Granulocyte Macrophage Colony Stimulating Factor Mediated Modulation of Polymorphonuclear Leukocyte Dysfunction in Chronic Kidney Disease and Hemodialysis Patients

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Abstract: Introductions: Protein factors accumulating in the sera of patients suffering from end stage renal disease interfere with nonspecific immune response by inhibiting essential functions of polymorphonuclear leukocytes (PMNLs). **Aim:** The study aimed to assess the neutrophil functions which may be responsible for bacterial infections encountered in chronic kidney disease (CKD) and hemodialysis (HD) patients. In addition to evaluation of the potential therapeutic impact of the survival factor GM-CSF on neutrophil functions in these patients. **Methods:** The study was conducted on 59 patients, 34 cases with CKD and 25 HD cases. In addition to 15 normal subjects served as a control group. Neutrophil function was evaluated (before and after culture, with and without GM-CSF), by flowcytometric study of β2 integrin (CD18) and unstimulated and GM-CSF stimulated Nitroblue Tetrazolium test (NBT). The chronic inflammatory state known to be present in CKD was evaluated by estimation of C-reactive protein (CRP) using latex agglutination and serum levels of soluble intercellular adhesion molecule-1 (sICAM-1) using ELISA technique. **Results:** Neutrophils CD18 expression was significantly higher in CKD and HD patients compared to controls. Also neutrophil NBT reduction was significantly increased in HD group compared to control subjects. GM-CSF resulted in a highly significant increase of surface CD18 expression in both HD and CKD groups compared to their corresponding culture levels without GM-CSF. Both HD and CKD groups showed a highly significant increase in GM-CSF stimulated NBT reduction. **Conclusions:** These data clarify the potential therapeutic benefit of GM-CSF in reducing the severity and course of superimposed bacterial infections in those immunocompromised patients.

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1. Introduction

The high frequency of bacterial infections, which are still the major cause of morbidity and mortality in end stage renal disease (ESRD) patients, suggests that PMNL dysfunction may be involved in the immune deficiency observed in these patients [1]. Disorders in PMNL function are exacerbated by the dialysis procedure, uremic toxins, iron overload, anemia of renal disease, and dialyzer bio-incompatibility [2].

Evidence supports the notion that uremia is associated with an acquired immune deficiency which involves both cellular and humoral immunity [3]. A multifactorial impairment of the immune function is diagnosed commonly in chronic renal failure (CRF), and is particularly exacerbated in HD patients [4]. Protein factors accumulating in sera of patients suffering from ESRD interfere with the nonspecific immune response by inhibiting essential functions of PMNLs [5]. Other expected factors might be the consequence of chronic and extensive challenge to immune cells by different uremic toxins, the use of bio-incompatible dialysis membranes, and the progressive worsening of the overall clinical conditions [4]. In addition, chronic systemic micro-inflammation is a common feature in patients maintained on HD treatment, which is evident by elevated levels of C-reactive protein [6].

CD18 is the 95 kDa integrin β2chain [7]. CD18 integrins are adhesion molecules which play a central role in the molecular mechanisms supporting leukocyte migration to zones of inflammation [8]. Signaling via adhesion molecules of the beta-2 integrin family (CD11/CD18) plays an essential role for PMNL recruitment and activation during inflammation [9]. The endothelial molecule ICAM-1 interacts with the neutrophil integrin CD11b/CD18 (also known as Mac-1) to strengthen adherence and subsequent cell migration.

Granulocyte macrophage colony stimulating factor (GM-CSF) is a potent PMNL priming agent and a chemotactic factor thus, promotes enhanced clearance of invading microorganisms [10]. GM-CSF can trigger proliferation, the activation state and improve functions of more mature cells such as, PMNLs, macrophages and eosinophils particularly during immune and inflammatory reactions [11,12, 13]. A phenomenon that likely further facilitates host defense [10]. In addition, it prolongs neutrophil survival via increase in the cellular levels of Mac-1[14].

The present work was designed to assess the neutrophil functions which may be responsible for the recurrent bacterial infections encountered in many cases of (CKD) and HD patients. The influence of GM-CSF on neutrophil functions was evaluated in an attempt to clarify whether its use could have a potential therapeutic benefit in reducing severity and course of superimposed bacterial infections which may be fatal especially in those high risk patients with immunocompromised status.

