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Research Article
Granulocyte Macrophage Colony Stimulating Factor as an Adjuvant in ESRD at High Risk of Bacterial Infection

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Abstract

Background and Objective: GM-CSF has previously been demonstrated to be important in glomerulonephritis (GN). As both renal parenchymal cells and infiltrating inflammatory cells produce GM-CSF. GM-CSF is now regarded to act at several levels in the generation and propagation of immune responses. The therapeutic potential of GMCSF as an adjuvant treatment administrated to ESRD patients at high risk of bacterial infection was evaluated. Evaluation of *in vitro* effect of GM-CSF on cytoplasmic expression of P53 and Bcl-2 in neutrophils isolated from CKD and HD patients. **Materials and Methods:** Fifty seven individuals enrolled in this work. About 22 patients suffered from CKD on conservative treatment, 20 patients maintained on regular hemodialysis; in the current study, P53 and Bcl-2 expression in neutrophils isolated from CKD and HD patients were estimated in absence and presence of rh-GM-CSF. All individuals included in the study were subjected to Automated complete hemogram liver function tests; kidney function tests; Hepatitis markers and HIV-Ab were analyzed. **Results:** Results showed significant higher P53 base line expression on freshly isolated neutrophils from CKD and HD patients ($p<0.01$) when compared with neutrophils harvested from healthy controls. While BCL-2 showed minimal expression in all studied groups. Culture of neutrophils in absence and presence of rh-GMCSF for 20 h significantly decreased expression of P53 in neutrophils cultured in absence of rh-GMCSF in comparison to cells cultured in presence of the stimulus. On the other hand Bcl-2 expression recorded non-significant differences between the results of the two culture conditions in all studied groups. **Conclusion:** It can be concluded that better understanding of the molecular mechanisms underlying neutrophil apoptosis. Ultimately, leads to explore the potential of novel therapeutic strategies to handle infection and inflammation in immuno-compromised patients as in case of CKD and HD patients.

Key words: GMCSF, apoptosis, neutrophil, CKD, HD, P53 and Bcl-2

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Granulocyte macrophage colony stimulating factor (GMCSF) and granulocyte colony stimulating factor (GCSF) are pleiotropic growth factors, with overlapping functions. Both GMCSF and GCSF are used widely to promote the production of granulocytes or antigen presenting cells (APCs). GMCSF was evaluated as an adjuvant with HBV vaccine in hemodialysis patients. It promoted APC function and Improved Hepatitis B vaccine efficacy in end stage renal disease (ESRD)[1].

Several molecules play an important role in regulating neutrophil apoptosis. The BCL-2 family contains pro and anti-apoptotic proteins that are found in the outer membranes of the mitochondria. The balance between these members control the opening and closing of permeability transition pores. The latter mechanism is a critical pathway of cytochrome C release from mitochondria and initiating apoptosis by activating caspase-9[2,3].

Apoptosis is a critical cellular process for modulating neutrophil number and function, hence essential in neutrophil homeostasis and the resolution of inflammation[4]. During infection, neutrophil production is accelerated by action of cytokines such as GMCSF, leading to significant neutrophilia. Chemotactic factors recruit circulating neutrophils to migrate to site of infection, they begin to phagocytose and kill ingested pathogens. To optimize neutrophil bactericidal function, its life span is extended by a range of inflammatory mediators including GMCSF and the short lived pro-survival BCL-2 family member Mcl-1. GMCSF slightly increased Mcl-1 level for a limited time period which is rapidly declined in absence of its support by the cytokine[5-7].

Once the infected site is rendered sterile, the correct regulation of the apoptotic program is vital to ensure the maintenance of neutrophil number in the circulation[8].

GMCSF also mediates anti-apoptotic effect in neutrophils and the survival effect is both time dependent and limited. In spite of the pro-survival activity of GMCSF in neutrophils, it does not prevent apoptosis. The pro-apoptotic activity is achieved by induction of pro-apoptotic BCL-2 family member (Bim) expression in human neutrophils particularly under conditions of survival factor exposure; GMCSF mediates increase Bim expression in the consequence of transcriptional activation of the Bim gene[9].

