Mononuclear Leukocyte Apoptosis and Inflammatory Markers in Patients with Chronic Kidney Disease

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Key Words
Apoptosis \cdot Inflammation \cdot Chronic kidney disease

Abstract
Background/Aim: Increased apoptosis along with enhanced inflammation has been reported in hemodialysis and predialysis patients. However, there is limited information at which stage during the progression of chronic kidney disease (CKD) the balance between pro- and anti-apoptotic mechanisms is disturbed and inflammatory response is activated. The aim of this study was to investigate possible alterations in apoptotic and inflammatory markers during CKD (stages 1–4) progression and the probable interactions between them. Methods: In a cross-sectional study, 152 steady-state CKD outpatients (83 males, 55%) with mean estimated glomerular filtration rate 46 (29–76) ml/min/1.73 m\textsuperscript{2} were studied. Apoptosis was assessed in peripheral blood mononuclear cells by estimating Bcl-2 expression, annexin V-propidium iodine staining and serum soluble Fas (sFas) and Fas-ligand. Serum levels of C-reactive protein, tumor necrosis factor-\alpha (TNF-\alpha), interleukin-6 and plasma levels of fibrinogen were measured as markers of inflammation. Results: Bcl-2 expression was found to decrease significantly in both lymphocytes and monocytes from CKD stage 1 to 4. In contrast, the activity of sFas increased significantly and so did the levels of TNF-\alpha and fibrinogen. The majority of these alterations occurred as soon as patients entered stage 3 of CKD. A multivariate regression analysis demonstrated that CKD remained a significant predictor of the aggregate of the assessed markers. Conclusions: Apoptosis appeared to increase across CKD stages 1–4, and this was associated with increased proinflammatory activity.

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Introduction

Increased apoptosis of leukocytes has been described in hemodialysis (HD) and non-dialysis patients, while miscellaneous factors, such as, uremia, acidosis, oxidative stress, iron overload and malnutrition have been implicated [1–3]. In addition, a significant percentage of HD patients demonstrate an activated inflammatory response in the absence of any apparent cause [4, 5]. Apoptosis and especially inflammation are established non-traditional, uremia-related cardiovascular risk factors implicated in the high incidence of cardiovascular mor
bidity and mortality in chronic kidney disease (CKD) patients [5]. However, there is limited information at which stage during the progression of CKD the balance between pro- and anti-apoptotic mechanisms is disturbed and inflammatory response is activated.

The aim of the study was to investigate possible alterations in apoptotic and inflammatory markers along with CKD stage 1–4 progression, the exact time in the course of CKD when these disturbances occurred and the probable interactions between them.

Subjects and Methods

Patients
One hundred and fifty-two successive CKD stage 1–4 patients (83 men, 55%) with a mean age of 62 years (range 28–88) were enrolled from two renal outpatient clinics. Primary renal diseases were hypertensive nephrosclerosis in 23 patients (15%), diabetic nephropathy (biopsy proven) in 18 (12%), chronic glomerulonephritis in 27 (18%), interstitial nephropathy in 19 (13%), polycystic kidney disease in 8 (5%), miscellaneous in 19 (12%) and unknown in 38 patients (25%).

Methods
Renal function was assessed by measuring estimated glomerular filtration rate (eGFR) using the Modification of Diet in Renal Disease formula [6]. Patient exclusion criteria were acute or chronic infection, active immunologic disease, malignancy, heart failure NYHA stage IV and recent major cardiovascular event, defined as stroke (ischemic or hemorrhagic), angina, myocardial infarction, requirement of coronary intervention and acute limb ischemia during the last 2 months. None of the patients was receiving immunosuppressive drugs at the time of the study.

Study protocol included recording of the demographic characteristics, smoking habits, body mass index (BMI), measurement of blood pressure (BP), comorbidity [hypertension, diabetes mellitus (DM), cardiovascular disease (CVD)] and medications. BP control was defined as 130 mm Hg or lower for systolic and 80 mm Hg or lower for diastolic BP. A full hematologic and biochemical screen was performed at recruitment. All blood samples were drawn after an overnight fasting. Serum biochemistry was determined by ELISA using commercially available kits (Quantikine, R&D systems) and stored at –80°C until assay.

