Increased myeloperoxidase enzyme activity in plasma is an indicator of inflammation and onset of sepsis

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Abstract

Introduction: Circulating lipopolysaccharides released from bacteria may activate both neutrophils and monocytes. The activated neutrophils release myeloperoxidase (MPO), a specific enzyme with strong oxidative activity. The aim of this study was to evaluate MPO enzyme activity in plasma of critically ill patients and to check the hypothesis that these concentrations in plasma would be higher in sepsis and systemic inflammatory conditions, as neutrophils release their contents before proliferating in response to stress.

Material and Methods: Blood samples were collected from 105 critically ill patients admitted to the intensive care unit, consisting of those with systemic inflammatory response syndrome (n = 42), sepsis (n = 37), and septic shock (n = 26). Plasma MPO enzyme activity was determined by o-dianisidine-H₂O₂ method, modified for 96-well plates.

Results: The plasma MPO enzyme activity in sepsis patients was significantly higher than that in the control group (mean, 2.4 ± 1.8 in sepsis and 1.86 ± 1.2 nmol per milligram protein per 10 minutes in systemic inflammatory response syndrome vs 0.32 ± 0.11 nmol per milligram protein per 10 minutes in healthy controls). Mean plasma lactate levels in sepsis (7.8 ± 1.2 mmol/L) and shock patients (9.5 ± 1.2 mmol/L) and cytokines like tumor necrosis factor–α, interleukin-8, and interleukin-1β were simultaneously evaluated to establish onset of inflammation and sepsis. These results show that neutrophil activation occurring during inflammation and sepsis could be detected by plasma MPO concentration.

Conclusion: The plasma MPO concentrations may be a marker of the neutrophil proliferation and severity of inflammation.

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1. Introduction

Various functions of neutrophils have been described in sepsis [1], including adherence, chemotaxis, degranulation, phagocytosis, and production of reactive oxygen intermediates [2-5]. In sepsis, the expected and appropriate inflammatory response to an infectious process becomes amplified, leading to organ dysfunction or risk for secondary infection [6]. As a clinical syndrome, sepsis occurs when an infection is associated with the systemic inflammatory response syndrome (SIRS). A continuum exists from a low-grade systemic response associated with a self-limited
infection to a marked systemic response with solitary or multiorgan dysfunction, that is, severe sepsis. Myeloperoxidase (MPO) is a heme enzyme of neutrophils azurophilic granules with a strong oxidative activity [7]. Myeloperoxidase present in the primary granules of polymorphonuclear leukocytes (PMNs) has been demonstrated to participate in the microbicidal activity of these cells. Together with the membranous NADPH oxidase, MPO is involved in the formation of reactive oxygen species (ROS) and oxidation of biological material [8,9]. In stimulated PMNs, NADPH oxidase reduces molecular oxygen to superoxide anion radical. This species and its dismutation product hydrogen peroxide are substrates for MPO. Myeloperoxidase has also been suggested to regulate the respiratory activity of PMNs during phagocytosis. During phagocytosis, PMNs undergo a burst in respiration; and through a series of single electron transfer, oxygen is reduced to superoxide anion (SO2) and hydrogen peroxide (H2O2) [10]. Although H2O2 alone is microbicidal, its bactericidal activity is greatly potentiated by the heme enzyme. Myeloperoxidase constitutes 5% of the human PMN by weight and is believed to represent a major pathway for O2-dependent microbicidal activity. The stimulation of PMNs results in a sudden increase in oxygen consumption, with the production of ROS and the release of enzymes such as elastase and MPO. In addition to its peroxidase activity, MPO catalyzes H2O2 and chloride anion (Cl−) reaction, forming hypochlorous acid (HOCl) [11]. Hypochlorous acid is a powerful oxidant and reacts with amines to form chloramines, which contribute to bacterial destruction inside the phagolysosome of the neutrophils [12]. Increased plasma MPO levels are a marker of neutrophil proliferation and degranulation in humans. This work was planned to test the hypothesis that patients with SIRS and sepsis would have neutrophils activation resulting in higher MPO enzyme activity in plasma.

