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TNF-α priming effect on polymorphonuclear leukocyte reactive oxygen species generation and adhesion molecule expression in hemodialyzed patients

Jacek Rysz[1], Maciej Banach[2], Robert A. Stolarek[1], Jarosław Pasnik[3], Aleksandra Ciałkowska-Rysz[4], Leszek Markuszewski[5] and Zbigniew Baj[6]

[1] 2nd Department of Family Medicine, Medical University Hospital No. 2, Medical University of Łódź, Poland,
[2] Department of Cardiac Surgery; Medical University Hospital No. 3, Medical University of Łódź, Poland
[3] Department of Pediatrics and Clinical Immunology; Medical University of Łódź, Poland
[4] Department of Palliative Care, Medical University of Łódź, Poland
[5] Department of Interventional Cardiology, Cardiodiabetology and Cardiac Rehabilitation, Medical University Hospital No. 2, Medical University of Łódź, Poland
[6] Department of Pathophysiology and Clinical Immunology, Medical University of Łódź, Poland

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Abstract

Introduction: The study aimed to assess reactive oxygen species generation and the expressions of some surface antigens on polymorphonuclear leukocytes (PMNs) in patients on regular hemodialysis (HD) treatment.
Materials and Methods: The respiratory burst of PMNs was determined with luminol-dependent chemiluminescence (CL) in resting cells and following N-formyl-methionyl-leucyl-phenylalanine (fMLP), phorbol 12-myristate 13-acetate (PMA), or opsonized zymosan (OZ) stimulation and expressed in arbitrary CL units times assay-time (aU×min). The expressions of CD11b/CD18, CD10, and CD13 receptors were determined with flow cytometry.
Results: Basal PMN CL was increased in HD patients to up to 1285±129 aU×min compared with 895±88 aU×min in healthy controls ($p<0.05$). The CL of unprimed PMNs increased after fMLP stimulation from 3085±746 to 4529±808 aU×min, and after OZ stimulation from 12945±1296 to 14678±1355 aU×min. PMA-stimulated CL of PMNs was similar to control values. The oxidative burst in PMNs from HD patients and healthy controls was similar in response to TNF-α alone. The CL of TNF-α-primed PMNs in HD patients was significantly lower than CL measured in healthy controls ($p<0.05$). The expressions of CD10 and CD13 metalloproteinase receptors were also increased ($p<0.05$). Although CD11b expression was significantly increased at rest and after fMLP stimulation, the expression of another β-integrin heterodimer compound, CD18, was not increased.
Conclusions: These results provide evidence that TNF-α priming of PMNs is down-regulated in HD patients despite constitutive up-regulation of resting cytotoxicity and enhanced expression of adhesion and metalloproteinase receptors.

Key words: hemodialysis, neutrophils, chemiluminescence, TNF-α, CD11b, CD18.

Corresponding author: Jacek Rysz, M.D. Ph.D., 2nd Department of Family Medicine, Medical University of Łódź, Żeromskiego 113, 90-549 Łódź, Poland, tel./fax. +48 42 639-34-65, e-mail: jacek.rysz@skwam.lodz.pl

INTRODUCTION

Hemodialysis (HD) patients face increased risk of infection [17] and increased in-hospital mortality related with infectious complications [19]. Chronic renal failure patients on maintenance hemodialysis present with diverse complications, including immune impairment [13]. Polymorphonuclear leukocytes (PMNs) are a crucial component of the immune response and play a major role in host defense through phagocytosis and the release of bactericidal enzymes and reactive oxygen species (ROS) [22, 32]. The profound alternations in inflammatory mediators and phagocyte activity in the uremic milieu are commonly related with detrimental immune defects in HD patients. These abnormalities in HD patients involve not only alterations in phagocyte cell signaling, impaired proliferation of PMNs, and altered regulatory interactions with T cells [11], but also

