Research Article

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Impaired intracellular signaling, myeloperoxidase release and bactericidal activity of neutrophils from patients with alcoholic cirrhosis ☆

Abdelali Boussif[1,2,3,4], Loïc Rolas[1,2,3], Emmanuel Weiss[1,2,3,5], Hamama Bouriche[6], Richard Moreau[1,2,3,7], Axel Périanin[1,2,3,*]

[1]*INSERM UMRS-1149, Faculté de Médecine X. Bichat, 75018 Paris, France;* [2]*CNRS ERL 8252, Centre de Recherche sur l'Inflammation, 75018 Paris, France;* [3]*Université Paris Diderot, Sorbonne Paris Cité, Laboratoire d'excellence INFLAMEX, 75018 Paris, France;* [4]*Université de Batna, Faculté des Sciences, Département de Biologie, Algeria;* [5]*Département d'Anesthésie Réanimation, Hôpital Beaujon, APHP, 92118 Clichy, France;* [6]*Laboratoire de Biochimie Appliquée, Département de Biochimie, Faculté des Sciences de la Nature et de Vie, Université Ferhat Abbas, Sétif 1, Algeria;* [7]*Département Hospitalo-Universitaire (DHU) Unity, Service d'Hépatologie, Hôpital Beaujon, APHP, 92118 Clichy, France*

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Background & Aims: Myeloperoxidase exocytosis and production of hydrogen peroxide via the neutrophil superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase contribute to efficient elimination of bacteria. Cirrhosis impairs immune functions and increases susceptibility to bacterial infection. We recently showed that neutrophils from patients with decompensated alcoholic cirrhosis exhibit a severe impairment of formylpeptide receptor (fPR)-mediated intracellular signaling and superoxide production. Here, we performed *ex vivo* studies with these patients' neutrophils to further investigate myeloperoxidase release, bactericidal capacity and signaling events following fPR stimulation by the formylpeptide formyl-met-leu-phe (fMLP).

Methods: Myeloperoxidase release was studied by measuring extracellular myeloperoxidase activity. Activation of signaling effectors was studied by Western blot and their respective contribution to myeloperoxidase release studied using pharmacological antagonists.

Results: fMLP-induced myeloperoxidase release was strongly impaired in patients' neutrophils whereas the intracellular myeloperoxidase stock was unaltered. The fMLP-induced phosphorylation of major signaling effectors, AKT, ERK1/2 and p38-MAP-Kinases, was also strongly deficient despite a similar expression of signaling effectors or fPR. However, based on effector inhibition in healthy neutrophils, AKT and p38-MAPK but not ERK1/2 upregulated fMLP-induced myeloperoxidase exocytosis. Interestingly, patients' neutrophils exhibited a defective bactericidal capacity that was reversed *ex vivo* by the TLR7/8 agonist CL097, through potentiation of the fMLP-induced AKT/p38-MAPK signaling axis and myeloperoxidase release.

Conclusions: We provide first evidence that neutrophils from patients with decompensated alcoholic cirrhosis exhibit a deficient AKT/p38-MAPK signaling, myeloperoxidase release and bactericidal activity, which can be reversed via TLR7/8 activation. These defects, together with the previously described severe deficient superoxide production, may increase cirrhotic patients' susceptibility to bacterial infections.

Introduction

Neutrophils play a key role in the elimination of invading microorganisms [1]. This innate defence function requires a fine coordination of two major neutrophil activities; the release of myeloperoxidase (MPO) from azurophilic granules (exocytosis) and production of reactive oxygen species (ROS) by the superoxide-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a phenomenon termed respiratory burst. MPO utilizes hydrogen peroxide (H_2O_2) derived from superoxide dismutation, and chloride to form hypochlorite and chloramine, which are toxic agents for bacteria [2,3]. MPO biological importance is further illustrated in the findings in *MPO*-knockout mice of increased infections by *Klebsiella* and *Candida*, increased mortality [4] and prolonged inflammation [5]. MPO release and ROS production are triggered by various pro-inflammatory mediators amongst which bacterial formylated peptides which also act as chemoattractants, thus alerting neutrophils in case of infection through stimulation of formylpeptide receptor (fPR), a G-protein

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* Corresponding author. Address: INSERM-1149, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, 75018 Paris, France. Tel.: +33 157277473; fax: +33 157277411.
E-mail address: axel.perianin@inserm.fr (A. Périanin).
Abbreviations: MPO, myeloperoxidase; fMLP, formyl-met-leu-phe; MAPK, mitogen-activated protein kinases; ERK1/2, Extracellular Signal-Regulated Kinases1/2; ROS, reactive oxygen species; fPR, formylpeptide receptor; TLR, Toll-like receptors.

coupled receptor [11]. Neutrophil antibacterial activities are tightly regulated by signaling events triggered via fPR including phospholipases, G-proteins, protein kinases such as protein kinase C (PKC), mitogen-activated protein kinases (MAPK) and mammalian target of rapamycin (mTOR) [6–8]. Signaling impairment under pathological situations or by drugs leads to neutrophil dysfunctions, which are detrimental to host-defence [9–11].