2. Material and methods

This study was carried out to assess MPO levels in plasma of 105 critically ill patients admitted to the intensive care unit (ICU) and managed following Surviving Sepsis guidelines [13]. Human ethical approval was granted by the institutional review board. Informed consents were obtained from control subjects and patients or their relatives. The control group was composed of the healthy relatives accompanying the patient. A total of 80 controls were taken; among them, 55 were men and 25 were women, with a mean (SD) age of 38.5 (8) years. Among the patient group, 67 were men and 38 were women, with a mean (SD) age of 37.4 (6) years (Table 1). The inclusion criteria were the following: (1) clinical evidence of infection, (2) hyperthermia (temperature >38°C) or hypothermia (temperature <35°C), (3) tachycardia (>100 beats per minute), (4) tachypnea (>30 breaths per minute) or need for mechanical ventilation, and (5) evidence of inadequate organ function or perfusion within 12 hours of enrollment. The exclusion criteria were the following: (1) patients older than 80 years, (2) cardiac failure (class III or IV), (3) liver insufficiency (Child C), and (4) immunosuppression (positive HIV, HBs Ag virus serologic result, cancer). Patients were followed up throughout their stays in the ICU. Age, sex, primary site of infection, infection-related organisms, and severity indexes including Acute Physiology and Chronic Health Evaluation Scores (APACHE II) and Sequential Organ system Failure Assessment score (SOFA) were recorded for each patient’s entry into the ICU. The plasma of these patients was tested for MPO levels at the time of study entry and then after every 24 hours until their stay in ICU. Besides MPO, plasma levels of tumor necrosis factor (TNF)−α, interleukin (IL)-1β, and IL-8 as markers of inflammation were also assessed using standard enzyme-linked immunosorbent assay (ELISA) technique. Blood lactate level was assessed for detection of metabolic acidosis due to inadequate tissue perfusion suggesting anaerobic metabolism in tissues. All the samples and information used in the study were coded, and patient confidentiality was preserved according to the guidelines for studies of human subjects.

2.1. Blood sample collection

First blood sampling was performed before antimicrobial, adrenergic, or steroid therapy. Blood samples were collected from central venous catheter (9 mL) into tubes containing
1 mL trisodium citrate upon admission of patient to ICU and subsequently. Plasma was separated by centrifuge at 13000 rpm for 15 minutes. The plasma was stored at −70°C for assessment of MPO enzyme activity, along with plasma TNF-α, IL-1β, and IL-8 levels. Repeated freeze thaw of samples was avoided to prevent degradation of plasma cytokines levels. Plasma levels of TNF-α, IL-1β, and IL-8 were assessed using standard ELISA technique.

2.2. MPO assay

Myeloperoxidase enzyme activity was determined by o-dianisidine-H2O2 method, modified for 96-well plates [14]. Briefly, plasma samples (20 μL) were added to 0.53 mmol/L o-dianisidine dihydrochloride Sigma-Aldrich, USA. and 0.15 mmol/L H2O2 in 50 mmol/L potassium phosphate buffer (pH 6.0). After incubation for 10 minutes at room temperature, the change in absorbance was measured at 460 nm (ε = 10 062/M/cm). Results were expressed as units of MPO per milligram protein per 10 minutes, whereby 1 unit of MPO was defined as the amount of enzyme degrading 1 nmol H2O2 per minute at 37°C [15].

2.3. Statistical analysis

All data were obtained in duplicate, and results of calculations are reported as means and standard deviation up to 2 decimal points. The data were analyzed by Bartlett test for nonparametric analysis of variance with Newman-Keuls multiple comparison posttest. The relation between plasma MPO value and total leukocyte count (TLC) was tested by determining the Pearson correlation coefficient (r). A P value < .05 was considered significant. All statistical analyses were performed with the Graph Pad InStat 5.0 demo program (Graph Pad Software, San Diego, CA).

3. Results

Among 105 patients admitted to the ICU during the period April 2009 to May 2010, 42 patients had SIRS, 37 patients were in sepsis, 12 patients were in a state of septic shock, and 14 patients were initially in the SIRS group but later developed sepsis. Sepsis was diagnosed on the basis of metabolic acidosis and specific culture reports from various possible sites of infection, including blood culture report. There were 67 male and 38 female patients, with a mean (SD) age of 37.4 (6) years (Table 1). We monitored the enzymatic activity of MPO in plasma as a marker of inflammation and sepsis. Other standard markers like TNF-α, IL-1β, and IL-8 were estimated for detecting onset of inflammatory activity. The MPO specific activity, expressed as nanomoles of H2O2 degraded per milligram protein per 10 minutes (Fig. 1), was increased 4- to 6-fold in plasma samples of SIRS and sepsis patients as compared with that of healthy controls (mean, 2.4 ± 1.8 in sepsis and 1.86 ± 1.2 in SIRS vs 0.32 ± 0.11 in healthy controls; range, 1.2-6.2 in sepsis and 0.14-4.3 in SIRS). Pearson correlation analysis identified a positive correlation between an increase in MPO specific activity and severity of sepsis assessed by SOFA and APACHE II scores recorded at the time of admission in ICU (Fig. 2; r² = 0.82, P < .01). In the SIRS group, MPO enzyme activity increased with severity of inflammation, showing a positive correlation (r² = 0.62, P < .01), whereas in patients of septic shock, no such correlation was observed (r² = 0.021, P = .45), indicating reduced MPO activity in severe sepsis. In state of septic shock, MPO activity was only 0.8 times increased, which was then correlated with reduced neutrophil count in cases of advanced sepsis (Fig. 3; mean, 0.6 ± 0.1; range, 0.1 to 2.5 nm per milligram protein per 10 minutes). This was then confirmed by reduced TLCs in patients of advanced sepsis due to bone marrow suppression.

The MPO enzyme activity was then compared with TLC, determined by automated cell counter. The overall increase in MPO activity in sepsis patients was associated with a corresponding increase in TLC (Fig. 3; Pearson correlation coefficient: r = 0.9, P < .001 for sepsis and r = 0.86, P < .01 for SIRS), whereas in patients of shock, cell count decreased with the progression of disease; thus, low MPO activity was observed.

