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JMJD3 is involved in neutrophil membrane proteinase 3 overexpression during the hyperinflammatory response in early sepsis

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ABSTRACT

Excessive production of pro-inflammatory cytokines in early sepsis causes high early mortality rates. Membrane proteinase 3 (mPR3) expression on neutrophils plays a critical role in pro-inflammatory cytokine production. However, the mechanism underlying mPR3 overexpression in early sepsis is unknown. Here, we explored mPR3 expression in early sepsis and its regulatory mechanism. Thirty-two patients with sepsis and 20 healthy controls were prospectively enrolled. On day 1 after the onset of sepsis, mPR3 and jumonji domain-containing protein D3 (JMJD3) expression levels were measured in peripheral blood neutrophils. Lipopolysaccharide (LPS) was employed to induce JMJD3 expression in vitro, and GSK-J4 was used to inhibit JMJD3. Neutrophils were divided into four groups, control, LPS, LPS + GSK-J4, and GSK-J4, and cultured with THP-1 cells respectively. Plasma and culture supernatant cytokine levels were measured by enzyme-linked immunosorbent assays. Neutrophil mPR3 levels were significantly higher in patients with early sepsis than in healthy controls. Plasma cytokine (IL-1β and TNF-α) levels were increased in patients with sepsis exhibiting high mPR3 expression. Additionally, JMJD3 expression levels in neutrophils were increased in early sepsis. In vitro, both mPR3 on neutrophils and IL-1β in culture supernatants increased in response to LPS stimulation. Neutrophil mPR3 expression and IL-1β levels were significantly reduced by GSK-J4 in cells treated with LPS. IL-1β level was significantly higher in LPS-stimulated co-culture supernatants than in the corresponding individual cultured cells. Thus, our results suggest that JMJD3 contributes to the high expression of neutrophil mPR3, which promotes the production of proinflammatory IL-1β in early sepsis.

1. Introduction

Sepsis, a complex clinical syndrome, is one of the most frequent causes of mortality in intensive care units [1,2]. Sepsis was initially defined as an infection together with a systemic inflammatory response syndrome [3]. It has since been redefined as a life-threatening organ dysfunction resulting from a dysregulated host response to infection [4]. Despite this revised definition, the activation of inflammatory signaling still has a crucial role in immunological activity and contributes to the pathogenesis of sepsis [5]. The hyperinflammatory stage causes multiple organ failure, which is the leading cause of mortality in early sepsis [6,7].

The hyperinflammatory stage in early sepsis is characterized by excessive production of pro-inflammatory cytokines, including interleukin (IL)-1β and tumor necrosis factor-alpha (TNF-α), by various cell types, such as neutrophils, macrophages, and endothelial cells [8–11]. Neutrophils are first mobilized to the site of infection or injury, where they are indispensable for the acute phase of inflammation and the innate immune response. Previous studies considered neutrophils as terminally differentiated cells with an approximate circulation time of 6–8 h in humans [12]. Few studies have examined neutrophil dysfunction in early sepsis. However, emerging evidence suggests that neutrophils have a longer lifespan in the circulation (about 5 days) and can display reverse migration and reenter the circulation [13,14]. Neutrophils also modulate the function of other immune cells by direct interactions via the expression of membrane-associated proteins [15]. Therefore, it is important to explore neutrophil membrane-associated proteins in early sepsis.

Proteinase 3 (PR3) belongs to the family of neutrophil serine proteases; it has various biological activities, including the degradation of matrix proteins, antimicrobial action, and regulation of myeloid cell differentiation [16–18]. It is primarily stored within azurophilic

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granules of neutrophils and is externalized to the plasma membrane under inflammatory conditions [19]. Neutrophil membrane proteinase 3 (mPR3) has well-characterized proinflammatory properties and unique substrates, including precursors of pro-inflammatory cytokines produced predominantly by monocytes [20–22]. Despite many studies on the roles of mPR3 in inflammatory diseases [23–25], its role in early sepsis is not clear.

Furthermore, the mechanisms underlying the high expression of mPR3 on neutrophils are poorly understood. PR3 expression may be regulated by changes in cytosine methylation at the PR3 locus and developmental stage-specific expression of transcription factors [26,27]. It is reasonable to assume that epigenetic factors control neutrophil mPR3 expression in systemic vasculitis [28]. Several studies have demonstrated that jumonji domain-containing protein D3 (JMJD3), identified as a demethylase targeting the trimethylation of histone 3 at lysine 27 (H3K27me3), has important roles in the epigenetic regulation of genes involved in the enhancement of inflammatory responses [29–31]. GSK-J4 is a potent, selective inhibitor of JMJD3 and has been employed to evaluate the effects of inhibiting H3K27me3 demethylation on the phenotypes and biological functions of neutrophils [32].

Epigenetic regulation of gene transcription has been identified as an important mechanism regulating myeloid cell function, resulting in excessive inflammation in sepsis [33]. However, the mPR3 regulatory mechanism in early sepsis is still unknown. Thus, in this study, we examined the expression of neutrophil mPR3 and explored whether JMJD3 is involved in neutrophil mPR3 expression during the hyperinflammatory response in early sepsis.