changes in the expressions of several genes, biologically relevant modifications of proteins, and accumulation of numerous uremic toxins. ROS generation depends mainly on the activity of the membrane-bound, multicomponent enzyme complex nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and the myeloperoxidase (MPO) system. The oxidase functional assembly facilitates the transfer of an electron from cytosolic NADPH-oxidase to molecular oxygen to produce superoxide anion [24]. Bioactive cytokines, such as tumor necrosis factor (TNF)-α, are involved in the regulation of NADPH-oxidase activity in PMNs [24]. TNF-α priming of human PMNs plays a crucial role in many functions of these cells *in vivo*. TNF-α leads to the release of large quantities of elastase from PMNs in the process of degranulation. TNF-α activity is increased in uremia and correlates with ongoing decline of renal function [23]. PMN priming with TNF-α or bacterial endotoxin, liposaccharide (LPS), leads to an exaggerated oxidative response to consecutive stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLP). Interestingly, the increase in stimulated oxidative burst after fMLP stimulation correlated significantly with creatinine clearance in uremic patients [32]. TNF-α, through one of its kidney receptor subtypes, appears to be a key mediator of LPS-induced acute renal failure [6]. TNF-α was shown to enhance respiratory burst and CD11b β integrin expression in PMNs from healthy subjects [10]. The oxidative burst response is primed for increased response with TNF-α, and the up-regulation of fMLP receptors and CD11b closely correlate with the oxidative response. CD11b up-regulation was recently proposed to reflect cytokine-induced priming [35].

The precise molecular patchwork involved in PMN priming by TNF-α is still being elucidated. Apparently, PMN priming results in the rapid mobilization of subcellular granules to the cell surface and in an increased expression of cell surface receptors, including the integrins CD11b and CD18 and the transmembrane metalloproteinases CD10 and CD13 [8, 14, 16, 28]. The CD11b/CD18 receptor (integrin β2 complement receptor 3, CR3) interacts with iC3b complement factor and is involved in leukocyte chemotaxis, adhesion [37], reactive species generation [30], aggregation, and dialysis-induced neutropenia [25]. In HD patients, neutropenia developed in parallel with increasing expression of CD10, CD11b, CD13, and CD18 on PMNs, which, in contrast to transient neutropenia, remained upregulated during the HD session [26]. CD10 is a single-chain glycoprotein with the properties of a neutral endopeptidase and a metalloproteinase, likely engaged in the inactivation of differentiation stimuli [31]. CD13 is a transmembrane zinc-dependent metalloproteinase with a role in MHC II antigen modification, chemokine binding, and coronavirus anchorage [29].

PMN function is significantly altered in HD patients; however, there are contradictory research data concerning these disturbances. In particular, knowledge about the relation between cell surface antigens and oxidative burst of PMNs is limited. Therefore, we studied the effect of TNF-α priming on the oxidative metabolism of PMNs in relation to the expressions of adhesion molecules (CD11b/CD18 heterodimer), transmembrane metalloproteinase receptors (CD10, CD13), and agonist-stimulated respiratory burst in HD patients.

MATERIALS AND METHODS

Patient population

The study included 10 chronic uremic patients (2 women, 8 men), aged 43.0±6.3 years, who all had been on regular HD treatment for 1–22 years (mean 6.6±3.6 years). Detailed patient characteristics are presented in Table 1. None of the study patients suffered from symptomatic hyperparathyroidism; their blood serum calcium and phosphate concentrations were effectively controlled by calcium carbonate, elimination of high-phosphate-containing food intake, and the adequate efficacy of HD. The patients were not given nitrates, arachidonic acid cyclooxygenase inhibitors, calcium antagonists, angiotensin-converting enzyme inhibitors, antiplatelet drugs, or any other drug known to affect immune functions. No symptoms of infection or aluminum toxicity were found in any patient. They had not received blood transfusions within the last 4 months. In all patients of this group, recombinant human erythropoietin (rHuEpo; Eprex; Cilag AG, Zug, Switzerland) was administered at a dose of 4000 U once a week to support a hematocrit level of approximately 30%. No iron supplementation was used during rHuEpo treatment and no study subject required blood transfusion. The patients received 4-h acetate HD procedures 3 times a week on an AK-100 machine (Gambro, Lund, Sweden) with single-use cuprophane membrane dialyzers (CU; Clirans C-101; Terumo Corp., Tokyo, Japan). Water for dialysate preparation was purified by reverse

