Role of GM-CSF in Normalizing the Effect of Uremic Toxins on Neutrophil Apoptosis and Function

Nariman Zahran¹, Ola Mahmoud¹, Manal Zahran¹, Azza Sayed¹, Manar Rafaat²

Departments of ¹Hematology, ²Nephrology, Theodor Bilharz Research Institute, Cairo, Egypt. <u>zahranmanal@yahoo.com</u>

Abstract: The retention of many uremic compounds, interact negatively with biologic functions. It is the interest of this study to evaluate the influences of uremic plasma from chronic kidney disease (CKD) patients on conservative treatment, with and without urinary tract infection (UTI), the effect of hemodialysis (HD) and recombinant human (rh)-GM-CSF addition on neutrophil apoptosis and activation. Annexin V and CD18 were studied using flowcytometry in normal neutrophils as an indicator of neutrophil apoptosis and activation respectively; after incubation with plasma from: CKD patients with (12 cases); and without (15 cases) UTI, HD patients (15 cases) and 15 healthy control subjects in absence and presence of rh-GM-CSF for 20h. The results revealed significant acceleration of neutrophil apoptosis incubated with uremic plasma from CKD patients with and without UTI compared to those incubated with normal and HD patients' plasma. Neutrophils cultured in presence of CKD patients' plasma with UTI showed significantly increased CD18 expression compared to CKD patients. Also delayed neutrophil apoptosis and; in parallel to increased neutrophil CD18 expression was observed in cells cultured with rh-GM-CSF compared to corresponding cultured cells without rh-GM-CSF in all studied groups. These results indicate that uremic toxins in CKD patients' plasma influence neutrophil survival and function by modulating neutrophil apoptotic cell death and activation. Neutrophils undergoing apoptosis are dysfunctional, this may contribute to high prevalence of infections among those patients. rh-GM-CSF down regulated apoptosis and up regulated activation of control neutrophils cultured in presence of uremic plasma.

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Key Words: Neutrophil apoptosis & activation, Annexin-V, CD18, GM-CSF, CKD, HD.

1. Introduction:

Uremia is associated with altered host defense mechanisms, which increase the risk of infection. Bacterial Infection is one of the leading causes of morbidity and mortality in patients with end-stage renal disease (ESRD). Indeed, bacterial infections account for 15% of deaths among ESRD patients on dialysis therapy [1]. The pathogenic mechanisms responsible for these immunological abnormalities have been ascribed in part to uremic toxins [2], malnutrition, iron overload [3], dialysis-related factors [4,5] and possibly apoptosis [6,7].

In the past several decades, apoptosis or programmed cell death (PCD) was the subject of intense investigation, in terms of morphology, sequence of events, mechanisms, and biochemistry. In contrast to necrosis or accidental cell death, apoptosis is a programmed, active, and highly selective mechanism of cell death allowing for the removal of cells that are redundant or excessively damaged [8]. In multicellular organisms, apoptosis is an essential component of development and cellular regulation. Apoptosis is initiated by a number of different stimuli, including DNA damage, toxins, or extracellular signals. Apoptosis in both excessive and reduced amounts has pathological implications. Consequently, control of the apoptotic mechanism may have significant pathophysiological implications [7,9].

Neutrophils are terminally differentiated cells that comprise the greatest cellular component of the immune system and play an important role in innate host defense against microbial infection [10]. *In vitro* studies have shown that neutrophils from uremic patients exhibit impaired chemotaxis, adherence and transmigration, reactive oxygen species (ROS) production, phagocytosis and undergo accelerated apoptosis [11-17]. However, the mechanisms responsible for altered neutrophil apoptosis and its contribution to neutrophil dysfunction in uremic patients are only partially understood.

The aim of this study was to evaluate the influences of uremic plasma collected from patients, with chronic kidney disease on conservative treatment with and without urinary tract infection and ESRD patients undergoing hemodialysis (HD), on neutrophil apoptosis and activation. The effect of HD and GM-CSF addition on neutrophil apoptosis and activation was also evaluated.

