Possible Role of G-csf on Neutrophil Activation, Endothelial Dysfunction and Haemostatic Changes, in Chronic Renal Failure Patients

Salwa M. Toima, Iman W. Bekheet, Manal Y. Zahran, Mona E. Madkour and Manar R. Raafat

Haematology and Nephrology Departments, Theodor Bilharz Research Institute.

Abstract: Cardiovascular disease remains the main cause of morbidity and mortality in maintenance haemodialysis patients. This study was designed to evaluate granulocyte colony stimulating factor (G-CSF) level in chronic renal failure patients, and to clarify its impact on neutrophil activation, endothelial dysfunction and haemostatic activation in these patients. Thirty patients on regular haemodialysis treatment were included in the study, as well as ten healthy controls. G-CSF was assayed by ELISA technique. Neutrophil activation was examined by surface expression of Mac1 (CD11b/CD18) using flowcytometry. Endothelial dysfunction marker (von Willebrand factor (vWF)) and the haemostatic activation marker (D-dimer) were measured by ELISA technique. Our results revealed a significant rise in G-CSF level, up regulation of neutrophil activation marker (Mac1) and endothelial dysfunction marker (vWF) in these patients. Also there is a significant haemostatic activation evidenced by high level of D-dimer. G-CSF showed a significant direct correlation with Mac1 (CD11b/CD18), vWF and D-dimer which incriminate G-CSF in the hypercoagulable state encountered in these patients. In conclusion, High level of G-CSF in CRF patients may play a role in cardiovascular disease events in these patients.

Keywords: chronic renal failure, neutrophil activation, endothelial dysfunction, G-CSF, vWF, D-dimer, CVD.

INTRODUCTION

Patients with end stage renal disease (ESRD), as well as those with mild renal insufficiency, in comparison with general population, are at increased risk for the development of cardiovascular disease (CVD), which can not be attributed entirely to traditional risk factors[47]. In addition, CVD remains the main cause of morbidity and mortality in maintenance haemodialysis (HD) patients[131]. Acute phase reaction markers were previously identified as independent predictors of CVD. Among the new factors that have been hypothesized, leukocytes might play an important role[61].

Serum granulocyte colony stimulating factor (G-CSF) levels in chronic renal failure patients were significantly higher than in normal individuals[62]. G-CSF enhances neutrophil functions in vivo and in vitro. It is known that neutrophil derived products can alter the haemostatic balance towards hypercoagulable state[22].

Several studies in vitro[35,55] and in vivo[10,15] have documented the neutrophil activating effect of G-CSF, indicating that it should be considered a potent immediate activator of circulating cells capable of priming respiratory burst, inducing the release of respiratory vesicles and cytoplasmic granules and modulating expression of surface adhesion molecules. Upon activation, polymorphonuclear leukocytes (PMN) release reactive oxygen species and intracellular proteases that possess several activities on endothelial cells and platelets, and may modify the haemostatic balance towards a prothrombotic state.

In vitro experiments have indeed shown that leukocyte elastase and cathepsin G can induce detachment[31] or even lysis[62] of endothelial cells. Endothelial dysfunction characterized by increased plasma concentrations of endothelium derived proteins has been demonstrated in end stage renal dialysis patients[63]. Endothelial dysfunction may be an important mechanism linking impaired renal function to cardiovascular disease, because healthy endothelium normally has anti atherothrombotic properties, such as promotion of vasodilatation and inhibition of vascular smooth muscle cell proliferation, thrombosis and inflammatory activity[48].

Independently from endothelial cell damaging effect, PMN-derived proteases may modify endothelial cell function involved in thromboregulation. It has been reported that PMN-derived proteases prevent thrombin-induced prostacyclin production by endothelial cells[22]. Cathepsin G has also been shown to induce the plasminogen activator inhibitor release by the endothelium[58]. Also proteolysis of components of the
endothelial surface, namely heparin-sulfated proteoglycans\(^ {30}\) or thrombomodulin\(^ {31}\) by elastase and cathepsin G, may contribute to impairment of endothelial non-thrombogenicity.

Furthermore, the potential thrombogenic effects of PMN-derived proteases include the direct potent platelet activation elicited by cathepsin G\(^ {91}\) and the enhancing effect of elastase\(^ {31}\). Finally, besides the cellular effects, elastase can directly proteolise and inactivate natural inhibitors of blood coagulation, including protein C\(^ {24}\), protein S\(^ {39}\), tissue factor pathway inhibitor\(^ {26}\), antithrombin\(^ {28}\) and heparin cofactor II\(^ {22}\), thus impairing the potent antithrombotic mechanisms.