2. Patients and Methods

Seventy four persons were the subjects of this study. Fifty nine patients selected from Nephrology Department and Hemodialysis unit, Theodor Bilharz Research Institute, in addition to fifteen healthy controls.

The study protocol was approved by the institutional committee for the protection of human participants and confirmed to the guidelines of the 1975 Declaration of Helsinki.

Patients were divided into:

Group A: Twenty five ESRD patients (16 males and 9 females, ages ranged between 33-65 years with a mean of 52±9.9 years), on regular HD treatment (3 sessions weekly: 4 hours each for a period more than 3months).

Group B: Thirty four CKD patients on conservative treatment (17 males and 17 females, ages ranged between 19-70 years with a mean of 44±14.9 years).

All patients were negative for HBs-Ag, HCV-Ab and HIV-Ab1+2.

Blood Sample Collection

Seven ml blood was collected from each subject into sterile endotoxin- free vacuum blood collection tubes under complete aseptic conditions. The withdrawn sample was distributed into the following:

- One ml was collected on potassium EDTA for complete hemogram using automated hemogram (ACT Differential, Beckman, France).
- Four ml of blood were delivered into vacuum tube provided with heparin lithium (15 IU/ml) for neutrophil separation and subsequent neutophil culture and determination of neutrophil function (CD18 and Nitroblue Tetrazolium {NBT}) test.
- Two ml were collected into plain tube and left to clot .The serum was separated by centrifugation at 2000 rpm for 10 min. Sera obtained were collected, for assay of liver and kidney function tests using (Hitachi 736, Hitachi, Japan). Also HBs-Ag, HCV-Ab and HIV1+2 Ab were performed by enzyme linked immunosorbent assay (ELISA) technique. In addition to determination of C-reactive protein by RAVITEX® CPR Latex serology test (Omega diagnostics, UK) and quantitative determination of sICAM-1 concentrations using a sandwich enzyme immunoassay technique (R&D Systems, Inc. Minneapolis, USA).

Neutrophil Isolation for Study of Neutrophil Function (CD18 Expression and NBT Test)

Leucocyte rich plasma (LRP) was separated on a gradient of Percoll after dextran (6 %) sedimentation of RBCs. Steps were carried out under the laminar flow and at 22°C to avoid blood contamination. LRP was used to detect CD18 expression on neutrphils and to perform NBT test.

The LRP was fractionated using a Percoll gradient: First 2 ml of Percoll (Density 1.077) were put using a sterile Pasteur pipette slowly on the wall of the tube , followed by putting 2 ml of Percoll (Density 1.113) in the same way. Two ml of LRP were over layered on the Percoll slowly and few millimeters above the surface of the Percoll without frothing.

The gradient performed by spinning in a swing out rotor centrifuge (Eppendorf 5024) at 1600 g for 20 min. PMNLs at the interface layer between Percoll and plasma were collected with a sterile Pasteur pipette in a sterile cell 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 and used for further manipulations. In all cases purity was about 97%.

PMNL count was adjusted according to the following equation:

$V2 = N/C – V1$

(C= the wanted concentration of cells, V2= the volume to be added, N = available number of cells, V1 = original volume {1ml})

Detection of CD 18 expression using flowcytometry [15] before and after culture with and without GM-CSF

Neutrophil Culture for Detection of CD18 Expression

PMNLs were resuspended at a final concentration of 2 $x10^6$/ml in complete RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (EuroClone, IBS, France) and 1ml penicillin streptomycin solution 100x (EuroClone, IBS. France) & mix well [16]. PMNLs were incubated for 20-22 hrs at 37°C in 5% CO_2 without addition (no stimulus) and with 100 ng/mL rh-GM-CSF (R&D systems Cat. No. 215-GM) [17].

Study of Neutrophil Metabolic Activity

This was achieved by NBT test. It was performed on freshly harvested neutrophils both in unstimulated and stimulated forms using rh-GM-CSF [18].

LRP was washed 3 times at 1000 rpm for 10 min. with Hank' s balanced salt solution (Sigma, Cat.

8389).The resuspended cells were divided into two tubes.

To prime neutrophils 100 ng/ml rh-GM-CSF were added to one tube and incubated for one hr. at 37°C before NBT staining. Then equal volumes of 0.1% of NBT solution and isolated neutrophils present in both tubes, incubated for 15 min at room temperature with shaking. Smears were prepared from the pellet, stained with Wright stain. A total of 100 cells per slide and at least 2 slides were counted for presence of formazan deposits (dark blue) in the cytoplasm, which represent reduced NBT dye.