2. Material and Methods:

Subjects

This study was enrolled on a group of 42 patients (26 males and 16 females) selected from Nephrology

Department and Dialysis unit of TBRI, Egypt. The patients were divided into:

- Group (1): Fifteen CKD patients on conservative treatment (10 males and 5 females with mean age of 49.65 ± 4.05).
- Group (2): Fifteen ESRD patients (9 males and 6 females with mean age of 40.53 ± 2.50) on regular HD; 3 sessions weekly, 4hrs each, for a period of ≥3 months using Fresenius 4008 B machine, Hemophane filters with 1.4 surface area and sodium acetate solution as a dialysate. The blood was collected immediately before session.
- Group (3): Twelve CKD patients with urinary tract infection (7 males and 5 females with mean age of 45.53 ± 3.05).

A group of 15 healthy subjects (10 males and 5 females with mean ages of 32.67 ± 2.79) were included to serve as controls.

Exclusion criteria included diabetes mellitus, acute infection caused by blood transfusion in the previous month, or chronic viral infections (e.g. hepatitis B, hepatitis C, human immunodeficiency virus), active immunological disease (e.g., systemic lupus erythematosus; rheumatoid arthritis & vasculitis); anti-inflammatory medication, antibiotic or antifungal treatment at the time of the study, previous transplants, or history of malignancy. The Ethical Committee of TBRI approved the study, and was conducted in accordance with Helsinki Declaration (1975). All participants gave written informed consent.

Blood collection

Blood samples were obtained under complete aseptic conditions from all patients and controls. EDTA blood for complete hemogram using automated hemogram (ACT Differential, Beckman, France). Sera for liver and kidney function tests using (Hitachi 736, Hitachi, Japan), HBs-Ag, HCVAb and HIV1+2 Ab by enzyme linked immunosorbent assay (ELISA) kits (Abbott laboratories, North Chicago, IL) and heparinized blood from healthy volunteers, for normal neutrophils separation.

Special investigations

Neutrophil apoptosis and activation were studied using normal neutrophils after incubation with patients' and controls' plasma in complete medium in a humidified atmosphere with 5% CO_2 at 37°C in absence and presence of recombinant human (rh)-GM-CSF for 20hrs.

Neutrophil isolation

Heparinized peripheral blood samples from control subjects were used to isolate normal neutrophils. The procedure was carried out under the laminar flow, at 22°C to avoid blood contamination. Firstly, heparinized blood; after adding 6 % dextran

{(mol wt 500,000 purchased from Parmacia (Upsala Sweden)}, sedimentation was centrifuged to separate leukocyte rich plasma (LRP) then fractionated using densitv gradient centrifugation with Percoll (Biochrom, AG) [18]. In brief, 2 ml of Percoll stock solution, (density 1.077), was added using sterile Pasteur pipette slowly on the tube wall, then 2 ml of Percoll (density 1.113) were put in the same way. Two ml of LRP were over layered on the Percoll slowly and few millimeters above the surface of the percoll. The gradient was performed by spinning in a swing out rotor centrifuge (Eppendorf 5024) at 1600 g for 20 min. After centrifugation, neutrophils were mostly recovered at the interface laver between Percoll and plasma. Neutrophils were collected in a culture tube, washed twice in phosphate buffer saline (PBS) at 1000 rpm for 10 min. The supernatant was discarded, and the cell pellet was resuspended in 1 ml complete RPMI 1640 medium with stable glutamine (EuroClone, IBS, France) for further manipulations. Examination of smears stained with May-Grunwald-Giemsa showed that neutrophils represented about 95% of the nucleated cells, cell viability, evaluated by trypan blue staining [19] was about 96%.

Neutrophil culture

Neutrophils (2 x10⁶/ml) were cultured in complete RPMI medium 1640 (Gibco, Grand NY, USA) supplemented with 10% heat inactivated fetal bovine serum (EuroClone, IBS, France); 1ml penicillin streptomycin solution 100x (EuroClone, IBS. France) and patients' or controls' plasma [20]. Cultures were performed for 20hrs at 37°C in 5% CO₂ in absence and presence of rh-GM-CSF (R&D systems Cat. No. 215-GM)(100 ng/mL) [21]. As a control for rh-GM-CSF, neutrophils were cultured in presence of rh-GM-CSF and absence of plasma.

Measurement of neutrophil apoptosis

Percentage of apoptotic neutrophils was assessed by Annexin V-FITC binding Kit, (BD PharmagenTM) using flowcytometry (COULTER[®] EPICS[®] XLTM, USA) after culture in absence and presence of rh-GM-CSF according to the manufacturer's specifications [22].

