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The Role of Neutrophil Myeloperoxidase in the Development of Inflammation Induced by Thermal Skin Burns

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Abstract—Luminol-dependent chemiluminescence (CL) of blood neutrophils stimulated with phorbol-12-myristate-13-acetate (PMA) and myeloperoxidase (MPO) activity of neutrophils and plasma have been investigated in children ($n = 16$) during the early period (1–7 days) after thermal skin burns exceeding 20% of total body surface. The CL level of stimulated neutrophils was higher in burn patients than in healthy children of the reference group ($p < 0.01$). Increased neutrophil MPO activity was found in 40% of patients, while increased plasma MPO activity was detected in 57% of patients. The albumin fraction isolated from plasma of burn patients increased the PMA-stimulated CL response of blood from healthy donors. These results suggest that the acute inflammatory response to the thermal burn causes neutrophil activation and MPO release into plasma. MPO-mediated modification of plasma proteins, particularly albumin, may stimulate neutrophil activation and provoke further inflammatory response of the body to the thermal injury.

Keywords: burns, chemiluminescence, neutrophils, myeloperoxidase, albumin

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INTRODUCTION

Thermal injury affecting ≥15% of total body surface area (TBSA) induces the systemic inflammatory response syndrome with a sharp increase in blood leukocyte chemiluminescence (CL) [1]. This suggests leukocyte activation, which involves cytokines such as tumor necrosis factor (TNFα), interferon (IFN), interleukin-8 (IL-8), granulocyte-colony stimulating factor (G-CSF) [2]. Infiltration of activated neutrophils into the mucosa (particularly, in intestinal and respiratory tract) is a risk factor for mucosal injury and the development of multiple organ failure [3]. Degranulation of activated neutrophils causes release of various bactericidal agents, including myeloperoxidase (MPO) [4]. MPO catalyzes oxidation of halides to corresponding hypohalous acids, mainly HOCl and HOBr. These acids are strong oxidants capable of modifying the structure and function of many biologically important molecules, including proteins [5]. For example, human serum albumin (HSA) treated with HOCl or HOBr increased neutrophil activity in vitro [6, 7].

The aim of this study was to investigate the interrelationship between MPO activity (in neutrophil and plasma) and blood neutrophil capacity to produce reactive oxygen and chlorine species evaluated by means of the CL method in children with thermal burns of the skin.

MATERIALS AND METHODS

Sixteen children (nine boys and seven girls aged from 4 years to 12 years) with thermal skin burns involving 20–60% TBSA (including 22 ± 17% deep burns) were examined. Patients were admitted to Speransky Clinical Hospital no. 9 (Moscow) within 1–3 days after thermal injury. During the observation period (1–7 post-burn days) all patients underwent excision of devitalized tissue with simultaneous or delayed autologous skin grafting. The patients included in the study did not have chronic diseases and any evident signs of infections. Complications induced by the thermal injury developed in seven patients. These included pneumonia ($n = 2$) and gastrointestinal ulcer ($n = 5$). Venous blood was collected in a 1–2 day interval on the basis of informed consent given by parents. A reference group included healthy children undergoing assessment prior to elective hernia surgery ($n = 24$).

Buffer salts, Krebs-Ringer bicarbonate (KRB) buffer, Histopaque gradient solutions (density 1.077 g/mL and 1.119 g/mL), human serum albumin (HSA), Triton X-100, *o*-dianisidine dihydrochloride,