Statistical Methods:

Statistical evaluation of results was done according to the methods described by Petei and Sabin [19]. SPSS computer program (version 12 windows) was used for data analysis. Comparison was performed using paired t and ANOVA tests.

3. Results

Results of studied parameters in different groups are illustrated in tables (1-3).

Assessment of the kidney function tests revealed that serum creatinine level reached the peak of elevation in the HD group followed by CKD patients (P<0.01) compared to control group (5.98 ± 0.40, 4.25 ± 0.42 and 0.76 ± 0.05 respectively) and also on comparing HD to CKD groups. At the same time, serum urea levels showed a highly significant increase (P< 0.01) in HD followed by CKD compared to control group (126.76 ± 9.40, 116.97 ± 7.96 and 21.07 ± 1.21, respectively).

Results proved a significant micro inflammatory state in both HD and CKD patients. A statistical significant increase in absolute neutrophil count, CRP and sICAM-1 was noted in HD (65.08±2.39, 59.00±16.34 and 24.53±1.54 respectively) and CKD patients (67.62±1.50, 78.69±12.99 and 20.14±1.06 respectively) compared to control subjects (53.27±2.77, 6.07±0.07 and 6.53±0.55 respectively). Moreover, CKD patients group showed a significantly lower sICAM level (P<0.05) compared to patients group on regular HD. While a non significant difference (P>0.05) was noted in absolute neutrophil count and CRP on comparing HD and CKD groups.

As regard CD18 (%) expression on neutrophils before culture, a highly significant statistical increase (P<0.01) was observed in both HD and CKD groups (48.41± 3.95 and 43.71± 2.64) compared to the control group (8.78± 1.05) as shown in (Table 1). On the other hand, comparison between HD and CKD revealed non significant difference (P>0.05).

The study showed that *in vitro* culture of neutrophils for 20 hours (Table 2) in absence of GM-CSF resulted in a highly significant decrease (P<0.01) of surface CD18 expression in both HD and CKD groups (32.98±2.99 and 29.85±2.46%) compared to their corresponding pre- culture levels (48.41± 3.95 and 43.71± 2.64). On the other hand, a highly significant increase (P<0.01) in neutrophil CD18 expression was detected in HD and CKD patients in presence of GM-CSF (40.26± 3.14 and 36.38± 2.62%) compared to expression on cells cultured without GM-CSF.

Evaluation of neutrophil metabolic activity (NBT reduction test) revealed that, HD group showed a highly significant increase (P<0.01) in unstimulated NBT compared to both control and CKD groups (15.20± 1.32, 7.33± 0.57 and 8.09± 0.65 respectively). But a non significant difference was noted on comparing CKD group with control group (P>0.05). Regarding stimulated NBT test (Table 3), both HD and CKD groups showed a highly significant increase (P<0.01) in NBT reduction after addition of GM-CSF (41.00±3.60 and 28.44±2.56) compared to unstimulated values.

Table (1): Results of parameters studied from fresh blood sample in different groups:

Parameter / Group	Control (n= 15)	HD (n= 25)	CKD (n=34)
Creatinine (mg/dl)	0.76 ± 0.05	5.98 ± 0.40 [a]	4.25 ± 0.42 [ab]
Urea (mg/dl)	21.07 ± 1.21	126.76 ± 9.40 [a]	116.97 ± 7.96 [a]
WBCs (x10^3/µl)	6.47 ± 0.44	7.98 ± 0.69	9.00 ± 0.74 [aa]
Neutrophils (x10^3/µl)	53.27 ± 2.27	65.08 ± 2.39 [a]	67.62 ± 1.50 [a]
CRP (mg/ L)	6.07 ± 0.07	59.00 ± 16.34 [a]	78.69 ± 12.99 [aa]
sICAM-1 (ng/ml)	6.53 ± 0.55	24.53 ± 1.54 [a]	20.14 ± 1.06 [ab]
CD18 (%) (before culture)	8.87 ± 1.05	48.41 ± 3.95 [a]	43.71 ± 2.64 [a]
NBT (%) (unstimulated)	7.33 ± 0.57	15.20 ± 1.32 [a]	8.09 ± 0.65 [b]

Values are expressed as mean ± SE.
[a a] p< 0.05 and [a] p< 0.01 relative to control group.
[b] p< 0.01 relative to HD group.