Determination of neutrophil activation

Using flowcytometry, immunostaining with R-phycoerythrin- conjugated antibodies (IOTest[®] CD18-PE), which were purchased from Immunotech, Beckman Coulter, Inc., together with specific isotypic control reagent. The density of CD18 on neutrophils, as an indication of neutrophils activation, was presented as mean fluorescence intensity of the gated leucocyte population within a chosen field.

Statistical Methods

All data were represented as means \pm standard error of mean (SE). SPSS computer program (version 12 windows) was used. Comparison between

mean values of post culture in absence and presence of rh-GM-CSF within the same group was performed using paired t test, while between different groups by one way analysis of variance (ANOVA) with post hoc using least significant difference [23].

3. Results

Data obtained are shown in tables (1, 2), figures (1, 2). Significant increase (P < 0.01) in neutrophil apoptosis (% of Annexin–V expression) on normal neutrophils incubated with uremic plasma from CKD patients with and without UTI compared to those incubated with both normal plasma and plasma from HD patients.

Significant decreased (P<0.01) in neutrophil activation (% of CD18 +ve cells) was found in normal

neutrophils incubated with both plasma from CKD patients and plasma from HD patients compared to those incubated with normal plasma. Moreover, Significant increase in CD18 expression (P<0.01) was noticed in neutrophils incubated with plasma from CKD with UTI patients compared to those incubated with plasma from CKD and HD patients.

Significant decrease in neutrophil apoptosis and increase in neutrophil activation (P < 0.05 and P < 0.01 respectively) was found by adding rh-GM-CSF to neutrophils incubated with uremic plasma from CKD patients. In addition, significant decrease in neutrophil apoptosis (P < 0.05) was also noticed by adding rh-GM- CSF to neutrophils incubated with uremic plasma from CKD with UTI patients.

Table (1): Creatinine.	Urea, W	BCs and Absolute	Neutronhil	Counts of Al	Studied Groups.
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	Controls $n = 15$	CKD patients n = 15	HD patients n =15	CKD patients with UTI n =12
Creatinine (mg/dl)	0.73± 0.19	4.05 ± 2.07^{ab}	6.85 ± 2.01^{a}	4.88± 2.60 ^{ab}
Urea (mg/dl)	21.07 ± 4.53	124.85 ± 46.59^{a}	136.36 ± 50.18^{a}	112.22± 40.05 ^a
WBCs (x10 ³ /µl)	6.86 ± 2.26	6.29 ± 1.94	5.83 ± 1.59	12.09±4.54 ^{abc}
Neutrophils(x10 ³ /µl)	3.79 ± 1.95	4.80 ± 1.57	3.58 ± 1.43	8.15 ± 3.73^{abc}

Values are expressed as mean \pm SD. ^ap < 0.01 significant difference compared to control group.

^bp < 0.01 significant difference compared to CKD group. ^cp < 0.01 significant difference compared to HD group.

Table (2): Results of	Special Studied Parameters in	Different Groups.
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	Normal neutrophils & plasma of different groups	Normal neutrophils, plasma of different groups& rh-GM-CSF
Control Annexin-V (%) CD18 (%)	$22.90 \pm 10.44 \\ 34.60 \pm 8.50$	$19.60 \pm 4.93 \\ 42.45 \pm 18.80^{\#}$
CKD Annexin-V (%) CD18 (%)	56.13 ± 22.13^{a} 14.97 ± 8.11 ^a	$41.08 \pm 16.73^{\#} \\ 38.53 \pm 15.48^{\#\#}$
HD Annexin-V (%) CD18 (%)	$29.94 \pm 5.40^{\rm b} \\ 23.26 \pm 9.95^{\rm a}$	25.69 ±16.91 33.66±15.65
UTI Annexin-V (%) CD18 (%)	53.67 ± 17.16^{ac} 34.74 $\pm6.80^{bc}$	25.62±10.25 [#] 39.69±14.25

^aP < 0.01 Significant difference compared to the control group. ^bP < 0.01 Significant difference compared to CKD group.