Alcoholic cirrhosis is a consequence of excessive alcohol consumption and represents a major cause of mortality worldwide with an estimated 3.8% of all global death [12]. This pathology combines different features of the liver disease including steatosis, inflammation, necrosis and fibrosis [13,14]. Cirrhosis is associated with immune dysfunctions and inability of host-defence systems to protect against infections [15]. Neutrophils contribute to the pathogenesis of cirrhosis through induction of liver injury in animal models (reviewed in [16]) as well in patients with alcoholic steatohepatitis [17]. Direct evidence for a role of neutrophils inducing liver injury was proposed by the observation of an intracellular oxidant stress in hepatocytes during neutrophil attack [18]. However, the view that neutrophils are systematically deleterious is not universally recognized. Indeed, Altamirano *et al.* showed that the higher neutrophil liver infiltration, the better the prognosis [19]. Neutrophils may exert their beneficial effects through production of hepatocyte growth factor [20], collagen degradation [21] or through granulopoiesis following G-CSF treatment [22]. Moreover, at least one study shows that G-CSF therapy is beneficial in patients with severe alcoholic hepatitis [23]. Finally, G-CSF therapy was found to improve survival in patients with acute-on-chronic liver failure, the most severe complication of cirrhosis [24]. Another common complication of cirrhosis is the development of sepsis, a major cause of death [12,25]. Although medical treatments exist to improve survival, about 35% of patients die within six months [26]. Of the numerous circulating host-defence mechanisms available, neutrophil production of ROS, microbicidal activity and phagocytosis are impaired in cirrhotic patients [27–35]. An impaired ROS production was also observed in liver transplanted recipients suffering from post-hepatitic cirrhosis [36]. However, the impact of alcoholic cirrhosis on receptor-mediated signaling events underlying neutrophil antibacterial activities remains largely unknown. We recently showed that neutrophils from patients with decompensated alcoholic cirrhosis, exhibit impaired signaling and ROS production induced by the bacterial tripeptide formyl-met-leu-phe (fMLP), which was further aggravated by the mTOR antagonist rapamycin [8]. This dysfunction was associated with a defective MAPK-dependent phosphorylation of p47phox, a major component of NADPH oxidase. Whether the defective signaling impacts other neutrophil defence activities is unknown.

In this study, we took advantage of these neutrophils from these cirrhotic patients to investigate possible alterations of MPO exocytosis induced by fMLP, and bactericidal activity. The state of activation of three major signaling effectors, AKT, p38-MAP-kinases and p44/42 MAP-kinases (ERK1/2), was also determined, and their respective contribution in MPO release was studied in healthy neutrophils using selective antagonists. Data reveal a severe deficient activation of the three signaling effectors in patients' neutrophils, impaired MPO release and bactericidal activity. Interestingly, these deficiencies can be reversed by a TLR7/8 agonist.

Patients and methods

Patients

Blood was obtained from patients hospitalized in the Liver Unit of Beaujon Hospital (Clichy, France). Inclusion criteria were age over 18 years, biopsy-proven cirrhosis, and Child-Pugh class B or C cirrhosis. Patients had a history of excessive alcohol ingestion (50 g/day), but no other causes of liver disease. Viral serologies for hepatitis B and C virus were negative. Alcohol consumption was stopped for at least 3 days. Clinical characteristics of patients are shown in Table 1. Patients with untreated or recently treated (less than one week) bacterial infection or gastrointestinal haemorrhage were not included. Cultures of ascites, urine and blood performed at the time of inclusion were all negative. Other exclusion criteria were treatment with corticosteroids, pentoxifylline or other immunosuppressive drugs in the past 30 days, and presence of hepatocellular carcinoma (HCC), other cancer, or human immunodeficiency virus infection. Healthy subjects (controls) were hospital employee volunteers or obtained from the blood bank (EFS, Paris, France). This study was approved by our institutional review board, and written informed consent was obtained from patients.

Materials and methods

Please see the Supplementary materials for details regarding neutrophil isolation [8], MPO exocytosis and assay [37], bactericidal activity [8], Western blot analyses of protein phosphorylation [8] and RNA quantification.

Statistical analysis

Unless otherwise stated, data represent means ± SEM. Statistically significant differences between means were identified using the Student's paired t test or Mann-Whitney U test, with a threshold of $p < 0.05$ and designated by *.

Results

Severe impairment of fMLP-induced MPO release and signaling in neutrophils from patients with advanced alcoholic cirrhosis

Stimulation of healthy neutrophils under optimal conditions by fMLP (1 μM) induced a weak release of MPO of approximately 10% of the total MPO content (Fig. 1A, C). Under these conditions, neutrophils from cirrhotic patients exhibited an impaired MPO release compared to that of healthy neutrophils. Optimizing the degranulation process by pretreating cells with the microtubule-disrupting agent cytochalasin B [37], potentiated fMLP-induced MPO release with the same efficacy, i.e. about 3–4 fold in both healthy and patients' neutrophils (Fig. 1B), suggesting no major alteration of the cytoskeleton mobilization efficiency. However, the fMLP-induced MPO exocytosis of neutrophils from cirrhotic patients, remained impaired relative to controls, while basal degranulation was not altered (Fig. 1B). To examine whether this deficient induction of MPO release was related to alteration of MPO intracellular stock, resting and stimulated cells were lysed, and MPO was quantified by measuring both MPO activity and expression by Western blot. In lysates of resting cells, the MPO activity was similar in both control and patients' neutrophils (Fig. 1C). In fMLP-stimulated cells, MPO activity decreased significantly in control neutrophils due to their MPO exocytosis (Fig. 1) but not in patients' neutrophils. MPO expression analyzed by Western blot in resting cells was not altered. Thus, the MPO intracellular pool was not impaired, which suggests that the deficient MPO release induced by fMLP in patients' neutrophils (Fig. 1A, B) may likely