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Low concentrations of neutrophil extracellular traps induce proliferation in human keratinocytes via NF-kB activation

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ABSTRACT

Introduction: Granulocytes play a pivotal role in innate immune response, as pathogen invasion activates neutrophils, a subclass of granulocytes, inducing the production of neutrophil extracellular traps (NETs). In this study, it has been evaluated how NETs could affect human keratinocytes (HaCaT cells) behaviour.
Materials and methods: HaCaT cells were treated with increasing NETs concentrations (0.01–200 ng/ml) and the effect on cell proliferation was evaluated by MTT assay. Inhibition studies were performed by pre-treating cells with dexamethasone, chloropromazine or amiloride. NF-kB pathway activation was evaluated by western blot.
Results: HaCaT cells stimulation with increasing concentrations of NETs (0.01–50 ng/ml) for 48 h resulted in a modulation of cell proliferation with a maximum increase corresponding to 0.5–1 ng/ml stimulation. NETs low concentrations not only increased cell proliferation, but were also able to induce a faster wound closure in an in vitro scratch assay. NETs scaffold, composed by histone proteins and DNA, is recognized by Toll Like Receptor 9 (TLR 9) that, in turn, activates the NF-kB pathway. In fact, NETs induced proliferation was inhibited by chloropromazine (1 nM), that blocks chlatrin vesicles formation, and by amiloride (50 nM) that inhibits macropinocytosis. Moreover, dexamethasone, an inhibitor of NF-kB, was able to abolish the NETs effect.
Discussion: This study thus demonstrates that low NETs concentrations undergo internalization finally resulting in a quick NF-kB pathway activation and HaCaT cells proliferation increase, suggesting a close relationship between first immune response and wound healing onset.

1. Introduction

Wound healing is a multistep process including a series of events starting from the epithelium itself as well as the temporal recruitment of immune system cells into the wound bed [1].

When the integrity of the epidermal barrier is threatened, pathogens can enter and keratinocytes can recognize their presence through the Toll Like Receptor (TLR) pathway [2]. TLRs mediate the activation of both innate and adaptive immune responses by modulating gene expression. Following TLRs pathway activation, in fact, keratinocytes produce chemokines involved in inflammatory status.

TLRs are able to recognize different molecules including lipopolysaccharide (LPS/recognized by TLR4), peptidoglycan (TLR2), lipoteichoic acid (TLR2), double-stranded RNA (TLR3), flagellin (TLR5), yeast zymosan (TLR2/6) and CpG motifs in DNA (TLR9) [2].

TLRs activate a series of phosphorylation events resulting in the activation of various nuclear factors inducing specific gene transcription, thus playing a key role in the regulation of many aspects of cell physiology.

TLR9 is a cell surface receptor expressed by CpG-responsive cells, such as keratinocytes and it is known to enhance vesicular uptake of CpG DNA fragments [3]. The direct target of TLR9 stimulation is NF-kB, a transcription factor involved in cell migration, proliferation and apoptosis [4]. From a physiologic point of view, it is known that after an injury, the first healing step

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is represented by inflammation, recalling granulocytes at the wound site.

Granulocytes play a pivotal role in innate immune response, as pathogen invasion activates neutrophils, a subclass of granulocytes, inducing the production of neutrophil extracellular traps (NETs). NET production (NETosis) has been identified as an immune defence strategy and neutrophils undergoing NETosis die through a death mechanism recognized to be different from both apoptosis and necrosis [5–7]. During NETosis the plasma membrane rupture results in the release of the neutrophil nuclear content into the extracellular space. NETs scaffold is composed of decondensed chromatin fibres with a 15–17 nm diameter, decorated with DNA and granular as well as cytoplasmic proteins. NETosis is a very quick process, occurring in a few minutes when induced by bacterial lipopolysaccharide or a few hours when induced by phorbol myristate acetate (PMA), Staphylococcus aureus or Candida albicans stimuli. Even if the molecular mechanisms underlying NETs formation are still poorly understood, there are evidences suggesting both reactive oxygen species (ROS) and autophagy involvement [8–10].

Historically, neutrophils have been described as an important player in acute inflammatory response and were considered as short lived antibacterial effector cells. However, recent evidences suggest that neutrophils are quite versatile and can perform previously unsuspected functions, including reverse transmigration and the ability to cross-talk with and regulate other innate and adaptive immune leukocytes [11]. Moreover, the circulatory half-life of neutrophils is now thought to be longer than previously estimated (several days opposed to hours). Besides their antimicrobial and cytotoxic mechanisms of action, such as the release of reactive oxygen species, α-defensins, cathelicidin, elastase, cathepsin G, matrix metalloproteinases and NETs, neutrophils display a remarkable, de novo biosynthetic capacity for C-X-C and C-C chemokines, cytokines with proinflammatory, anti-inflammatory, or immunoregulatory properties as well as angiogenic and fibrogenic factors [12].