^C P < 0.01 Significant difference compared to HD group. [#]P < 0.05 Significant difference compared to the corresponding tube without rh-GM-CSF. ^{##}P < 0.01 Significant difference compared to the corresponding tube without rh-GM-CSF.

4. Discussion

The high prevalence of bacterial infections among patients with end-stage renal disease suggests that "professional" phagocytes such as neutrophils are functionally impaired [1]. The team of the present work has previously demonstrated that cultured neutrophils harvested from uremic patients had undergone accelerated apoptosis and disturbed function when compared with cells harvested from healthy volunteers [16, 17]. The mechanisms responsible for altered neutrophil apoptosis and function in chronic kidney disease are only partially understood and have been loosely ascribed to several factors [24]. In the present study we investigated the influences of uremia on neutrophils' apoptosis and function. This was achieved by assessment of apoptosis and activation of neutrophils from healthy volunteers cultured in complete medium supplemented with plasma obtained from CKD, HD, CKD with UTI patients and control subjects.



Fig (1): Annexin expression on normal neutrophils cultured with plasma of different studied groups with and without rh-GM-CSF.



Fig (2): CD18 expression on normal neutrophils after culture with plasma from different groups of the study with and without rh-GM-CSF.

Studies conducted on healthy individuals, demonstrated that culture of whole blood samples or freshly harvested neutrophils for 20 hours contained a markedly higher percentage of apoptotic neutrophils compared to that before culture. This post culture increased apoptotic rate was interpreted by the fact that, preserved cytokines and other factors naturally present in plasma could modulate apoptosis [25, 17]. Results of this work revealed highly significant acceleration in apoptosis of normal neutrophils incubated with uremic plasma from both CKD patients and CKD patients with UTI compared to those incubated with normal plasma. This impairment might be the result of the impact of uremic retention solutes on the balance between apoptotic and antiapoptotic process of neutrophils. It seems that some uremic retention solutes delay apoptosis and others promote this process. When apoptosis is delayed. neutrophils survive longer, thereby increasing host-defense capabilities against infections. However, such neutrophils are more prone to undergo necrosis, which is associated with a release of numerous pro-inflammatory molecules, leading to a state of low-grade inflammation. On the other hand,

induction of apoptosis decreases necrosis-induced

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inflammation but at the same time diminishes the response against infections [26]. In accordance with the present data are the findings of Cendoroglo et al. [6] and Glorieux et al. [26]. They demonstrated that the net effect of the uremic milieu accelerates the rate of normal neutrophil apoptosis. Neutrophil apoptosis is triggered by several mechanisms that include deathinduced receptors (tumor necrosis factor receptor or Fas), stress stimuli (UV irradiation, temperature shift), and phagocytosis as well as reactive oxygen species (ROS) production [27]. The Fas protein and Fas ligand (FasL) are the leading candidate for a molecular trigger of spontaneous apoptosis in neutrophil. Neutrophil constitutively release FasL, thus providing an autocrine/paracrine pathway for neutrophils to mediate their own programmed cell death [28]. Abundant expression of Fas and FasL on neutrophils was detected among patients with chronic renal failure [14]. Jaber et al. [7] also found that uraemic serum increased the expression of neutrophilassociated Fas and FasL protein, and that Fasstimulated apoptosis strongly correlated with creatinine clearance. Furthermore, ESRD gives rise to various cytokine disturbances and to a state of hypercytokinemia involving "death" factors consist of anti-inflammatory cytokines, such as IL-10 [29,30]. The net effect of patients' plasma included in the present work, whatever the combined causes, was to promote apoptosis.

In the present study, normal neutrophils incubated with plasma from the HD patients revealed significantly lower Annexin-V expression than that of CKD patients' plasma. The decrease of the apoptoticinducing potential of uremic plasma on normal neutrophils following HD may be explained by suggestions of Majewska *et al.* and Sardenberg *et al.* [13, 31] that uremic toxicity plays an essential role in neutrophil apoptosis and that the apoptosis inducing molecules are significantly cleared by dialysis or that they may be counter balanced by the generation of antiapoptotic factors.