2. Materials and methods

2.1. Study design

The patient group consisted of 32 patients who met the criteria for sepsis according to the Surviving Sepsis campaign definitions [3] from the intensive care unit of Ruijin Hospital, Shanghai Jiao Tong University (Shanghai, China) School of Medicine. All patients enrolled in the study were required to provide a blood sample within 24 h of the diagnosis of sepsis. The study exclusion criteria were patients aged < 18 years, > 24 h from sepsis diagnosis to blood collection, preexisting cancer or hematologic malignancy, inflammatory or metabolic disease such as Crohn's disease or diabetes, recent administration of an immunosuppressor or immunopotentiator, and patients with human immunodeficiency virus (HIV) or hepatitis B virus (HBV) infection. Twenty healthy volunteers, matched by sex and age, were enrolled in the control group. The following information was collected and recorded: demographic characteristics (age and sex), Acute Physiology and Chronic Health Evaluation II (APACHE II) score, temperature, heart rate, respiratory rate, white blood cell count, site of infection, microbiological findings, and outcome after 28 days (nonsurvival or survival).

2.2. Ethics statement

All patients and healthy volunteers provided informed consent before participation in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ruijin Hospital Ethics Committee, Shanghai Jiao Tong University School of Medicine, China (released on 25 February 2015).

2.3. Isolation of neutrophils from human peripheral blood

On the day of inclusion in this study, peripheral vein blood samples were collected from patients with sepsis and healthy donors. Heparinized blood was collected by venipuncture of one forearm vein under aseptic conditions, and all samples were processed within 1 h of collection. First, the whole blood was centrifuged at 3000 × *g* to separate plasma from blood cells, and the plasma was collected for subsequent tests. The blood cells were diluted with phosphate-buffered saline, and then neutrophils were separated by Polymorphprep (AXIS-SHIELD PoC AS, Oslo, Norway) after centrifugation at 500 × *g* for 30 min. Residual erythrocytes in the granulocyte cell pellet (after density centrifugation) were destroyed using ACK lysing buffer (Gibco, Life Technologies, Grand Island, NY, USA). Cell purity was determined by Giemsa/Wright staining (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Cell viability was > 99% for every cell preparation, as determined by trypan blue exclusion.

2.4. Cell culture and in vitro stimulation

THP-1 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were maintained at 37 °C under a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with penicillin G (10 U/mL), streptomycin (10 μg/mL), L-glutamine (2 mM), and 10% fetal bovine serum (FBS) (Gibco, Life Technologies). Freshly isolated neutrophils from healthy donors were resuspended in RPMI-1640 medium at a final concentration of 2×10^6 cells/mL. Neutrophils were stimulated with 100 ng/mL bacterial lipopolysaccharide (LPS; *Escherichia coli* 0111:B4; Sigma-Aldrich) alone or in combination with 30 μM GSK-J4 (Selleckchem, Houston, TX, USA) for 4 h at 37 °C under a 5% CO_2 atmosphere. GSK-J4 was added to the culture at the beginning of LPS treatment to inhibit JMJD3. GSK-J4 was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a stock concentration of 10 mM. In parallel, neutrophils were incubated with DMSO or GSK-J4 alone. For general co-incubation, neutrophils were incubated in the presence or absence of a four-fold lower quantity of THP-1 cells. After co-incubation, the supernatants and cells were analyzed for protein release and gene expression. All incubations were performed in duplicate.

2.5. Flow cytometry

After red cells were lysed with ACK lysing buffer, neutrophils were gated according to relative size (forward scatter) and relative granularity (side scatter). They were then stained with a fluorescein isothiocyanate (FITC)-labeled anti-PR3 antibody (Clone PR3G-2). Isotype-matched irrelevant antibodies (all from Abcam, Cambridge, UK) were used for control staining. The stained cells were analyzed using a FACSCalibur flow cytometer and CELLQUEST software (BD Biosciences, San Diego, CA, USA).

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from neutrophils using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. For cDNA synthesis, RNA (0.5 μg) was reverse transcribed using reverse transcriptase with random hexamers as primers (PrimeScript RT-PCR Kit; Takara, Kyoto, Japan). Real-time PCR was performed using the SYBR Green PCR Master Mix (Takara) and the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. *TBP* was used as an endogenous control to normalize *JMJD3* RNA levels [34]. All data were analyzed using the $2^{-\Delta\Delta CT}$ method and expressed as fold changes relative to reference control samples. The primer sequences used are listed in Table 1.

2.7. Cytokine measurements

The concentrations of IL-1β and TNF-α in the plasma of patients and healthy controls and co-cultured cell supernatants were estimated in duplicate using commercially available ELISA kits (Anogen, Mississauga, Canada). The lower detection limits were 0.008 pg/mL for IL-1β and 4.0 pg/mL for TNF-α.