Bcl-2 Expression
A 3-ml blood sample infused in an EDTA vial was incubated at room temperature for 20–24 h without adding culture medium. Erythrocytes were removed by adding 2 ml erythrocyte lysis solution. After 10 min, the sample was centrifuged, washed with phosphate-buffered saline (PBS) and resuspended in 500 µl permeabilization solution. After 15-min incubation in the dark, the sample was washed and 10 µl Bcl-2-PE QuantiBRITE® (1:1; Becton Dickinson) were added to the cells. The samples were incubated for 20 min in the dark, washed with PBS and finally resuspended with 500 µl PBS.

The quantitative expression of Bcl-2 was calculated by QuantCALC®, an analysis software that enables visualization of flow cytometry data in terms of antibodies bound per cell (molecules/cell) [7]. Bcl-2 binding to the cells was expressed as antibodies/cell in three zones: high, medium and low. The median value of the medium zone was used for the analysis, since about 90% of the positive cells were found in this area.

Annexin V – PI Staining
Annexin V detects phosphatidylserine expression on the outer surface of cells during the early apoptotic phase [8]. Once isolated from blood samples anticoagulated with citrate, peripheral blood mononuclear cells were washed with PBS, and density was adjusted to 1 × 10⁶ cells/ml. 5 µl annexin V-FITC (fluorescein isothiocyanate) and 10 µl of PI stock solution were added to 100 µl of cell suspension. After 15 min of incubation in the dark at room temperature, cells were washed and resuspended in 400 µl permeabilization solution. The following controls were used: unstained cells, cells stained with annexin V-FITC (no PI) and cells stained with PI (no annexin-FITC). Cells staining positive for PI were considered as dead cells (necrosis or late apoptosis), cells staining positive only for annexin V were considered as apoptotic (annexin V-FITC+/PI–), and cells negative for both were considered as viable. Cell viability was assessed 1 h after preparation of samples and expressed as percentage of positive apoptotic cells (FACScan flow cytometry instrument).

Serum Markers of Apoptosis and Inflammation
Serum levels of soluble Fas (sFas), Fas ligand (sFasL), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured by ELISA using commercially available kits (Quantikine Research and Diagnostic Systems Europe Ltd, Abington, UK). Serum samples were separated from clotted blood by immediate centrifugation (3,000 g for 10 min for sFas, sFasL and IL-6; 1,500 g for 10 min for TNF-α) and stored at −80°C until assay. For sFas and sFasL, sera were diluted 1:10 for quantification of both parameters, and were not diluted for the estimation of TNF-α and IL-6. The sensitivity of the ELISA system was <30 and 20 pg/ml for sFas and sFasL, respectively, and 0.5 pg/ml for TNF-α and IL-6. The concentrations of these molecules were calculated by reference to standard curves performed with the corresponding recombinant molecule. All serum samples were tested in duplicate.

Statistical Analysis
Continuous variables were expressed as mean ± standard deviation or median with interquartile range. χ² or Fisher’s exact test was used for categorical variables, whereas comparisons of means among the 4 CKD stages were analyzed using one-way analysis of variance and Kruskal-Wallis test as appropriate. Variables exam-
Results

The distribution of patients in the 4 CKD stages, eGFR, demographic and somatometric characteristics, comorbidty and treatment, hematological and biochemical parameters are presented in Table 1. The median eGFR was 46 (29–76) ml/min/1.73 m². There were significant differences in age (p < 0.05) and pulse pressure (p = 0.006) across the 4 CKD stages. Hemoglobin was lower, and Ca²⁺ × PO₄⁻ product was higher in stage 4 compared with the other stages (p < 0.001, p = 0.008). Urine protein, PTH and uric acid increased significantly from CKD stage 1 to 4 (p < 0.001).

Apoptosis and Inflammation across CKD Stages

According to our results, Bcl-2 expression was found to decrease significantly both in lymphocytes and monocytes as CKD stages were advancing (p < 0.001 and p < 0.04, respectively; Table 2). Conversely, sFas levels increased significantly (p < 0.001), and so did serum levels of TNF-α and plasma levels of fibrinogen from CKD stage 1 to 4 (p < 0.003 and p < 0.001, respectively). Serum levels of IL-6 showed a trend to increase with the deterioration of renal function, while CRP, sFasL levels and annexin V staining did not change within CKD stages.