In addition to PMN endogenous products release, G-CSF activated neutrophils present phenotypic changes involving the expression of surface adhesion molecules\(^ {30,10}\) including integrins (e.g. Mac1 \(\{CD_{11b}/CD_{18}\}\)) and selectin (e.g. L-selectin), which mediate PMN adhesion to endothelial cells and platelets\(^ {31}\). It has been shown that G-CSF can increase PMN adhesive function in vitro and in vivo\(^ {23}\).

Taking into account all the observations reported above we aimed to evaluate the level of G-CSF in chronic renal failure patients and to investigate its impact on neutrophil activation and haemostatic changes to clarify its possible role in high cardiovascular diseases incidence encountered in these patients.

**MATERIALS AND METHODS**

This study was conducted on forty subjects. Thirty patients (16 males and 14 females, age ranged 45-63 years) suffering from end stage renal disease on regular haemodialysis treatment at haemodialysis unit, Theodor Bilharz Research Institute (3 sessions weekly, 4 hours each for a period of more than 6 months, using Fresenius 4008 B machine, cuprohane membrane dialyzer with 1.1m² surface area and sodium acetate solution as a dialysate). In addition, ten age and sex matched healthy volunteers were studied as a control group. Patients with acute and chronic infections, active immunological disease, previous transplantation, immunosuppressive therapy or history of malignancy were excluded from the study.

The study protocol was approved by the institutional committee for the protection of human subjects and conformed to the guidelines of the 1975 declaration of Helsinki.

Six ml fasting blood samples were collected from ESRD patients in the morning, before the start of HD session, under complete aseptic conditions from the arterial outlet of the arteriovenous fistula using an arteriovenous needle. For healthy controls fasting venous samples were collected from the antecubital vein with minimal stasis and without frothing, using standard equipment. 1ml EDTA blood was used for complete blood count, and another 1ml of blood was delivered into a sterile tube containing EDTA for flowcytometric study. 2ml blood was delivered into a clean dry tube, allowed to clot at room temperature, centrifuged at 2000 rpm for 10min and serum was separated and used for laboratory assessment of G-CSF, kidney function tests (including blood urea and serum creatinine), liver function tests and hepatitis markers to exclude patients with acute or chronic hepatitis. 1.8ml (9 volumes) blood was delivered into a tube containing 0.2ml (1 voume) Na citrate, centrifuged at 2000 rpm for 20 min. plasma was separated, aliquoted and stored at -70°C until used for assay of vWF and D-dimer.

**Laboratory Assay:**

- The flowcytometric Epics R Elite Coulter System was used for the study of surface granulocyte expression of Mac1 (CD\(_{11b}/CD_{18}\)) using fluorescein labeled anti Mac 1 (CD\(_{11b}/CD_{18}\)) FITC (Sero tec)\(^ {31}\).
- 10ml of FITC labeled anti Mac 1 was incubated with 100ml of EDTA anticoagulated whole blood for 30 min. The samples were washed 3 times using phosphate buffer saline and analyzed by flowcytometry. Five thousand gated cells were counted.

Appropriate concentration of isotope matched control antibody were used to define the cutoff for positive fluorescence which was the 99th percentile of the distribution of cells labeled with control antibody (FITC conjugated IgG2).

Granulocytes were represented by a well separated cluster based on forward and side scatter properties. Discrimination frame were placed around granulocytes field. The actual number of cells and the mean fluorescent intensity of the cell population within the field were measured.

- Serum level of G-CSF was determined using Quantikine G-CSF ELISA kit (R and D systems, USA).
- Plasma vWF antigen level was assayed as a marker of endothelial dysfunction by ELISA technique using an Asserachrom vWF kit (Diagnostica Stago, France).
- Plasma D-dimer level was measured as a marker of in vivo clotting activation by ELISA technique using an Asserachrom D-dimer kit, (Diagnostica Stago, France).
- All specific assays were carried out according to manufacture’s guidelines.
- Cardiovascular disease status was defined as
Results: A significant increase in serum G-CSF, plasma vWF-Ag and plasma D-dimer levels were observed in chronic renal failure group compared to the control group (P<0.001). Also a significant rise in granulocyte expression of Mac 1 (CD11b/CD18) was noted on comparing the ischemic and non ischemic groups (P<0.001) (Table 3).

The above mentioned results suggest a possible role of G-CSF in cardiovascular disease events encountered in chronic renal failure patients via granulocyte activation and endothelial dysfunction with subsequent haemostatic activation evidenced by high D-dimer level as a marker of on going prothrombotic state.

On the other hand logistic regression revealed that G-CSF is not a direct dependent factor for cardiovascular disease in chronic renal failure patients (odd ratio estimated for G-CSF was considered insignificant, P <0.05).