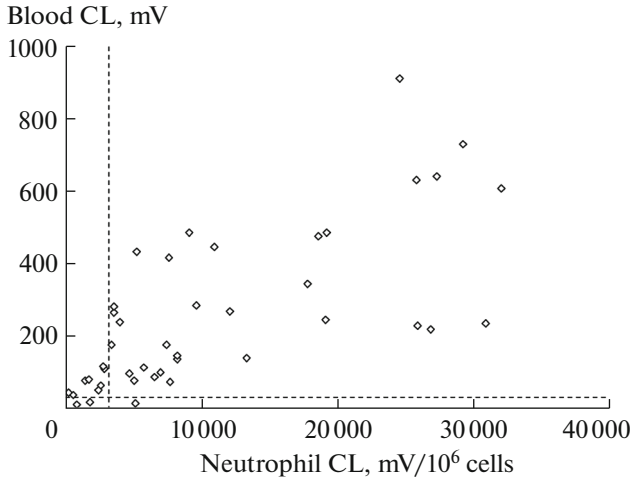

Fig. 1. Correlation between the PMA-induced CL response of whole blood and isolated neutrophils from patients with thermal injury ($n = 16$) during first seven post burn days ($R = 0.72$, $p < 0.001$). The dotted lines show the upper limit of the examined parameters in the reference group ($n = 24$).

hydrogen peroxide (H_2O_2) solution (30 wt %), phorbol-12-myristate-13-acetate (PMA), luminol, polyethylene glycol (PEG) 3000 were purchased from Sigma-Aldrich (USA).

Venous blood collected into heparinised vacutainer tubes was immediately used for isolation of neutrophils and measurement of whole blood CL. Neutrophils were isolated by layering blood over 1.077/1.119 double density gradient followed by centrifugation at 400 *g* for 35 min. Neutrophils were collected, washed with KRB buffer, and counted with a Goryaev counting chamber. Plasma was separated by centrifugation at 900 *g* for 15 min.

The albumin fraction of plasma was prepared using 30% solution of PEG 3000 in 10 mM phosphate buffer (pH 7.4) as described earlier [6]. Protein concentration was determined by the method of Lowry.

The CL analysis was performed using whole blood (10 μL) or neutrophil suspension (100 μL) containing 5×10^6 cells/mL; the aliquots of blood or cell suspension were added into a cuvette of the Wallach 1251-Luminometer (LKB), containing 1 mL of 0.2 mM luminol in KRB buffer. CL was monitored at 37°C and constant stirring. After registration of spontaneous CL for 5 min a neutrophil activator, phorbol-12-myristate-13-acetate (PMA), was added to the final concentration 100 ng/mL and stimulated CL was recorded. In experiments with blood of healthy volunteers PMA was added after a short (5 min) preincubation of the analyzed blood sample with a 10 μL-aliquot of plasma or its albumin fraction (or human serum albumin, HSA) used in the final protein concentration of 0.5 mg/mL. The CL response to PMA was evaluated as a difference between maximal levels of stimulated and spontaneous CL (in mV).

MPO activity was assayed in plasma and neutrophil lysate by *o*-dianisidine oxidation after H_2O_2 addition [2, 8]. Neutrophils were lysed with 0.1% Triton X-100; protein concentration in the lysate was measured with the method of Lowry. The amount of oxidized *o*-dianisidine formed during 5 min was calculated using the molar extinction coefficient of $\varepsilon_{560} = 20040\ M^{-1}cm^{-1}$ [8]. Enzyme activity was expressed in units (U). One unit was defined as the amount of enzyme catalyzing formation of 1 μmole of oxidized *o*-dianisidine per 5 min. Specific MPO activity was expressed per 1 g of cell lysate protein (U/g) or per plasma volume (U/L).

Data represent mean ±SD. Differences in mean values of examined groups analyzed using the Mann–Whitney U test were considered as statistically significant at $p < 0.05$. Correlation coefficients were calculated by the method of Spearman. Statistical treatment was performed using Statistica 6 software.

RESULTS AND DISCUSSION

According to the complete blood count, an acute inflammatory reaction within first days after injury (1–6 days before the beginning of the surgical treatment) was characterized by increased ESR (23 ± 12 mm/hr versus the normal rate of 2–10 mm/h), increased number of leukocytes (12.3 ± 2.5 mln./mL versus the normal range of 4–9 mln./mL) and stab leukocyte content (17.2 ± 9.4% versus the normal range of 1–6%). Starting from first days after the thermal injury, the level of the whole blood CL response to PMA was significantly higher than in the reference group and correlated ($R = 0.72$, $p < 0.01$) with the level of the CL response of neutrophils isolated from the same blood sample (Fig. 1).