Table 1. Study subject characteristics

	HD patients	Healthy controls
Age	43.0±6.3	37.0±3.4
Female (%)	20.0	40.0
Creatinine (μmol/l0)	664.2±292.8*	93.0±9.0
Urea (μmol/l)	18.4±9.0*	5.2±0.6
Uric acid (μmol/l)	337.2±44.5*	195.0±38.0
Na^+ (mM)	140.9±3.0	141.1±2.0
K^+ (mM)	4.2±0.7	4.0±1.4
Cholesterol (μmol/l)	4.4±2.3	4.0±1.2
HDL (μmol/l)	1.2±0.5	1.4±0.4
WBC (G/L)	5.9±2.9	6.3±1.7
RBC (T/L)	3.0±0.5*	4.7±0.4
Hb (g/dl)	9.4±1.3*	14.5±1.4
HCT (%)	28.3±4.4*	42.4±4.6
ESR (mm/h)	61.7±35.4*	8.5±2.1

HD patients: chronic renal failure patients on chronic HD therapy. Results are expressed as mean ±SD; *$p<0.05$ compared with healthy reference group.

osmosis using the WRO-10 system (Gambro). Heparinized venous blood samples were taken on a fasting basis in the morning, in the patients just before the consecutive HD procedure. The reference group consisted of 10 healthy volunteers (4 women and 6 men), aged 37.0±3.4 years. The protocol of the studies was reviewed and approved by the Bioethics Committee of the Medical University of Łódź and informed consent was obtained from each subject participating in the study.

Cell preparation

Peripheral blood PMNs were isolated, in case of the HD patients just before an HD session in the morning, by one-step density gradient centrifugation using Gradisol G (1.115 g/cm^3, 440 mOsm/kg H_2O; Polfa, Kutno, Poland) [39]. The obtained PMNs were washed twice and resuspended at a concentration of 1×10^6 cells per one ml in RPMI 1640 (Sigma Chemical Co., St. Louis, MO, USA) culture medium supplemented with 2 μM L-glutamine (Flow Laboratories, UK), 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal calf serum (Gibko, Paisley, UK). The purity of the PMNs was 92.7±3.8% and their viability was 97.6% for normal and 96.7% for HD patients as determined by trypane blue exclusion.

Cell incubation

Suspensions of PMNs (10^6 cells/ml) were incubated for 30 min at 37°C in RPMI medium. To start the priming, the PMNs samples were incubated with TNF-α (10 ng/ml) for 15 min at 37°C. Control cells of each donor were incubated with the medium only.

Measurement of chemiluminescence

The capacity to trigger (NADPH)-oxidase and MPO dependent activities was measured by luminol-amplified chemiluminescence (CL). A luminometer 1251 (Bio-Orbit, Turku, Finland) coupled with an IBM-PC AT-compatible computer was used for the measurement of CL. Phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co, St. Louis, MO, USA), fMLP, and opsonized zymosan (OZ; Sigma Chemical Co, St. Louis, MO, USA) were used for the different modes of phagocyte stimulation. fMLP was dissolved in dimethyl sulfoxide to a final concentration of 2×10^{-2} M and stored at –80^{0}C until assay. Luminol solution was prepared by dissolving 25 mg luminol in 90 ml of 0.1 M Na_2HPO_4, then the pH was adjusted to 7.4 with 1 N HCl and the volume adjusted to 100 ml with distilled water. Then the solution was filtered through a 0.2 μM Millipore filter and stored at 4°C in the dark for no longer than 2 weeks. Concentration curves were used to assess the dose-effect relationships based on responses from PMN cells isolated from healthy controls. The agonist concentrations used for the measurements were PMA 200 ng/ml, OZ 0.3 mg/ml, and fMLP 0.2 mg/ml. Ten serial dilutions of each agonist were tested in these measurements. Each agonist was added to the PMN suspension with an automatic, software-controlled dispenser. The assays were carried out at 37°C. The samples contained 2×10^5 PMNs (unprimed or primed with TNF-α) in 200 μl of PBS, 20 μl of luminol and 20 μl of fMLP or PMA or 30 μl of OZ, and PBS to a final volume of 1 ml. The response of the resting cells was also investigated over 15 min. Each measurement was conducted in triplicate and expressed as the area under the obtained curve of CL over the time (integrated light emission 0–30 min.).