Discussion: Cardiovascular disease is a frequent complication and a major cause of mortality in chronic haemodialysis patients, accounting for more than half of all deaths[24]. In addition, chronic haemodialysis patients suffer from atherosclerotic complications at a relatively young age. The increased cardiovascular risk is probably multifactorial in origin and already observed in the predialysis phase[23].

Several studies have demonstrated that inflammatory markers such as C-reactive protein, interleukin-6, fibrinogen and soluble adhesion molecules are associated with increased cardiovascular risk[21,27,42,45,14]. Additional pathway that might mediate the association between renal dysfunction and cardiovascular risk is neutrophil activation and endothelial dysfunction mediated by high level of G-CSF[56].

In the present study serum G-CSF level in CRF patients is significantly higher than in normal controls. High serum G-CSF level in CRF patients was reported by Wu et al. (2001)[52]. Also Saionji et al. (1997)[41], reported that G-CSF levels in chronic ambulatory peritoneal patients were higher than in healthy volunteers. On the other hand Sato et al. (1994)[44], reported that serum G-CSF is not affected by CRF, although haemodialysis may induce an increase of G-CSF level. High value of G-CSF may be caused by a decrease in G-CSF clearance and/or an increase in G-CSF release[52]. The neutrophil activating effect of high G-CSF and the release of neutrophil derived products may play a role in haemostatic activation and predisposition of CRF patients to thrombotic complications.

Study of leucocytes confirmed the occurrence of activated PMN in the circulation of CRF patients. There is a significant increase in Mac1 (CD11b/CD18) expression on granulocytes in CRF patients in comparison to the controls. The altered behavior of PMN integrin pattern in these patients is likely prevalent cardiovascular disease, subclinical cardiovascular disease and no cardiovascular disease. Prevalent cardiovascular disease included a history of myocardial infarction, revascularization procedure, angina or stroke. Assessment of patients without prevalent cardiovascular disease was performed depending on ECG (12 leads), stress test, echocardiography [left ventricular hypertrophy (left ventricular mass index ≥ 100g/ (height in meters)² in women or ≥ 131g/ (height in meters)² in men)] and borderline or abnormal left ventricular function, coronary catheter and thallium isotopic scan.

Statistical Methods: Data were analyzed by computer using the statistical package SPSS for Windows version 13.

Data were summarized as mean ± SD. Groups were compared by one way analysis of variance followed by multiple comparisons using Student t test. Non Parametric (Mann-Whitney U) test was used for analysis of quantitative data, as data was not symmetrically distributed. Pearson correlation coefficient 'r' was used to measure the relationship between two variables. Logistic regression test was used for detection of risk factor for cardiovascular disease in chronic renal failure patients. For all tests P-value was considered significant if <0.05*.

RESULTS AND DISCUSSIONS
reflecting a state of PMN activation, which might have a pathophysiological significance considering the high incidence of cardiovascular events in ESRD[36]. Up regulation of adhesion molecules in CRF patients are multifactorial and have been detected by many authors[40,32,50]. In addition, Combe et al. (1994)[13], suggested that increased granulocyte expression of adhesion molecules may be caused by complement activation during haemodialysis using cellulose dialysis membranes with consequent deposition of C3, activation and degranulation products on the membrane surface[11]. Also neutrophil activation could be attributed to proinflammatory cytokines which are a common phenomenon in CRF patients which up regulate the expression of adhesion molecules[49,29].

Granulocyte activation in haemodialysis patients could also be due to some substances accumulated or generated by renal failure e.g. uremic toxins or complement products[17]. Bacterial products in addition to dialysis membrane may trigger and even perpetuate an abnormal cell activation with increased expression of surface adhesion molecules[13]. Also, Yoon et al. (2007)[34], reported recently a direct evidence of spontaneous leukocyte activation and increased reactive oxygen species generation (hence the link between oxidative stress and inflammation) in end stage renal disease patients. Lastly, the direct relation between G-CSF and granulocyte expression of Mac1 in the present study suggest that high G-CSF in haemodialysis patients may play a role in neutrophil activation.

A significant high level of vWF as a marker of endothelial cell damage and activation were found in plasma of CRF patients in comparison to the control group, suggesting endothelial cell dysfunction condition. vWF is a specific marker of endothelial perturbance and is 80-90% derived from the endothelium, with the remaining being from platelets[35,41]. Plasma level of vWF is directly correlated with the level of neutrophil activation marker (Mac1), and serum level of G-CSF. Depending on the basis of the in vitro results showing that neutrophil elastase is able to stimulate vWF release from the endothelial cells[22], we can speculate a cause effect relationship between neutrophil activation and endothelial dysfunction. Also increased production and release of inflammatory cytokines e.g. tumor necrosis factor-α in CRF patients may contribute to the increment of plasma vWF. Finally, a direct effect of G-CSF on endothelial vWF release could be hypothesized on the basis that endothelial cells express G-CSF receptors[22].