Neutrophil MPO activity deviated from the parameter of the reference groups towards both higher (40% of the samples) and lower (26% sample) values. In neutrophils from blood samples with high CL responses a tendency to a decrease in MPO activity was found (Fig. 2).

The inverse dependence between the magnitude of the whole blood CL response and MPO activity in neutrophils was observed in most patients. For example, Fig. 3 shows results obtained for patient B (thermal skin burn of 50% TBSA). It can be seen that at basically stable (but increased) blood neutrophil content, the change in blood CL activity was well pronounced and was accompanied by the simultaneous opposite change in neutrophil MPO activity.

During the early period after thermal injury (days 1–3) plasma peroxidase activity exceeded the normal level in 50% of patients (median of 227 U/L in patients and 77 U/L in the reference group) and directly depended on the injured area (Fig. 4).

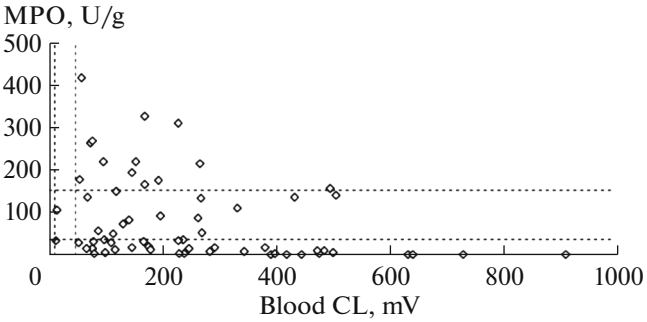

Fig. 2. Correlation between the PMA-induced CL of whole blood and MPO activity of isolated neutrophils from patients with thermal injury (n = 16) during first seven post burn days (R = −0.35, p < 0.05). The Y axis shows MPO activity U/g (units per g of neutrophilic protein). The dotted lines show the upper and lower limits of the examined parameters in the reference group (n = 24).

In the presence of plasma aliquots from patients with thermal injury the CL response of blood samples from healthy volunteers to PMA stimulation was higher; however, this effect did not reach the level of statistical significance. Addition of the albumin fraction isolated from the patient's plasma to the blood sample of healthy volunteers resulted in the increase of the CL response to PMA stimulation to 189 ± 46% as compared with the CL response observed in the absence of albumin; this increase correlated with peroxidase activity assayed in the same blood sample (R = 0.5; p < 0.05). PEG or HSA did not cause any statistically significant increase in the CL response of blood samples of healthy volunteers to PMA stimulation.

Leukocytosis in inflammation is a normal reaction of innate immunity. The decrease in neutrophils' activity causes septic complications, while their hyperactivation results in the development of organ failure [9]. The process of neutrophil activation accompanied by assembly of membrane NADPH-oxidase triggers reactions involving MPO, the enzyme of azurophilic granules; the activation results in production of reactive oxygen species (ROS: $^{\bullet}O_2^-$, H_2O_2, $^{\bullet}OH$-radical, etc.), and reactive halogen species (RHS: HOCl, HOBr, etc.) by neutrophils [5]. Luminol-dependent CL of neutrophils is mainly induced by luminol oxidation by HOCl, the main reaction product of MPO [10].

Results of this study indicate that in patients with thermal burns, neutrophils both in whole blood and as isolated cells exhibited increased susceptibility to activation and in 40% of patients' blood samples we observed increased level of neutrophil MPO. At the same time, our study revealed inverse correlation between CL activity of neutrophils and MPO activity in isolated neutrophils. In 26% of analyzed blood samples MPO activity was below the normal values. It is possible that increased activity of circulating neutrophils is accompanied by their degranulation in blood as evidenced by increased peroxidase activity detected in more than 50% of patients' plasma samples. It is also possible that degranulation of some proportion of neutrophils occurs during their isolation and this should be taken into consideration during analysis of CL of isolated neutrophils. Extracellular MPO may be implicated in modification of plasma proteins as shown in a model system containing HSA [11]; in this context, chlorinated albumin added to neutrophils promoted exocytosis of MPO [6, 7].