Table (2): Comparison of CD18 (%) expression on neutrophils before and after culture with and without GM-CSF in patients groups

Group / Parameter	**Before culture DAY 1**	**After culture without GM-CSF DAY 2**	**After culture with GM-CSF DAY 2**
HD group	48.41 ± 3.95	32.98 ± 2.99 [a]	40.26 ± 3.14 [ab]
CKD group	43.71 ± 2.64	29.85 ± 2.46 [a]	36.38 ± 2.62 [ab]

[a] $p < 0.01$ relative to CD18 before culture.
[b] $p < 0.01$ relative to C18 after culture without GM-CSF.

Parameter / Group	**Unstimulated**	**GM-CSF Stimulated**
HD group	15.20 ± 1.32	41.0 ± 3.60[a]
CKD group	8.09 ± 0.65	28.44 ± 2.56[a]

[a] $p < 0.01$ relative to unstimulated NBT test.

4. Discussion

The depression of the immune response in the uremic patient is global. Accordingly, chronic hemodialysis patients are highly susceptible to infection with its sequel of morbidity and mortality [3]. In fact, during dialysis treatment, cytokines and many other soluble uremic toxins may be partly responsible for a variety of functional disturbances that contribute to an increased risk of infection by interfering with essential functions of the non specific immune response such as chemotaxis, phagocytosis and oxidative metabolism [20]. Moreover, the infections are sustained not only by opportunistic microorganisms but also by common bacteria [3].

Total WBC and absolute neutrophil counts are considered to be the neutrophil derived inflammation markers [21]. In this study, the absolute neutrophil counts showed a highly significant increase in both HD and CKD groups when compared to control group. Although the mean values didn't exceed the upper normal range. This may be attributed to accelerated apoptosis among those patients [22].

Previous studies have documented the existence of oxidative stress and chronic inflammatory process in CKD with or without ongoing dialysis treatment. This process reflected by elevated CRP and expression of adhesion molecules. Chronic micro inflammatory process is caused by the continuous exposure to an inflammatory stimulus (uremia, ROS and endotoxin exposure), while the chronic inflammation in HD patients is induced by exposure to different external stimuli such as high/ low flux dialysis, bio incompatible cellulosic membranes, dialysate contamination, intravenous iron therapy and access site infection [22].

CRP is said to independently predict mortality in CKD patients [23]. In the present work, estimated CRP in the studied patients showed that both HD and CKD groups had highly significant increase compared to control group. On the other hand; comparison between HD group and CKD group revealed non significant difference. In accordance with present data are those reported by many investigators [21,24,25]. The authors referred elevated CRP to exposure to inflammatory stimuli in CKD group and due to external stimuli in HD as mentioned before [22]. Therefore, the association of a micro inflammatory state and uremia could be speculated, and might further be aggravated by the superimposed infection.

In the current study, the serum level of inflammatory biomarker, sICAM-1, showed a highly significant increase in both HD and CKD groups compared to control group. Also, a statistically significant difference was found between HD and CKD patients. Increased sICAM-1 levels observed in ESRD might be a consequence of decreased renal elimination and/or increased generation following induction by uremic toxins, oxidative stress, volume overload and co morbidities.

sICAM-1 binds competitively to ligends of membrane bound ICAM-1, such as LFA-1, MAC-1 and therefore might have potential ability to block leukocyte endothelial interactions and to regulate the enhanced leukocyte priming under the effect of uremic milieu. In addition, sICAM-1 activates pro-inflammatory cascades. These suggested that sICAM-1 might be involved in the progression of chronic inflammatory diseases associated with ESRD. The elevated serum levels of CRP in patients sharing in the current study might also be related to corresponding increase in sICAM-1.

In accordance with the present results, are those of Vaccaro *et al.* & Gad *et al.* [24,25]. They found elevated levels of the endothelial cell injury and inflammatory biomarkers sICAM-1and CRP in all ESRD groups; HD patients exhibited a significant increase in sICAM-1 as compared with the CKD group. They proposed that uremia is associated with

low grade chronic inflammation and enhanced oxidative stress. The dialysis procedure per se might aggravate the already present inflammation. Moreover, HD patients experienced longer disease duration and more obvious decline of renal function than the CKD.