However, studies addressing links between vWF as an endothelial dysfunction marker and cardiovascular disease in haemodialysis patients showed that increased vWF Ag levels are associated with alterations in the microcirculation characterized by decreased post ischemic vasodilatation[36], and activated intravascular blood coagulation[35,16,37]. Also, vWF has prothrombotic properties through its involvement in platelet adhesion/aggregation and in blood coagulation[24].

<p>| Table 1: Statistical comparison of different studied parameters in the patients and control groups. |</p>
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group (n=10)</th>
<th>CRF group (n=30)</th>
<th>U</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF (ng/l)</td>
<td>467±156.2</td>
<td>1314±445.3</td>
<td>4.000</td>
<td>0.001*</td>
</tr>
<tr>
<td>CD11b/CD18 (%)</td>
<td>93±12.6</td>
<td>126.9±13.4</td>
<td>6.500</td>
<td>0.001*</td>
</tr>
<tr>
<td>CD11b/CD18 (%)</td>
<td>67.9±58.4</td>
<td>120.8±46.8</td>
<td>60.000</td>
<td>0.01'</td>
</tr>
<tr>
<td>D-dimer (ng/ml)</td>
<td>135.1±88.6</td>
<td>1019.2±764.4</td>
<td>2.000</td>
<td>0.001'</td>
</tr>
</tbody>
</table>

* Sign. differences (P<0.05)

<p>| Table 2: Correlation analysis between different studied parameters in patients’ group. |</p>
<table>
<thead>
<tr>
<th>Parameters</th>
<th>CD11b/CD18</th>
<th>vWF</th>
<th>D-dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>r=0.868**</td>
<td>r=0.810**</td>
<td>r=0.793**</td>
</tr>
<tr>
<td>CD11b/CD18 (%)</td>
<td>-</td>
<td>r=0.720**</td>
<td>-</td>
</tr>
<tr>
<td>vWF</td>
<td>-</td>
<td>-</td>
<td>r=0.912**</td>
</tr>
</tbody>
</table>

** Highly significant (P<0.001).

<p>| Table 3: Statistical comparison of different studied parameters in relation to cardiovascular disease. |</p>
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ischemic group (n=21)</th>
<th>Non-ischemic group (n=9)</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF (ng/l)</td>
<td>1503±3093</td>
<td>873.7±409.5</td>
<td>4.603</td>
<td>0.001'</td>
</tr>
<tr>
<td>CD11b/CD18 (%)</td>
<td>132±12.1</td>
<td>115.2±7.7</td>
<td>3.628</td>
<td>0.0001'</td>
</tr>
<tr>
<td>vWF (%)</td>
<td>138.9±44.8</td>
<td>78.8±9.1</td>
<td>3.043</td>
<td>0.0001'</td>
</tr>
<tr>
<td>D-dimer (ng/ml)</td>
<td>1272.1±784.7</td>
<td>428.9±137.8</td>
<td>3.176</td>
<td>0.0001'</td>
</tr>
</tbody>
</table>

* Sign. differences (P>0.05)
Depending on the significant high level of plasma marker of clotting activation (D-dimer), together with its direct correlation with G-CSF, vWF and granulocyte expression of Mac1, we can hypothesize that the haemostatic balance is altered towards a hypercoagulable state. Whether this effect is due to direct or an indirect action of high G-CSF is not established with both possibilities being plausible. Although logistic regression analysis revealed that G-CSF is not a direct dependent factor for cardiovascular events in chronic renal failure patients, we can hypothesize indirect effect of G-CSF to take place in the network of cellular interactions occurring in the circulation or it may be induced by other cytokines as tumor necrosis factor-a produced by activated granulocytes[39,53,20]. Also platelets possess functional G-CSF receptors that seems to transduce a priming effect and makes the platelets more responsive to additional agonists[66]. Accordingly, it is possible to speculate that platelets per se or via the interaction with neutrophils and monocytes[9] may contribute to the imbalance of the haemostatic system in these patients.

In conclusion, this study provides evidence that high level of G-CSF in chronic renal failure patients may have its impact on the activation status of PMN and may be associated with changes of endothelial cells and clotting activation markers. These changes may have a pathophysiological significance considering the high incidence of cardiovascular disease encountered in these patients, which can help in launching new preventive and therapeutic modalities.

REFERENCES


17. Dou, I., P. Brunet, F. Dignat-George, J. Sampol