Detection of metabolic acidosis in arterial blood gas analysis was evaluated further by blood lactate estimation (Fig. 5). Blood lactate levels were increased in the SIRS group (5.2 ± 1.1 mmol/L) with range from 4.9 to 5.5mmol/L. Similarly, blood lactate levels in sepsis (7.8 ± 1.2 mmol/L) and septic shock group (9.5 ± 1.2 mmol/L) were significantly high (95% confidence interval [CI]: sepsis, 7.4-8.2; 8.9-10.1 mmol/L). In the control group, mean values were 0.93 ± 0.3 mmol/L (95% CI, 0.8-1.0). Plasma cytokine levels evaluated by ELISA were also elevated in SIRS, sepsis, and septic shock patients (Fig. 4). In SIRS, mean (SE) value of TNF-α, IL-8, and IL-1β were 77.99 (14.6), 68.25 (27.4), and...
99.52 (14.3) pg/mL, respectively; in sepsis, mean (SE) values were 187.1 (88.3), 225.9 (56), and 175.8 (10.7) pg/mL, respectively; in septic shock, mean (SE) values were 107 (15.03), 252.9 (74.14), and 320.2 (33.06) pg/mL, respectively. It was observed that the difference in plasma values of cytokines between control, sepsis, and septic shock groups was statistically significant ($P < .05$).

4. Discussion

Polymorphonuclear leukocytes are the first cell type in human beings that is activated in host immune defense against infection [16]. These cells driven by chemotactic gradients migrate to inflammatory loci, where they recognize and phagocytose bacteria and other extrinsic microorganisms by release of hydrolytic enzymes and bactericidal proteins prestored in granules as well as newly generated ROS [17,18]. Vascular leakage and recruitment of circulating PMNs to the site of injury represent the early phase of the host defense mechanism and response to tissue injury or sepsis. This response is common to all organs and tissues. Clinically, the increased number of PMNs in blood is generally used to determine the development of inflammation/sepsis [19]. A more feasible and quantitative approach is the use of the biochemical assay of PMNs-associated MPO enzyme activity. This enzyme is highly
enriched in the azurophilic granules of PMNs recruited to injured tissue to mediate the acute phase of the inflammatory response [20].

In this study, we report the association between severity of inflammation and plasma MPO enzyme activity. Neutrophils are considered major contributors to the tissue damage that occurs in inflammatory diseases. Myeloperoxidase, a major granule enzyme in neutrophils, accounts for 5% of the total neutrophil protein and is responsible for the production of HOCl oxidant. Activated neutrophils produce ROS (O₂⁻ and H₂O₂) via NADPH oxidase as part of their antipathogen response [21-23]. The release of ROS and HOCl by neutrophils may cause damage to important biological structures such as proteins, carbohydrates, lipids, and nucleic acids and may enhance inflammatory responses.

Development of sepsis and inflammatory response in patients was further assessed by detecting markers of inflammation (TNF-α, IL-1β, and IL-8) in patient blood samples [24]. These inflammatory cytokines significantly increased in SIRS, sepsis, and septic shock groups (Fig. 4), thus establishing the episodes of inflammation [25]. However, increased lactate levels, as a marker of tissue hypoxia in critically ill patients, remain a matter of debate. Given the many processes that may affect the ultimate concentration of lactate, both individual lactate levels and the change in level over time may well reflect the general homeostasis of the critically ill patient [26]. In our study groups, blood lactate levels were found to be significantly raised in sepsis and septic shock patients (Fig. 5). Our data in this study validate the observations that MPO activity and cell count are increased in patients with SIRS and sepsis (Fig. 3). The finding of a positive correlation between the increase in MPO activity and TLC suggests that trauma- or bacteria-induced inflammatory or infective responses are pathological and that patient with sepsis sustain MPO-dependent protein oxidative damage.
Myeloperoxidase activity was expected to increase with the progression of sepsis and development of septic shock, but it was constantly observed to be on the lower side in patients with advanced stages of septic shock [27]. Because MPO enzyme activity reflects the neutrophil function, blood levels of neutrophils were then evaluated to look for TLC [28,29]. Neutrophil count was found to be low in shock patients; further evaluation revealed pancytopenia in these patients due to bone marrow suppression in advanced stages of septic shock [30]. Evaluation of plasma MPO activity in sepsis and septic shock patients could help us in assessing neutrophil status and its functioning; our data further point toward the use of plasma levels of MPO enzyme activity as biomarkers of inflammatory oxidative pathology.

5. Conclusion

The study reported here reveals that neutrophil activation occurs in a high percentage during oxidative stress in critically ill patients, especially during SIRS and sepsis; these cells proliferate during stress, and MPO is released into blood. Owing to the high variability of MPO levels, we could not establish a cutoff point in MPO enzyme activity levels to distinguish survivors from nonsurvivors. In summary, we demonstrate that plasma MPO enzyme activity is a good biomarker of inflammatory responses in patients with SIRS and sepsis.

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