Multivariate Analysis

CKD remained the only independent predictor of Bcl-2Med levels, with CKD stage 4 patients having significantly lower levels compared to stage 1 and 2 patients (Table 3). An eGFR < 60 ml/min/1.73 m², and serum albumin and CRP levels were found to predict Bcl-2Med expression. sFas levels were significantly altered in CKD stages.
3 and 4 and from treatment with statin. TNF-α levels were significantly increased in CKD stages 2–4 and by IL-6 level. Regarding fibrinogen, an eGFR of 60 ml/min/1.73 m² was the threshold for significantly higher levels among CKD patients. Moreover, CRP and serum albumin significantly influenced plasma fibrinogen levels.

**Discussion**

In this study, we investigated levels of specific apoptotic and inflammatory markers across CKD stages 1–4. Interestingly, we were able to demonstrate that the majority of these alterations occurred early in the course of CKD, as soon as patients entered stage 3 of CKD and even earlier. At the same time, we demonstrated that CKD remained a significant predictor for the aggregate of the assessed markers after adjusting for potential confounders.

Uremia has been linked to acquired immune deficiency, and dysregulation of apoptosis in leukocytes has been suggested as a regulatory pathway. Existing data suggest that enhanced apoptosis appears in the progression of CKD and uremic milieu per se is partly responsible for the phenomenon [1–3, 9, 10]. We demonstrated that expression of Bcl-2 decreased significantly in the course of CKD which remained the only independent predictor. A change in Bcl-2 expression in vitro has been shown in predialysis and dialysis patients compared with healthy controls, which was linked with immune defects, but not with renal function [3, 11]. The mechanism that leads to gradually lower Bcl-2 expression in CKD as well as its clinical importance are poorly understood. It has been speculated that the diminished expression of Bcl-2 is responsible for the failure of uremic lymphocytes to rescue from apoptosis by neglect [11].

The Fas/FasL system is a key regulatory apoptotic pathway. In our CKD population, sFas levels were significantly higher in patients with an eGFR<60 ml/min/1.73 m², possibly due to gradually decreasing renal clearance. In accordance to our results, Perianayagam et al. [10] observed a significant inverse correlation between sFas levels and creatinine clearance. Moreover, those of our patients who received statins had lower serum sFas levels compared with those who did not. Studies in populations with different cardiovascular risk factors and in uremic patients have shown that sFas concentrations are elevated, suggesting that sFas may be a novel marker of atherosclerotic vascular damage [12, 13]. Furthermore, the ACTFAST study concluded that atorvastatin lowered sFas level mainly in patients with diabetes or metabolic syndrome through anti-inflammatory effects on the vascular wall. These data are of particular interest considering that Fas/FasL interaction can induce the expression of proinflammatory cytokines implicated in the development of atherosclerosis [14]. Finally, regarding serum sFasL, we observed no significant correlation with renal function or with sFas, and a possible explanation could be that serum sFas may act as a protecting mechanism by binding circulating sFasL and thereby minimizing mediation of cellular apoptosis [10].

We noted gradually increasing levels of TNF-α and fibrinogen with progression of CKD. TNF-α is a key mediator of inflammation and plays a role in apoptosis as well. In animal models, its effects on kidneys include reduction of GFR and increase of albumin permeability, enhanced renal vasoconstriction and hypofiltration via reducing activity of NO [15]. Enhanced TNF-α level has been found in predialysis and dialysis patients compared to healthy controls [16], while two independent groups of investigators have shown an inverse correlation between renal function and TNF-α levels [17, 18]. Several previous studies have demonstrated that increased production