Since the albumin fraction of burn patients' blood potentiated the CL response of blood samples from

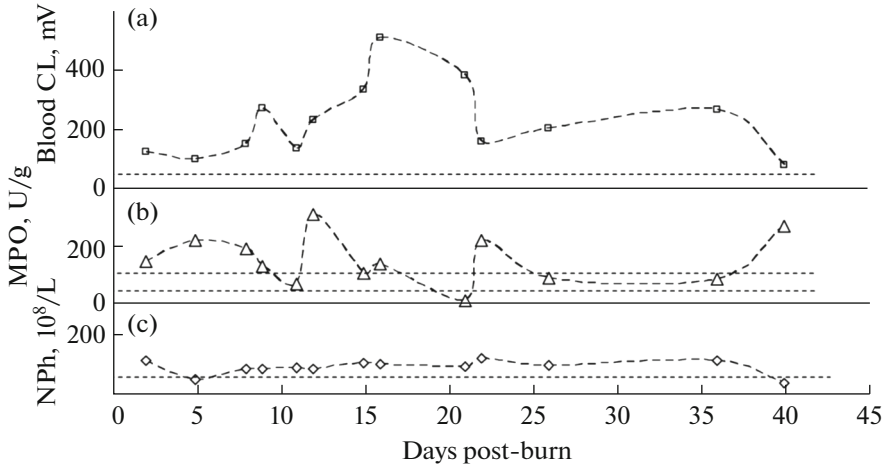

Fig. 3. The time course of inflammatory parameters in patient B. with thermal skin burn of 50% TBSA: (a) PMA-induced whole blood CL; (b) MPO activity in isolated neutrophils; (c) neutrophil (NPh) count. The Y axis shows whole blood CL (in mV); MPO activity in neutrophils in U/g (units per g of neutrophilic protein); NPh, 10^8/L—blood concentration of neutrophils. The dotted lines show limits of the examined parameters in the reference group (n = 24): the upper limit for (a) and (c) and both the upper and lower limits for (b).

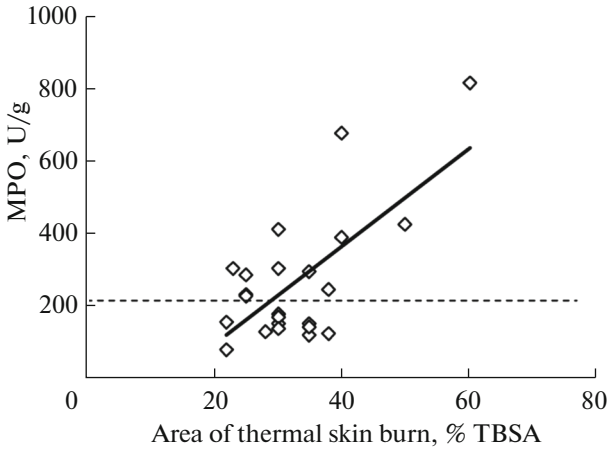

Fig. 4. Correlation between the area of thermal skin burn and MPO activity in plasma of patients ($n = 16$) during the early period after thermal trauma (1–3 post burn days) ($R = 0.68$; $p < 0.05$). The *X* axis shows area of thermal skin burn, % TBSA. The *Y* axis shows MPO activity in plasma (U/L, units per L of plasma). The dotted lines shows the upper limit in the reference group ($n = 24$).

healthy volunteers to PMA it is reasonable to suggest that albumin modified during development of the inflammatory reaction will further activate blood neutrophils, thereby increasing the risk of multiple organ damage.

CONCLUSIONS

Measurements of neutrophil MPO activity or content of this enzyme in blood plasma, as well as the whole blood CL response to the stimulus may serve as informative parameters reflecting severity of the inflammatory response of the body in thermal skin burns.

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