Assay of surface receptor expression on PMNs

The determination of PMN cell surface receptor expression was performed in whole blood. The commonly used techniques were applied for the immunofluorescent labeling of the blood cell samples [38]. One hundred μl of blood was mixed with monoclonal antibodies (Dako, Copenhagen, Denmark) to visualize the appropriate surface molecules and incubated at room temperature according to the manufacturer's prescription. Erythrocytes were removed by the addition of Lysing Solution (Becton-Dickinson, Heidelberg, Germany) to the experimental medium. After short incubation followed by washing, the samples were fixed with 1% paraformaldehyde. A FACScan flow cytometer (Becton-Dickinson, Heidelberg, Germany) with a 488 argon laser was used to test the labeled PMNs. Lymphocytes and granulocytes were gated based on morphological and physical features of the cells. Monocytes were identified with anti-CD14 antibodies and differentiated as CD14-positive and low-SS (side scatter) cells. Neutrophils were differentiated in whole blood cytometry analysis as high-FS (forward scatter) and high-SS objects in morphological gates. The LYSIS II software was used for the analysis and the results were expressed as the percentage of analyzed cells which expressed the receptor (CD10, CD13).

Quantitative evaluation of surface receptor CD11b/CD18 density with the QIFIKIT test

QIFIKIT is intended for the quantitative determination of cell surface antigen with flow cytometry using indirect immunofluorescence assay [2]. The antibody-binding capacity of the analyzed cells was calculated by interpolation on the calibration curve [18]. QIFIKIT was used to evaluate the number of CD11b/CD18 molecules on a single PMN and presented in antibody binding-capacity units. Data were collected and analyzed accordingly using a analysis program Tally Call for Windows provided by the manufacturer (Dako).

Statistical analysis

Data were analyzed for statistical significance using the Mann-Whitney test for comparisons of groups. The data were presented as the mean ±SD. A p value of ≤0.05 was considered significant.

RESULTS

The relation between clinical and experimental data

The patient group in the study differed from the healthy reference group with respect to the biochemical parameters of chronic renal failure and anemia as depicted in the study population characteristics (Table 1). Transient HD-related neutropenia, expressed as the percentage of the decrease in neutrophil concentration (neutrophil percentage multiplied by total white blood count before and after the HD session) was 72±32% and it returned to the normal range in each case (n=10). Analysis of the data from the CL assays of respiratory burst and the flow cytometry data did not correlate the hemoglobin levels, the magnitude of anemia in HD patients, the creatinine clearance levels, or sedimentation rate. Also, probably due to the study group size, there was correlation between respiratory burst activity and surface integrins neither in HD patients nor healthy controls.

The resting and stimulated luminol-dependent CL of PMNs in HD patients

The luminol-dependent CL of resting PMNs and those stimulated with fMLP and OZ was significantly increased ($p<0.05$). The highest increase of CL was found after cell surface receptor stimulation of uremic PMNs with fMLP; however, there were no significant differences between receptor-independent PMA stimulation of polymorphonuclear leukocyte CL in HD patients and control subjects (Table 2).