The P53 protein has a pro-apoptotic function in contrast to BCL-2, which possesses a pro-survival activity. P53 is a transcriptional master regulator of DNA repair, cell cycle arrest, senescence and apoptosis[10]. P53 inhibition boasts oxidants generation by human neutrophils[11]. Irreversible DNA damage-induced P53/BCL-2 interaction at the mitochondrial membrane results in BCL-2 conformational changes with loss of its anti-apoptotic activity in interleukin-3-dependent myeloid cells. Activation of protein phosphatase 2A (PP2A) dephosphorylates BCL-2 and/or increase P53/BCL-2 binding with subsequent apoptotic cell death[12]. P53 has been extensively studied as a tumour suppressor and more recently shown to be an inflammation suppressor[11].

The serum glucocorticoid inducible serine/threonine protein kinase gene (sgk) acts as a direct transcriptional target of P53. Many transcriptional target genes of P53 are involved in important cellular processes including apoptosis (e.g., bcl-2 and bax)[13].

Although induced by the pro-apoptotic protein P53, SGK acts as a pro-survival factor. Thus, this P53-dependent SGK induction can be seen as compensatory mechanism. This negative feedback loop may also explain why the neutrophils are not significantly more sensitive to P53 apoptotic pathway[14].

The precursor of potent oxidants, Superoxide anion (0^{-2}) is essential for bacterial killing and potentiate inflammatory reactions in response to variety of stimuli, neutrophils release large[15] quantities of O^{-2}. High levels of oxidative stress may cause genotoxic injury; DNA damage triggering the P53 pathway, thereby leading to apoptosis[16].

DNA repair enzymes regulate the innate immune response[17]. On the other hand, bacteria and their products induce DNA damage and compromise DNA repair[18,19]. The mentioned recognitions have led to suggestion that defense of the host and the genome may be intrinsically interconnected to a degree not previously appreciated[11]. The later authors speculated that P53 may be centrally positioned to integrate the mentioned two fundamental responses to the environment and that thus carries wide ranging-infections for human diseases.

Several studies using RT-PCR had noted dysfunctional apoptosis state and/or response in chronic kidney disease (CKD) and hemodialysis (HD) patients. Decrease and increase of bcl-2 and bak genes expression levels respectively were reported in neutrophils isolated from CKD and HD patients compared to healthy individuals[20]. On the other hand, other studies reported that plasma obtained from CKD and HD patients accelerated apoptosis in neutrophils isolated from healthy subjects[21].

Neutrophils play a central role in host defense and fight against infection and tissue injury. The CKD and HD patients are known to suffer from recurrent bacterial infection and chronic inflammatory state[22,23].

In the current study, P53 and BCL-2 expression in neutrophils isolated from healthy subjects, CKD and HD

patients were estimated in absence and presence of rh-GMCSF. In order to, evaluate the expected potential value of administration of the cytokine as an adjuvant therapy to such patients at high risk of bacterial infection.

MATERIALS AND METHODS

Subjects: Fifty seven individuals enrolled in this work. About 22 patients suffering from CKD on conservative treatment, 20 patients maintained on regular hemodialysis; 3 sessions weekly, 4 h each, for a period of $\geq$3 months using a Fresenius 4008 B machine, Hemophane filters with 1.4 m^2 surface area and sodium acetate solution, patients was selected from those admitted to the Nephrology Department and the Haemodialysis Unit in The odor Bilharz Research Institute (TBRI), Giza, Egypt. In addition, 15 healthy subjects served as a control group. None of the patients had transfusion transmitted infection in the previous month; chronic infections (e.g., hepatitis B, hepatitis C and human immunodeficiency virus or osteomyelitis), active immunological diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis or vasculitis), was on immunosuppressive therapy or any anti-inflammatory medication at the time of the study had previous transplantation or history of malignancy. Informed written consent was obtained from all participants.

The procedures used in the study were approved by TBRI ethics committee according to Helsinki Declaration TBRI-IRB number 01/16. The study was conducted in the period from January, 2016 to June, 2017.