It is now well established that neutrophils are not solely involved in acute infections and inflammation but are also implicated in chronic inflammatory disorders or aging-related diseases, such as atherosclerosis, psoriasis, rheumatoid arthritis, inflammatory bowel disease, diabetes, and cancer [11–13]. In fact, excessive activation of neutrophils may lead to the development of multiple organ dysfunction syndrome, and lungs are the main target of this syndrome (acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS)) [14,15]. Although elevated amounts of NETs were observed in several pathophysiological conditions especially in vivo, to the best of our knowledge, the direct effect of low NETs concentrations on cells behaviour have not been investigated in vitro.

Ex vivo experiments demonstrated that neutrophils activation leading to NETosis induce nuclear and granular membrane dissolution, followed by nuclear content decondensation into the cytoplasm [16,17].

NETs can have a “bad” role when involved in occlusion of vessel and ducts as well as thrombus formation and acute inflammation. Moreover, in diabetes it has been demonstrated that hyperglycaemia induced NF-kB down regulation and NETs over production delay wound closure [4–20].

On the other hand, the “good” NETs are able to solve inflammation as demonstrated for gouty arthritis [8].

The aim of this study was to evaluate, in an in vitro cellular model, if low concentrations of NETs can influence human keratinocytes behaviour.

2. Materials and methods

2.1. Plasma and granulocytes separation

Human peripheral venous blood (40 ml) was obtained from 5 healthy donors (30–40 years) using Lithium/Heparin (Vacutainer, Becton Dickinson, Frankling Lakes, NJ, USA) as anticoagulant. Granulocytes were separated from whole blood using a modification of Boyum's method. Briefly, blood was diluted 1:1 with phosphate buffer (PBS, pH = 7.4) and centrifuged onto Lympholyte-H (Cedarline, Burlington, Canada) cell separation medium for 20 min at 2000 rpm. Erythrocytes were lysed using ammonium chloride lysis solution (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH = 7.4) for 20 min in ice until complete haemolysis. Isolated granulocytes were washed in sterile PBS, counted and suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium (Carlo Erba Reagents srl, Cornaredo, Italy).

2.2. NETs production

Granulocytes were incubated in the presence of PMA (Sigma Aldrich, Saint Louis, MO, USA) (100 nM) for 240 min in a humidified atmosphere containing 5% CO_2 at 37 °C to induce NETs release. PMA stimulation resulted in a 226.3 ± 139.5 μg/ml NETs production.

2.3. NETs quantification

NETs were quantified according to Vong and coworkers' protocol [21]. Briefly 1×10^5 cells/well were seeded in HBSS buffer in a 96 well plate and stimulated with 100 nM PMA (to activate NETs formation), Triton X-100 (to evaluate total DNA content) or left untreated (control and DNase digested samples). After 2 h 5U DNAse were added to all wells (excluding controls and Triton X-100 treated samples). 45 min after DNAse addition, Sytox Green dye (Thermo Fisher Scientific, Waltham, MA, USA) was added to each well and the plate was incubated for further 15 min. At the end of the incubation, Sytox Green fluorescence (λ_{ex}504 nm/λ_{em}523 nm) was quantified using a microplate reader (Victor X4, Perkin Elmer, Waltham, MA, USA). Extracellular DNA content was expressed as percentage of total DNA obtained by subtracting the fluorescence intensity of the DNase containing wells from the comparative control and dividing the obtained result by the fluorescence intensity of Triton X-100 containing wells.

2.4. Cell culture

Human spontaneously immortalized keratinocytes (HaCaT) [22] were purchased from Cell Lines Service GmbH (Eppelheim, Germany) and cultured in DMEM medium with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/l streptomycin (Euroclone, Milan, Italy) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.5. Cell treatment

Confluent cells were trypsinized, counted and 5×10^3 cells/well were seeded in a 96 multiwell culture plate and allowed to adhere overnight. Unadherent cells were removed by gentle wash in PBS before NETs stimulation. Cells were incubated for 48 h with NETs (0–200 ng/ml). NETs original solution was diluted in complete cell culture medium to the tested concentrations. At the end of the incubation time (48 h, corresponding to HaCaT cells duplication time) cell proliferation was evaluated by MTT assay.