 μM at the beginning of adhesion. Then extracellular medium was separated by centrifugation (10 min, 600*g*). Lysozyme activity in the supernatant was assessed by the lysis of *Micrococcus lysodeikticus* monitoring by the decrease in absorbance at 450 nm for 5 min at 25 °C using a spectrophotometer PV1251(Solar, Minsk, Belarus) (Shugar, 1952; Styrt et al., 1985). Enzyme release was expressed as the percent of total enzyme

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