Assay methods: Routine investigations, all individuals included in the study were subjected to Automated complete hemogram (ACT Diff, Beckman, Coulter, France), liver function tests; alanine aminotransferase (ALT), aspartate aminotransferase (AST) and kidney function tests; serum creatinine and urea (Hitachi 736, Hitachi, Japan). Hepatitis markers (HBs-Ag and HCV-Ab) and HIV-Ab were analyzed by means ADVIA Centaur immunoassay system (Siemens, Germany).

Special investigations concerned with neutrophil apoptosis including neutrophil intracellular P53 and BCL-2 proteins expression, which were estimated in absence and presence of recombinant human rh-GMCSF using flow cytometry (FC).

Isolation of neutrophils: About 4 mL of heparinized peripheral blood were collected from all subjects. Leucocyte rich plasma (LRP) was separated after 6% dextran sedimentation then fractionated using density gradient centrifugation with Percoll (Biochrom, AG)[24]. In brief, 2 mL of LRP were over layered on the 2 mL Percoll. The gradient was performed by spinning in at 1600 g for 20 min. Neutrophils at the interface layer between Percoll and plasma 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 re-suspended in 1 mL of complete RPMI 1640 medium with stable L-glutamine (Euro Clone, IBS. France), stained with May Grunwald-Giemsa (MGG). Cell viability was evaluated by Trypan blue staining[25]. Intracellular BCL-2 /P53 proteins estimation in neutrophils[26,27] using FC.

Neutrophil culture: Neutrophils were re-suspended at a final concentration of 2×10^6/mL in complete RPMI 1640. Neutrophils were incubated for 20 h at 37°C in 5% CO_2 in absence and presence of 100 ng mL^{-1} rh-GMCSF Cat No 215-GM (R and D systems)[28].

Quantification of apoptosis-related molecules P53 and BCL-2 in neutrophils by FC: The percentage of neutrophils expressing cytoplasmic proteins; BCL-2 and p-53 proteins was assessed by FC (COULTER® EPICS® XL™, USA): FC Fixation and Permeabilization kit I Cat No FC009 (R and D Systems, Inc.), mouse anti-human BCL-2FITC antibody Cat no BMS1028FI (E-Bioscience) and mouse anti-human P53 antibody cat No ab1101 (Abcam) P53 unconjugated, secondary antibody for P53, PE goat anti-mouse IgG (H+L) F(ab') 2 fragment antibody Cat No F0102B (R and D Systems, Inc.). An isotype matched IgG1 (E bioscience) and IgG1-RPE monoclonal antibodies (R and D Systems) were used in every experiment as negative controls for BCL-2 and P53, respectively.

Statistical analysis: Results were expressed as Mean$\pm$standard deviation (SD). According to test of normality, data were not normally distributed so comparison between values of different variables in the three groups was performed using Kruskal Wallis test followed by Mann Whitney test if significant results were recorded. Pairwise comparison within the same group (culture in presence or absence of rh-GM CSF) was performed using Wilcoxon Signed Ranks test. Correlation between parameters was performed using Spearman's rank correlation coefficient. SPSS computer program (version 19 windows) was used for data analysis. p-value$\leq$0.05 was considered significant and less than 0.01 was considered highly significant.

RESULTS

Comparison between mean values of age, hemogram and kidney function tests in the patients and the control groups revealed a statistically significant difference between the three groups concerning age distribution, creatinine, urea and hemoglobin levels in the studied patient groups (Table 1).

Neutrophil intracellular staining of P53 in the patients and the control groups showed statistically significant increase CKD and HD group compared to control group. Comparison between CKD group and HD group revealed non-significant difference between the two groups with a higher P53 expression in CKD (Table 2).

Neutrophil intracellular staining of BCL-2 showed low expression in all studied groups. There was no statistically significant difference between the two patient groups regarding expression level of BCL-2 however a lower expression level of BCL-2 was elicited in HD group (Table 2).

Neutrophil culture for 20 h in presence of rh-GMCSF revealed a statistically significant increase in P53 expression in all studied groups. There was no statistically significant difference in expression level of BCL-2 with addition of rh-GMCSF to culture however the lowest expression level was noticed in control group than other two groups (Table 3).