Our data also revealed increased apoptotic rate in neutrophils cultured with plasma from CKD with UTI compared to those cultured with normal plasma and plasma from HD patients. Several lines of evidence indicate that bacterial pathogens induce neutrophil apoptosis differentiation program [32]. Phagocytosis elicits production of ROS, which plays a critical role in bacteria-induced cell death or phagocytosis-induced cell death [33]. Moreover, genes encoding proapoptotic factors were upregulated and genes encoding antiapoptotic proteins were down-regulated after phagocytosis of all pathogens by human neutrophils [32].

Neutrophil apoptosis is associated with marked downregulation of a number of neutrophil functions (including phagocytosis, stimulated granule secretion, and chemotaxis) [16,33], many of which show marked adhesion-dependency [34]. CD11b/CD18 is an important adhesion molecule that mediates leucocyte recruitment [35]. Our Data revealed lower CD18 surface expression on normal neutrophils exposed to CKD and HD plasma compared to those exposed to heterologous normal plasma. These results mirror the functional impairments that are commonly seen in neutrophils undergoing apoptosis. Increased CD18 expression was previously detected on cultured neutrophils separated from CKD patients [16]. This may indicate the presence of in vivo factors that affect CD18 expression. Fukuda and Schmid-Schönbein. [36] reported that fluid shear stress serves to directly regulate the nature of the CD18 expression on neutrophils. CD11b/CD18 expressed on the apoptotic neutrophil surface was observed to be functionally inert in spite of their maintained expression on the apoptotic neutrophil surface [37]. So delaying the apoptotic changes is required during infection episodes to promote neutrophils' functions.

Regarding neutrophils activation. neutrophils cultured in presence of plasma from CKD patients with UTI showed significantly increased CD18 expression compared to CKD patients. CD18 is considered a marker of neutrophils activation. The previous obtained results could be explained by the declaration of Aida and Pabst. [38] that bacterial lipopolysaccharide {LPS (endotoxin)} has been shown to be a major mediator of neutrophil activation. Also Lehr et al. [39]; Simms and D'Amico. [40] reported that expression of CD18-containing integrins on neutrophil cell surface increases substantially within few minutes after the cell comes into contact with bacteria or endotoxins.

Since its purification, molecular cloning and expression in different vectors in mid-1980s; considerable experience has been gained with rh-GM-CSF in vitro and in vivo animal models, in patients with neutropenia and in non neutropenic subjects [41]. In the current work the in vitro effect of rh-GM-CSF in regulating neutrophil apoptosis and function is indicated by the significant delayed normal neutrophil apoptosis and; in parallel increased neutrophil CD18 expression observed on cells cultured with rh-GM-CSF compared to corresponding cultured cells without rh-GM-CSF in all studied groups. Delayed neutrophil apoptosis was significant in neutrophils incubated with plasma from CKD patients with and without UTI. In addition, significant increased CD18 expression was found on neutrophils incubated with plasma from CKD patients. The exact mechanisms

that mediate the prosurvival effects of GM-CSF seem to happen through the mitochondrial pathway by preventing Bax translocation, cytochrome C release and subsequent caspase3 activation [42,43]. In accordance with our findings; several in vitro studies demonstrated that rh-GM-CSF delay normal neutrophil apoptosis, enhanced a number of neutrophil functions with improvement of their bactericidal activity [16, 17, 44-48]. The mentioned studies were performed on neutrophils belongs to normal volunteer, chronic liver disease and CKD patients.

In view of all the proceeding data, we can sum up that neutrophils are affected by the uremic environment; uremic toxins accumulating in the plasma of patients with CKD influence neutrophil survival by modulating the rate of apoptotic cell Neutrophils undergoing apoptosis death. are dysfunctional, this contributes to the high prevalence of infections among these patients. rh-GM-CSF down regulating apoptotic process and up regulated neutrophil activation of normal neutrophils cultured in presence of uremic plasma which was reflect by decreased annexin- V and increased CD18 expression changes found compared to corresponding cultures devoid of the growth factor. In addition, rh-GM-CSF had been shown to exert activity on monocytes, eosinophils as well as neutrophils and hence it seems to be beneficial in supporting host defense mechanisms. Further extended studies are however needed to emphasize the importance of developing additional management strategies to evaluate the impact of rh-GM-CSF on improving the outcome of CKD patients suffering from infection and septicemia in a trial to manage the causes for deranged immune system in CKD patients under conservative or HD treatment.

Conflict of interest:

The authors have declared that no Conflict of interest exists.

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