| Table 2. Apoptosis and inflammation markers for all patients and across CKD stages |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | All patients    | CKD 1           | CKD 2           | CKD 3           | CKD 4           |
| Bcl-2LMed, molecules/cell | 1,579 ± 44      | 1,801 ± 115     | 1,799 ± 97      | 1,508 ± 59      | 1,356 ± 81      |
| sFas, pg/ml           | 10,900 ± 275    | 9,176 ± 746     | 8,542 ± 369     | 11,423 ± 403    | 13,462 ± 461    |
| TNF-α, pg/ml          | 1.97 (1.4–4.4)  | 1.43 (0.9–1.98) | 1.68 (1.3–2.5)  | 1.9 (1.4–2.7)   | 2.8 (1.9–3.5)   |
| Fibrinogen, mg/dl     | 377 (301–480)   | 300 (278–323)   | 365 (291–433)   | 385 (294–481)   | 461 (377–630)   |
| IL-6, pg/ml           | 2.9 (2.0–4.37)  | 2.4 (1.6–3.4)   | 2.9 (1.8–4.1)   | 2.8 (2.0–4.8)   | 3.7 (2.3–5.1)   |
| CRP, mg/l             | 2.0 (0.9–4.4)   | 1.6 (0.6–2.9)   | 2.2 (1.0–5.0)   | 2.6 (1.0–5.8)   | 2.0 (0.5–4.2)   |
| sFasL, pg/ml          | 79 ± 32         | 72 ± 23         | 79 ± 31         | 80 ± 34         | 81 ± 35         |
| Annexin V-FITC+/PI–, % | 12.6 ± 8.6      | 12.5 ± 6.8      | 14.9 ± 9.1      | 12.2 ± 8.3      | 10.7 ± 8.9      |

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Apoptosis appeared to increase across CKD stages, and this was associated with increased proinflammatory activity. CKD stage remained a significant predictor for most of the studied apoptotic and inflammatory markers. Further longitudinal studies are needed to clarify the complexity of interaction between apoptotic and inflammatory disorders in non-dialysis CKD patients.

Conclusion

Apoptosis appeared to increase across CKD stages, and this was associated with increased proinflammatory activity. CKD stage remained a significant predictor for most of the studied apoptotic and inflammatory markers. Further longitudinal studies are needed to clarify the complexity of interaction between apoptotic and inflammatory disorders in non-dialysis CKD patients.

Table 3. Adjusted multivariate regression analysis of apoptosis and inflammation markers in CKD patients

<table>
<thead>
<tr>
<th>CKD stage</th>
<th>Bcl-2Med</th>
<th>Bcl-2LMed</th>
<th>sFas</th>
<th>Fibrinogen</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95% lower CI</td>
<td>95% upper CI</td>
<td>p</td>
<td>95% lower CI</td>
<td>95% upper CI</td>
</tr>
<tr>
<td>1 vs. 2</td>
<td>1.82</td>
<td>-160.4</td>
<td>164.12</td>
<td>0.98</td>
<td>-1.83</td>
</tr>
<tr>
<td>1 vs. 3</td>
<td>107.29</td>
<td>-47.5</td>
<td>262.03</td>
<td>0.17</td>
<td>282.08</td>
</tr>
<tr>
<td>1 vs. 4</td>
<td>176.47</td>
<td>14.83</td>
<td>338.10</td>
<td>0.001</td>
<td>455.92</td>
</tr>
<tr>
<td>2 vs. 4</td>
<td>174.65</td>
<td>44.15</td>
<td>305.19</td>
<td>0.001</td>
<td>453.69</td>
</tr>
<tr>
<td>3 vs. 4</td>
<td>69.18</td>
<td>-51.83</td>
<td>190.19</td>
<td>0.15</td>
<td>173.85</td>
</tr>
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<td>sAlb</td>
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<td>-875.2</td>
<td>-200.9</td>
<td>0.001</td>
<td>-131.8</td>
</tr>
<tr>
<td>CRP</td>
<td>-419.4</td>
<td>-712.9</td>
<td>-125.9</td>
<td>0.004</td>
<td>15.2</td>
</tr>
</tbody>
</table>

An adjusted multivariate regression analysis of apoptosis and inflammation markers in CKD patients.
Acknowledgements

The authors thank Aleka Papageorgiou for her excellent secretarial assistance, and Dr. Vianda Stel, Department of Medical Informatics, Academic Medical Center, Amsterdam, for her statistical guidance.

References


Disclosure Statement

The authors state no conflict of interest and have no financial disclosures to report.