Table 2. The luminol-dependent CL of unprimed neutrophils

Protocol	Normal	HD patients
PMNL (no agonist)	895±88	1285±129*
PMNL+fMLP	3085±746	4529±808*
PMNL+PMA	5146±1047	5947±895
PMNL+OZ	12945±1296	14678±1355*

Human PMNLs were stimulated with fMLP, PMA, and OZ. Results are expressed as mean ±SD in arbitrary CL units multiplied by the time of an assay in minutes; *$p<0.05$ compared with healthy reference group.

The luminol-dependent CL of TNF-α-primed PMNs in HD patients

TNF-α-primed PMNs from both HD patients and healthy controls produced similar levels of CL (Table 3). Preincubation of PMNs with TNF-α followed by fMLP stimulation resulted in a synergistic response: the response was greater than the sum of responses to either TNF-α or fMLP alone. This synergy was observed in uremic and normal patients. The CL of TNF-α-primed PMNs observed in HD patients was significantly lower than the CL measured in healthy controls (Table 3).

Table 3. The luminol-dependent CL of TNF-α-primed neutrophils

Protocol	Normal	HD patients
PMNL+TNF-α	1468±168	1325±99
PMNL+TNF-α+OZ	16438±3287	18352±2976
PMNL+TNF-α+PMA	5956±1423	6852±933
PMNL+TNF-α+fMLP	7285±939	6247±739*

Human PMNLs were stimulated with OZ, PMA, and fMLP following priming with TNF-α. Results are expressed as mean±SD in arbitrary CL units multiplied by the time of an assay in seconds; *$p<0.05$ compared with healthy reference group.

Surface expressions of CD11b/CD18, CD10, and CD13 on PMNs from HD patients

Moreover, there was a significant increase in CD10 and CD13 receptor expression on the surfaces of PMNs from HD patients in comparison with healthy controls (Table 4). The expression of CD11b adhesion molecule was also higher on the surface of PMNs in HD patients in resting conditions and after fMLP stimulation. There was no significant difference between the two groups in relation to the expression of CD18 on the surface of PMNs (Table 4).

Table 4. Expressions of CD10, CD11b, CD11b/fMLP, CD13, and CD18 on the neutrophil cell surface

Molecule	Normal	HD patients
CD11b	968±297	1346±32*
CD11b/fMLP	1665±306	1968±207*
CD18	346±86	306±68
CD10	68±30	107±23*
CD13	135±65	211±54*

Results are expressed as mean ±SD as mean fluorescence intensity; *$p<0.05$ compared with healthy reference group.

DISCUSSION

Although both resting and stimulated PMN chemiluminescence was increased in our study HD patients, preincubation with human recombinant TNF-α was related with decreased PMN response to stimulation with the same agonist compared with the response in cells from healthy controls. Additionally, increased expressions of CD10, CD13, and CD11b receptors were found in the PMNs of HD patients.

In HD patients, the elevated serum activities of superoxide anion, MPO, and superoxide dismutase were even further increased by a single HD procedure [5], but other research data indicated that an HD session could normalize increased hydrogen peroxide (H_2O_2) production [13]. Moreover, an HD session could decrease H_2O_2 release from unstimulated and PMA-stimulated PMNs [13]. The uremic milieu was demonstrated to

suppress PMA-, but not fMLP-elicited PMN chemiluminescence, indicating the presence of serum compounds able to suppress oxidative burst in these cells [21]. The increased oxidative resting activity and the enhanced response to agonist stimulation of PMNs in HD patients is currently supported by immense research data, and our finding may only confirm previous reports [22, 32, 35]. The increased PMN oxidative burst in CL assays in HD patients is most probably related with advanced glycation end-products accumulated both in uremic, non-dialyzed [12], and HD patients [34] that are able to stimulate NADPH-oxidase [36].