DISCUSSION

Neutrophil death is mediated by a complex network of intracellular signaling pathways and extracellular stimuli such as pro-inflammatory cytokines[4]. Several reports indicated that gene toxic effect can induce P53 translocation to the outer mitochondrial membrane where it may interact with and inactivate BCL-2 anti-apoptotic function. High levels of oxidative stress may cause DNA damage injury triggering the P53 pathway leading to apoptosis[16,29,30].

Table 1: Comparison between mean values of age, hemogram and kidney function tests in the studied groups

Parameters	Control	CKD	HD
Age (years)	37.55±13.65	51.27±11.62[a]	52.62±11.72[a]
WBCs (×10^3/μL)	7.51±2.31	6.35±2.05	7.25±2.29
Neutrophil (%)	60.36±8.13	68.44±9.96[a]	69.22±8.41[a]
Lymphocyte (%)	32.49±7.25	25.12±9.49	25.72±7.79
Monocyte (%)	6.15±2.61	6.45±1.70	5.68±1.16
Haemoglobin (g dL^{-1})	13.62±1.39	9.52±2.04[a]	9.57±2.08[a]
Platelet (×10^3/μL)	236.73±82.35	206.91±85.56	195.85±62.32
Urea (mg dL^{-1})	20.36±2.20	126.49±75.07[aa]	125.62±25.83[aa]
Creatinine (mg dL^{-1})	0.67±0.16	7.78±8.92[aa]	8.33±2.05[aa]

Data expressed as Mean±SD. [a]p<0.05 and [aa]p<0.01 relative to control group. [b]p<0.05 relative to CKD group

Table 2: Comparison between mean values of P53, BCL-2 and co-expression of both proteins in the studied groups before culture

Parameters	Control	CKD	HD
P53 (%)	0.56±0.22	11.11±3.78[aa]	10.43±2.38[aa]
BCL-2 (%)	0.06±0.07	0.50±0.35[aa]	0.48±0.31[aa]
Co-expression (%)	0.23±0.13	1.05±0.31[aa]	1.22±0.28[aa]

Data are expressed as Mean±SD. [a]p<0.05 and [aa]p<0.01 relative to control group. [b]p<0.05 relative to CKD group

Table 3: Comparison between mean values of P53, BCL-2 and co-expression of both proteins in neutrophils after culture for 20 h in absence and presence of rh-GM CSF within the studied groups

Parameters	Culture in presence of rh-GM CSF	Culture in absence of rh-GM CSF	p-value
Control group			
P53 (%)	8.10±2.39	7.87±2.30	0.018*
BCL-2 (%)	0.22±0.27	0.37±0.27	0.328
Co-expression (%)	0.90±0.37	0.62±0.18	0.016*
CKD group			
P53 (%)	13.08±3.49	12.45±3.47	0.013*
BCL-2 (%)	0.49±0.28	0.54±0.30	0.448
Co-expression (%)	1.12±0.30	0.92±0.30	0.001**
HD group			
P53 (%)	14.92±2.11	13.52±1.49	0.004**
BCL-2 (%)	0.70±0.55	0.58±0.26	0.552
Co-expression (%)	1.30±0.25	1.17±0.26	0.001**

Data expressed as Mean±SD. *p≥0.05: Significant, **p≥0.01: Highly significant

The purpose of the present study was to assess the effect of rh-GMCSF in modulating neutrophil intracellular BCL-2 (anti-apoptotis) and P53 (pro-apoptosis) regulating proteins in cultured neutrophils isolated from CKD, HD patients and healthy controls using FC.

The results obtained from the current study concerning neutrophil BCL-2 expression using FC are comparable with results obtained by Santos Beneit and Mollinedo[31]. The authors used reverse transcriptase-polymerase chain reaction technique (RT-PCR), cloning and sequencing techniques and found that peripheral blood neutrophils expressed may play a role in the acquisition of the neutrophil apoptotic features. Moreover, published research investigated the acute effects of hemodialysis on pro/anti-apoptotic genes in leukocytes using RT PCR analysing transcript levels of the bcl-2, bcl-2L2 and other genes. There was no significant increase in gene expression levels of the analyzed bcl-2 and bcl-2L2 during the HD treatment. The authors stated that neutrophils contain only two anti-apoptotic BCL-2 proteins, Mcl-1 and A1 (also called BCL-2A1 or Bfl-1) of which Mcl-1 in particular is essential for cell viability[32].