Regarding CD 18 (%) expression in the present study, the patients' had highly significant increase before culture compared to healthy individuals. The phenotypical groups' expression of CD18 reached the peak of elevation in HD group followed by CKD in comparison to control. This reflected the state of neutrophil activation met in those patients mainly due to uremic toxin solutes and/or bacterial infections. In accordance with the current data, [26] showed similar increase in CD18 expression in CRF and HD patients, an observation which characterize the state of PMNL activation.

It is worthy mention that uremia may exhibit influence on neutrophils, it induces a state of neutrophilic activation under the effect of uremic toxins.

Neutrophils are unique in expressing all the three β2-integrin receptor complexes [LFA-1 (CD11a /CD18), MAC-1 (CD11b / CD18) and CR4 (CD11c/CD18)] receptors complexes. These complexes are known to play an important role in normal microbicidal function since (CD11/CD18) is an important adhesion molecule mediating recruitment of leukocytes [27].

It had been established that CD18 mediates neutrophil cytotoxicity, through releasing azurophilic granules and augmenting superoxide generation. CD18 has also been implicated as a triggering protein in tyrosine phosphorylation and may also mediate intracellular signaling through the intimate association of its intra cytoplasmic portion with α-actinin. Subsequently, CD18 plays an essential role for PMNL recruitment and activation during inflammation via adhesion, migration and phagocytosis [9].

In this study, cultured neutrophils for 20 hours in absence of GM-CSF resulted in a highly significant decrease of surface CD18 expression in both HD and CKD groups compared to their corresponding pre-culture levels. Indeed, the obtained data reflected the deteriorated functional capacities of cultured PMNLs, whether the diseased groups or the control group.

Evaluation of the effect of GM-CSF on cultured PMNL CD18 expression in the present study revealed a significant increase in both HD and CKD groups compared to those cultured without GM-CSF. These results suggested the potential role of GM-CSF in delaying some of the functional disabilities of PMNLs in uremic patients. Accordingly, this cytokine preserve PMNL function; a phenomenon that likely facilitates host defense against increasing liability to bacterial infections in CKD and HD dependent patients.

In agreement with these data [28] found that both G-CSF and GM-CSF had a promoting effect on PMNL function and survival. The authors reported that G-CSF augments neutrophil function by up-regulating both CD11b expression and ROS activity.

In agreement with the present results Wolach *et al.* [29] found that cultured neutrophils' chemotactic capacity was greatly reduced when GM-CSF was lacking. On the other hand neutrophils cultured in presence of GM-CSF had preserved chemotactic abilities in a dose dependant manner. They suggested that functional capacity of neutrophils aging in vitro is reduced or completely lost, but this loss could be postponed by GM-CSF.

In the same context, evaluation of GM-CSF to improve patients' immune response to invading microorganisms, NBT test both in the unstimulated and GM-CSF stimulated forms were performed. NBT test was utilized to assess phagocytic and ROS production in neutrophils. NBT reaction indirectly reflected the ROS generating activity in the cytoplasm of cells and therefore could help determining the cellular origin of ROS.

NBT test results in the present work, showed a highly significant increase of basal (unstimulated) ROS production in neutrophils of both HD and CKD groups compared to control group. While, GM-CSF stimulation resulted in a highly significant increase of PMNL respiratory burst activity in both HD and CKD groups compared to basal ROS production levels.

The present findings could be attributed to the suggestion of Granata *et al.*[30] who reported that both CKD and HD patients had significantly higher ROS levels compared to healthy subjects. However, they observed non significant differences between CKD and HD patients. They suggested that the increased production of ROS in uremia might be due to the effect of pro inflammatory mediators which cause a profound inhibition of the oxidative phosphorylation system leading to a compensatory hypertrophy of its components. Moreover, a hypertrophic and impaired oxidative phosphorylation system might prime a vicious circle, causing a continuous release of ROS. CKD and HD patients might have an impaired mitochondrial respiratory system and this deregulation of the mitochondrial respiratory machinery which is known to be closely associated with an enhanced oxidative stress.

Conclusion

It could be concluded that the survival factor GM-CSF has an important impact in improving neutrophil functions in HD and CKD patients. Further clinical trials to evaluate GM-CSF in reducing the severity and course of superimposed bacterial infections in these patients are recommended.

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5/5/2012