Immunobiology 216 (2011) 334–342

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.de/imbio

Stimulation of Fas signaling down-regulates activity of neutrophils from major trauma patients with SIRS

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ARTICLE INFO

Article history:
Received 8 March 2010
Received in revised form 1 July 2010
Accepted 1 July 2010

Keywords:
Caspases
Chemotaxis
Neutrophils
Oxidative burst
Phagocytosis
SIRS

ABSTRACT

Posttrauma apoptosis resistance of neutrophils (PMN) is related to overshooting immune responses, systemic inflammatory response syndrome (SIRS) and multiple organ failure (MOF). Recently, we have shown that the apoptosis resistance in circulating PMN from severely injured patients which is known to be mediated by high serum levels of pro-inflammatory cytokines can be overcome by the activation of Fas death receptor. Here, we aimed to study whether stimulation of surface Fas leads to the inactivation of hyperactivated PMN from critically ill patients with SIRS. PMN from 23 multiple trauma patients (mean injury severity score (ISS) 34 ± 1.9) were isolated at day 1 after admission to the trauma center. PMN from 17 volunteer blood donors served as controls. Neutrophil activity has been determined after *ex vivo* short (1 h) and long-term (4 h) stimulation of freshly isolated PMN with immobilized agonistic anti-Fas antibodies. We found neutrophil chemotactic migration in response to IL-8, phagocytosis and oxidative burst to be significantly inhibited in control cells already after short-term (1 h) Fas stimulation. In contrast, inactivation of trauma PMN by agonistic anti-Fas antibodies was found to be efficient only after long-term (4 h) incubation of cells with agonistic antibodies. Thus, in trauma PMN down-regulation of neutrophil activity seems to be delayed when compared to cells isolated from healthy controls, suggesting impaired susceptibility for Fas stimulation in these cells. Interestingly, whereas Fas-mediated inhibition of phagocytosis and oxidative burst could be prevented by the broad range caspase inhibitor t-butoxycarbonyl-aspartyl(O-methyl)-fluoromethyl ketone (BocD-fmk), the chemotactic activity in response to IL-8 was unaffected. In conclusion, we demonstrate that stimulation of neutrophil Fas does not only initiate apoptosis but also induces inhibition of neutrophil functions, partially by non-apoptotic signaling.

Introduction

Polymorphonuclear leukocytes (PMN) play a major role in the first line of defence and their amount increases rapidly during inflammation. Normally, the neutrophil half life in the circulation is limited to 8–10 h because aged PMN undergo spontaneous apoptosis in the absence of cytokines or other inflammatory agents, followed by their removal by macrophages (Edwards, 1994; Savill et al., 1998). However, in acute inflammation and after trauma, the spontaneous neutrophil apoptotic pathway is delayed by the action of local inflammatory mediators and PMN numbers within tissues can be extremely high. The extended neutrophil survival within tissues may result in persistent inflammation and tissue damage by secretion of cytotoxic molecules, such as reactive oxygen species (ROS) and proteases (Edwards, 1994; Edwards and Hallett, 1997). Excessive systemic PMN activation as seen after trauma is part of a systemic inflammatory response syndrome (SIRS). It is widely accepted that the increased cytotoxic potential of PMN is a sign of an uncontrolled inflammatory reaction, which causes damage to tissues and leads to early multiple organ failure (MOF) (Botha et al., 1995). In this context, it has been shown in experimental models that blocking or depletion of PMN results in a reduction of organ failure in the early phase after trauma (Fabian et al., 1994). Neutrophil death by apoptosis and safe removal

Abbreviations: AIS, abbreviated injury scale; ANOVA, one-way analysis of variance; BE, base excess; BocD-fmk, t-butoxycarbonyl-aspartyl(O-methyl)-fluoromethyl ketone; DHR, dihydrorhodamine; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell scan; FasL, Fas ligand; FADD, Fas-associated death domain protein; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; c-FLIP, cellular FLICE-inhibitory protein; GM-CSF, granulocyte macrophage-colony stimulating factor; ICU, intensive care unit; ISS, injury severity score; MFI, mean fluorescence intensity; MAPK, mitogen-activated protein kinase; MOF, multiple organ failure; MPO, myeloperoxidase; PBS, phosphate-buffered saline; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes, neutrophils; PMNE, neutrophil elastase; PU, polyurethane; RFU, relative fluorescence units; RLU, relative luminescence units; ROS, reactive oxygen species; SEM, standard error of the mean; SOFA, sequential organ failure assessment; SIRS, systemic inflammatory response syndrome.

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doi:10.1016/j.imbio.2010.07.005

by phagocytic cells thus helps to limit tissue damage during inflammation.

The Fas–FasL system represents a major pathway for the induction of apoptosis in cells and tissues (Peter and Krammer, 1998). Activation of the death receptor Fas induces the recruitment and activation of caspase-8, which in turns initiates apoptosis by the activation of downstream caspases, such as caspase-3 (Kischkel et al., 1995; Boatright et al., 2003; Pop et al., 2007). As it is well established that human PMN express functional Fas on their surface, this molecule has emerged as a critical pathway for the induction of neutrophil apoptosis, and may be involved in the regulation of acute inflammation (Iwai et al., 1994; Marsik et al., 2003). Previous work has stated that activation of the Fas receptor by soluble agonistic anti-Fas antibody (clone CH-11) leads to a strong stimulation of neutrophil apoptosis *in vitro* (Himpe et al., 2008). However, since Fas is expressed on many cell types in different organs, systemic application of anti-Fas revealed to have severe toxic effects, e.g. in lung and liver (Hagimoto et al., 1997; Ogasawara et al., 1993). Therefore, to avoid systemic application of anti-Fas antibodies the concept of challenging circulating PMN with an agonistic anti-Fas IgM antibody that is immobilized on an open porous biocompatible polyurethane (PU) foam (Fas+ foam) has been proposed (Scholz et al., 2004). The goal of the present study was to investigate whether Fas targeting might also be a useful strategy for the inhibition of neutrophil activity. We further questioned if down-regulation of cellular activity might be linked to the well known apoptotic process triggered by Fas ligation or might occur by non-apoptotic signaling pathways.