In the present study, a significant higher P53 base line expression on freshly isolated neutrophils from CKD and HD patients ($p<0.01$) when compared with neutrophils harvested from healthy controls. This notion was more obvious regarding CKD group than HD group ($p>0.05$). These results indicated influences of uremia and HD treatment on expression P53 molecules denoting parallel effects on the gene. Hence increased expression of P53 gene was produced. This finding indicates a quiescent state of survival/apoptosis homeostasis with lowest expression of both P53 in the control group. This state was disturbed due to the pathological condition which seems to affect P53 more than BCL-2 pushing towards apoptosis in CKD and HD patients[33].

On the other hand, the current investigation denoted activation of P53 induced apoptosis pathway in neutrophils from uremic patients. In consensus with other study on neutrophil apoptosis by PCR that demonstrated increased Bak (pro-apoptotic) gene expression. The trend for abnormal mRNA expression was consistent with the increase in kidney function tests in CKD patients whether on conservative treatment or HD[20]. Therefore, in this study, neutrophil apoptosis pathway in this group of patients might be the intrinsic mitochondrial pathway. Likewise, the present findings concerned with increased P53 expression and enhanced apoptosis are in accordance with another published research. The authors studied other markers for apoptosis; E-cadherin, extracellular matrix metalloproteinase inducer (EMMPRIN) and matrix metalloproteinase-8 (MMP-8) in serum using ELISA technique. They demonstrated elevation of E-cadherin, EMMPRIN and MMP-8 concentrations in children with CKD. Higher levels were recorded in pre-dialysis patients than in controls and increased progressively with renal failure aggravation[34].

Neutrophils cultured for 20 h in absence and presence of rh-GMCSF recorded non-significant differences between the results of the different culture conditions in BCL-2 expression in all studied groups. On the other hand, P53 values showed significant decreased expression on neutrophils cultured in absence of rh-GMCSF in comparison to cultures containing the stimulus. Intracellular co-expression denoted significant increase in cultures containing rh-GMCSF, which means that the results trend appeared very close to those of P53 in cultures under the same culture conditions.

Results of neutrophil co-expression of both proteins suggests that P53 took the upper hand in cultured neutrophils survival/apoptosis regulation depending on studied BCL-2 and P53 proteins and under the current culture conditions. These results could be explained by the observations from other research group who reported that GMCSF and other neutrophil hematopoietins initiate a proapoptotic counter regulation. The authors attributed this proapoptotic activity to up regulation of Bim which is a proapoptotic member of the BCL-2 family; its increased expression is mediated by GMCSF[9].

Furthermore, they reported that GMCSF survival effect is both time dependent and limited. So GMCSF not only prolongs neutrophil life span and improves neutrophil functions but also induces counter reaction of their actions. Bim was increased after 8 h Bim levels were maintained up to 24 h. In the same study the workers did not observe increase in BCL-2 under their conditions of the study[9].

Global gene expression in human neutrophils during spontaneous apoptosis was compared with that in cells cultured with rh-GMCSF. Genes encoding proteins that inhibit apoptosis such as mcl-1, bcl-2 and sgk were down-regulated coincident with neutrophil apoptosis. In contrast, those encoding bcl-2-like 1 and sgk were up-regulated in neutrophils cultured with GMCSF. Correspondingly, GMCSF delayed neutrophils apoptosis, increased cell viability and prolonged neutrophil phagocytic capacity. In addition, expression of SGK protein diminished during neutrophil apoptosis but was restored by culture with GMCSF suggesting SGK is involved in leukocyte survival[35].

CONCLUSION

The survival action of rh-GMCSF is both time dependent, short acting and does not inhibit apoptosis. As soon as the

clinical cure and control of infection the cytokine is stopped and it is possible to use neutrophil survival pharmacological inhibitor to direct neutrophils to apoptosis and promote resolution of inflammation.

SIGNIFICANCE STATEMENT

The present study shed the light on the possibility of using rh-GMCSF as an adjuvant therapy in cases of bacterial infection in patients with CKD and HD.

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