The refractory response to TNF-α priming has been previously assumed as an inherent feature of abnormal function of PMNs in non-dialyzed, uremic, and HD patients [27, 33]. This effect has been related with some soluble factors in uremic plasma; however, more in-depth research revealed that the candidate soluble TNF-α circulating receptors p55 and p75 that accumulate in uremic plasma are only partially responsible for the decreased priming effects. Apparently, neutrophils in HD patients are chronically stimulated at baseline by high circulating TNF-α levels and that this chronic stimulation makes them "refractory" to additional *in vitro* stimulation with TNF-α. An intracellular abnormality was, however, assumed to be a crucial factor underlying diminished TNF-α priming [33]. PMN priming with TNF-α involves receptor up-regulation and mobilization of receptor-storing organelles, including activation of signal processing via G-protein-coupled receptors [4]. In human PMNs, the mechanism of TNF-α priming and enhanced reactive oxygen generation was specifically related with activation of p38 MAPK, leading to increased expression of CD11b and CD18 and tyrosine phosphorylation of p72 subtype [9]. A similar pattern of priming and phagocyte response in HD patients was observed in monocytes [20]. Interestingly, research evidence indicates that TNF-α and the uremic milieu act through separate mechanisms of priming. PMNs from uremic patients were previously reported to be merely unresponsive to TNF-α priming [22]. TNF-α priming and subsequent stimulation with fMLP was demonstrated to induce phosphorylation of p47. TNF-α, via its p55 receptor, induces a protein tyrosine kinase-dependent selective phosphorylation presumably engaged in TNF-α priming mechanisms [7].

The increased expressions of CD10 and CD13 antigens with transmembrane metalloproteinase activities might hypothetically be related with the overall increase in the activity of numerous metalloproteinases during HD sessions, favoring or initiating inflammatory or fibrotic response in HD patients. Our findings are supported by previous reports on CD11b expression, which was found to be increased approximately 3-fold and related with overall cellular activation during the HD procedure [15]. The α (CD11b, and also CD11a, CD11c) and β (CD18) chains of β2 integrins form heterodimers and the CD18 number is presumed to be equal to the sum of α chains. The disproportionate increase in CD11b without an increase in CD18 forming the CD11b/CD18 heterodimer on the PMN surface does not provide any straightforward conclusions on the protein stoichiometry, as CD18 forms heterodimers also with CD11a and CD11c. Indeed, similar patterns of increased CD11b expression without accompanying CD18 increase was previously reported in flow cytometry analysis [1]. Despite the transient changes in PMN surface phenotype, reportedly related to complement activation in the course of reversible PMN sequestration during HD with cuprophane membranes, the up-regulated expressions of CD10, CD13, and CD11b were permanent, even though other CD receptor types returned to baseline following initial down- or up-regulation, in addition to those unaffected by the HD session [26]. Our results, also from HD patients dialyzed with cuprophane membranes, on the increased expressions of CD10, CD13, and CD11b in HD patients prior to regularly scheduled HD session could imply that repeated HD sessions actually lead to a cumulative increase in PMN surface integrins. However, the magnitude of this increase and the enhancement of the expressions of these particular surface antigens cannot be compared with their increase during the session, probably due to other factors affecting PMNs such as complement activation, neutropenia itself, and the leukosequestration known to occur during HD sessions. Long-term up-regulation seems more credible considering that the expression of CD11b in PMNs was increased and unrelated with HD sessions in another study [3].

The current evidence that TNF-α priming of PMNs is down-regulated in HD patients, despite constitutive upregulation of resting cytotoxicity and the expressions of adhesion and peptidase receptors, may imply significant inefficiency of molecular or signaling events underlying TNF-α priming in PMNs. This is the first study to demonstrate the contemporaneous abnormalities in phagocyte oxidative metabolism and the overexpression of surface receptors indicative of enhanced resting and agonist-elicited activity along with an impaired ability to respond to TNF-α priming. However, the design of our experiments cannot definitively demonstrate whether the differences observed are a result of high-molecular-weight uremic toxins or the dialysis procedure. Further studies on patients just prior to HD therapy could address this issue and further research may identify and rationalize the most promising approaches to TNF-α--based therapies.

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