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Neutrophil extracellular traps in patients with sepsis

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ABSTRACT

Background: Release of neutrophil extracellular traps (NETs) has been identified as an important aspect of innate immunity. We examined whether sepsis had any influence on *ex vivo* generation of NETs by neutrophils.

Materials and methods: We isolated neutrophils from consecutive patients with sepsis ($n = 17$) and without sepsis ($n = 18$) admitted to the intensive care unit. Neutrophils were activated by incubation with phorbol-12-myristate-13-acetate (PMA) to induce release of NETs, and NET formation was assessed by measuring the extracellular DNA level. Immunolabeling and fluorescence imaging were also performed. Extracellular killing of bacteria by NETs was studied by co-culture of *Escherichia coli* and neutrophils in the presence of a phagocytosis inhibitor. To assess *in vivo* NET formation, plasma levels of cell-free DNA and histones were measured.

Results: After stimulation with PMA, neutrophils isolated from septic patients released 4.08 ± 1.02% of their total DNA, whereas neutrophils from nonseptic patients released 29.06 ± 2.94% ($P = <0.0001$). Immunofluorescent staining of released DNA, elastase, and myeloperoxidase also revealed similar results. Neutrophils from nonseptic patients showed effective extracellular killing of *E coli* through NETs, whereas neutrophils from septic patients did not ($P < 0.001$). Plasma levels of cell-free DNA and histones were higher in septic patients than nonseptic patients ($P < 0.001$).

Conclusions: The *ex vivo* generation of NETs is downregulated in neutrophils isolated from patients with sepsis. However, it is unclear whether *in vivo* NET formation is also impaired during sepsis, so further investigation is necessary.

1. Introduction

Neutrophils are one of the important cellular components of innate immunity [1] because these cells are rapidly recruited to sites of infection and can eliminate pathogens by multiple methods. Neutrophils engulf microorganisms in a process called phagocytosis. Then the microorganisms are encapsulated in phagosomes and are killed by nicotinamide adenine

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dinucleotide phosphate oxidase-dependent mechanisms or by antibacterial granular proteins such as cathepsins, defensins, lactoferrin, and lysozyme [1].

In 2004, Brinkmann *et al.* [2] described a novel antimicrobial mechanism of neutrophils, reporting that stimulation caused the extrusion of a meshwork of chromatin fibers coated with antibacterial proteins such as elastase, cathepsin G, and myeloperoxidase (MPO). These structures are called neutrophil extracellular traps (NETs), and their role is to trap, immobilize, and kill invading microorganisms [2]. Formation of NETs is triggered by a variety of stimuli, including microorganisms, activated platelets, and proinflammatory cytokines such as interleukin (IL)-8 and tumor necrosis factor-α [2–4]. NETs can bind and kill both gram-negative and gram-positive bacteria, as well as fungi and parasites [2,5–7]. However, the detailed mechanism of NET formation is still unknown.

Sepsis is a deleterious host response to infection that can lead to organ failure and circulatory shock. It is a major health problem affecting millions of people worldwide each year with an overall mortality rate of 25% and its incidence is increasing [8]. Despite intensive research and much clinical effort, the mechanisms underlying the deleterious pathophysiological consequences of sepsis remain unclear. After the onset of sepsis, the processes of neutrophil adhesion to opsonized bacteria, phagocytosis, and bacterial killing by release of oxygen radicals or cytotoxic granular proteins into phagosomes have been variously reported to be impaired [9,10] or to be augmented [11,12].

In the present study, we explored the possibility that NET formation by neutrophils is altered in sepsis. The primary aim of this study was to investigate differences in the *ex vivo* generation of NETs by neutrophils from septic and nonseptic patients. We also examined the plasma levels of cell-free DNA (cf-DNA) and histones to assess *in vivo* NET formation.

2. Materials and methods

2.1. Patients

The subjects were patients admitted to the acute intensive care unit (ICU) of Fujita Health University Hospital from April 2012–September 2013. Approval for this study was obtained from the Ethics Review Board of Fujita Health University (#12-196). Written informed consent was obtained from each patient. Thirty-five patients aged between 18 and 80 y old were enrolled. Sepsis was defined by the presence of a systemic inflammatory response syndrome and microbiologically proven or clinically proven or suspected infection. Diagnosis of systemic inflammatory response syndrome required at least two of the following: temperature <36°C or >38°C; heart rate >90/min; respiration rate >20/min or arterial PCO_2 <32 mm Hg; and white blood cell (WBC) count >12,000/mm^3 or <4000/mm^3, or shift to the left of the differential WBC count with band forms ≥10% [13]. To evaluate organ dysfunction and the severity of illness, the sequential organ failure assessment score [14] and the acute physiological and chronic health evaluation score [15] were determined. All septic patients were managed according to the Surviving Sepsis Campaign protocol 2008 [8] including fluid resuscitation, administration of vasopressors, transfusion, and timely initiation of antibiotic therapy. Some patients also received hemofiltration.

2.2. Blood sampling and testing

Collection of blood samples from septic patients was performed within 24 h after the diagnosis of sepsis, whereas blood samples were obtained from nonseptic patients on the day of admission. Blood samples were obtained before or after the interventions and treatments. Venous blood was collected aseptically from each patient into heparinized pyrogen-free tubes to determine the WBC count and for isolation of neutrophils. Part of each blood sample was centrifuged at 1500*g* for 10 min at 4°C, and the plasma thus obtained was frozen at −80°C for assay of cytokines. Before measurement of histones and cf-DNA, plasma samples were again centrifuged at 16,000*g* for 10 min to remove any residual cells [16]. The WBC count and the neutrophil count were determined with an automated analyzer (XI-1800i, Sysmex Corp, Kobe, Japan). Peripheral blood smears were prepared immediately, stained with Leishman stain, and examined under a light microscope using an oil immersion lens at a magnification of ×1000. Neutrophil differentiation was assessed by counting at least 200 cells in each smear, with immature cells (promyelocytes, myelocytes, metamyelocytes, and band forms) being identified. The immature polymorphonuclear leukocyte (PMN) count and the immature-to-total PMN ratio were calculated [17].

2.3. Isolation of neutrophils

Neutrophils were isolated by discontinuous density gradient centrifugation on 1-step Polymorphs (Axis-Shield, Oslo, Norway). Purity of the neutrophil populations was checked by CD45 fluorescence combined with side scatter [18] and was found to be >98% in all experiments. The viability of the neutrophils was generally >95% as assessed by trypan blue dye exclusion.

2.4. Quantification of NET release by activated neutrophils

Quantification of NET release was performed according to the method of Fuchs *et al.* [3]. Briefly, freshly isolated neutrophils were resuspended at a final concentration of 5×10^5–10^6 cells/mL in RPMI-1640 medium (phenol red-free) containing 2 mM glutamine supplemented with 4% heat-inactivated fetal bovine serum. Then the neutrophils were seeded into tissue culture plates and stimulated with 25 nM phorbol-12-myristate-13-acetate (PMA) for 4 h at 37°C under a humidified atmosphere containing 5% CO_2. Next, the neutrophils were digested with 500 mU/mL micrococcal nuclease (Worthington Biochemical Corp, Lakewood, NJ) for 30 min, after which nuclease activity was stopped with 5 mM EDTA and the culture supernatant was collected for storage at 4°C until use [19]. Total DNA was extracted from untreated neutrophils using DNAzol (Life Technologies, Carlsbad, CA) supplemented with 1% polyacryl carrier (Molecular Research Center, Cincinnati, OH) and solubilized in 8 mM NaOH. cf-DNA from the