Materials and methods

Study population

Twenty-three primarily admitted patients (17 males, 6 females), mean age: 45.9 ± 3.5 years (range 18–80) were enrolled in this prospective study. Study approval was obtained from the Ethics Review Board of the University of Duesseldorf, Germany. Patients with blunt or penetrating multiple injuries who were admitted to our Trauma Center Level I during a 17-month period (June 2007–October 2008) with an injury severity score (ISS) greater 16 were enrolled in this study. Exclusion criteria were death of the patient on day of admission and patients withdrawing consent. In addition, patients with known preexisting immunological disorders or systemic immunosuppressive medication were excluded. The severity of injury was assessed by the ISS, based on the abbreviated injury scale (AIS; Greenspan et al., 1985). SIRS and sepsis were defined using the criteria outlined in the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference (Calandra and Cohen, 2005). Patients were determined as septic if they fulfilled criteria for SIRS and had a definite source of infection. SIRS was defined by two or more of the following criteria: temperature > 38 °C or < 36 °C; heart rate > 90 beats per minute; respiratory rate > 20 breaths per minute or arterial carbon dioxide tension ($PaCO_2$) < 32 mm Hg; and white blood cell count >12,000 cells/mm^3 or <4000 cells/mm^3, or with >10% immature (band) forms. Furthermore, in order to evaluate early organ dysfunction/failure, the sequential organ failure assessment (SOFA) score was determined (Marshall et al., 1995). Seventeen volunteers (10 males, 7 females) from the staff of the hospital aged between 26 and 49 years (mean age 33.1 ± 1.7) were used as controls. All were free of infection at the time of blood sampling.

Heparinized, citrated and serum blood samples (each 5–10 ml) were collected from healthy volunteers and from patients at day 1 (during the first 24 h, 8:00 to 9:00 a.m.) after admission to the trauma center. Heparinized blood was immediately used after collection for neutrophil isolation. In parallel, sera and plasma were harvested by centrifugation and stored at −80 °C until further processing.

Isolation and incubation of PMN

Human PMN were isolated by discontinuous density-gradient centrifugation on Percoll (Biochrom) as previously described (Maianski et al., 2002). Isolated PMN were suspended in RPMI 1640, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% autologous serum at a final concentration of 1×10^6/ml. Purity and viability were routinely above 95% as assessed by flow cytometry.

A volume of 400 μl of freshly prepared neutrophil suspension (1×10^6 cells/ml) was incubated with PU foams (1 cm^3) that carry immobilized agonistic anti-Fas IgM (clone CH-11) or with PU foams alone, without antibody (Leukocare, Munich). Control cells (without PU foams) were incubated in parallel under same conditions. In some experiments PMN were pre-cultured in medium containing 100 μM of the broad-spectrum caspase inhibitor t-butoxycarbonyl-aspartyl(O-methyl)-fluoromethyl ketone (BocD-fmk; Calbiochem) for 30 min at 37 °C.

For quantification of neutrophil apoptosis cells were incubated overnight (18 h) in 24-well cell culture plates at 37 °C in a humidified atmosphere containing 5% CO_2.

Flow cytometric analysis of the surface expression of Fas and FasL

The expression of Fas and FasL on PMN was determined as previously described (Paunel-Görgülü et al., 2009). Briefly, cells (1×10^6/100 μl) were incubated with mouse anti-human Fas IgM (clone CH-11; MBL, Woburn, MA) or mouse anti-human FasL IgG (BD Pharmingen) for 20 min on ice. Mouse IgM or IgG1 to *Aspergillus niger* glucose oxidase (Dako) were used as negative controls. After washing with PBS cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM or IgG (Dianova), respectively for further 20 min on ice protected from light. Finally, PMN were washed twice with PBS and analyzed by flow cytometry.

Neutrophil chemotaxis

Human neutrophil chemotaxis was measured in 12-well plates containing transwell inserts with 3 μm pore size (Transwell Permeable Supports, 3.0 μm Polycarbonate Membrane, Costar, Corning NY). Transwell inserts containing 100 μl (1×10^5) of cells were placed in wells containing RPMI 1640, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS and 25 ng/ml of IL-8 (R&D Systems) as chemoattractant. After incubation of plates for 60 min at 37 °C, the transwell inserts were removed and fluorescent CountBright counting beads (Invitrogen) were added to samples to quantify absolute cell numbers by flow cytometry. Chemotaxis of Fas−/Fas+ foams-treated cells is expressed as relative percentage of cells migrated in response to IL-8.

Neutrophil oxidative burst

To determine the percentage of PMN that produce reactive oxidants with or without Fas stimulation, the Phagoburst™ kit (Orpegen Pharma) was used according to the manufacturer's instructions with some modifications. In brief, a suspension of 100 μl was suspended in RPMI medium supplemented with 10% autologous serum and incubated for 20 min with phorbol 12-myristate 13-acetate (PMA) as high stimulus or without stimulus at 37 °C. The burst activity was determined by flow cytometry as the conversion of dihydrorhodamine 123 (DHR-123) to green fluorescent rhodamine 123 (R-123) by oxidative burst products. Results