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

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Key Words

Apoptosis • Inflammation • Chronic kidney disease

Abstract

Background/Aim: Increased apoptosis along with enhanced inflammation has been reported in hemodialysis and pre-dialysis 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 m² 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- α (TNF- α), 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- α 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 screening was performed at recruitment. All blood samples were drawn after an overnight fasting. Serum biochemistry was determined using an automated analyzer (Olympus AU580). Intact parathormone (iPTH) was measured with an N-IRMA. C-reactive protein (CRP) was measured by immunoturbidimetry (Integra 800, Roche), and plasma fibrinogen levels were measured by visual agglutination (Thrombolyzer Biomerieux). Urine protein excretion was measured in a 24-hour urine sample (Olympus AU580).

The study was approved by the local ethical committee, and patients participated in the study after being informed and having signed a formal consent form.

Peripheral Blood Mononuclear Cell Apoptosis

Apoptosis was assessed in peripheral blood mononuclear cells (lymphocytes and monocytes) at recruitment by measuring Bcl-2 expression, annexin V and propidium iodide (PI) staining using flow cytometry.

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 μ l permeabilization solution. After 15-min incubation in the dark, the sample was washed and 10 μ l 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 μ l PBS.

The quantitative expression of Bcl-2 was calculated by QuantiCALC®, 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 1×10^6 cells/ml. 5 μ l annexin V-FITC (fluorescein isothiocyanate) and 10 μ l of PI stock solution were added to 100 μ l of cell suspension. After 15 min of incubation in the dark at room temperature, cells were washed and resuspended in 400 μ l 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 $\pm$ standard deviation or median with interquartile range. χ^2 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-

Table 1. Demographic and clinical characteristics, eGFR, comorbidity and treatment, hematological and biochemical parameters of the study population

	All patients (n = 152)	CKD 1 (n = 19)	CKD 2 (n = 39)	CKD 3 (n = 54)	CKD 4 (n = 40)	p
Males, %	83 (55)	8 (42)	21 (54)	33 (61)	21 (53)	NS
Age, years	64 ± 12	58 ± 11	63 ± 10	65 ± 13	68 ± 11	<0.05
eGFR, ml/min/1.73 m ²	46 (29–76)	106 (94–118)	75 (69–81)	42 (36–49)	22 (19–27)	<0.001
BMI	29 ± 5	30 ± 4	29 ± 5	29 ± 5	29 ± 5	NS
Current smokers, %	40 (26)	4 (21)	8 (21)	15 (28)	13 (33)	NS
Hypertension, %	136 (90)	17 (90)	33 (85)	50 (93)	36 (90)	NS
Mean BP, mm Hg	100 ± 11	96 ± 10	101 ± 11	100 ± 15	101 ± 15	NS
Pulse BP, mm Hg	57 (46–70)	55 (40–65)	50 (40–64)	60 (50–70)	63 (50–82)	0.006
BP control, %	52 (34)	9 (47)	11 (28)	18 (33)	14 (35)	NS
DM, %	45 (30)	9 (48)	6 (15)	18 (33)	12 (30)	NS
CVD, %	40 (26)	4 (21)	7 (18)	16 (30)	13 (32)	NS
RAA blockers, %	92 (61)	14 (74)	25 (64)	34 (63)	19 (48)	NS
Statins, %	55 (36)	9 (48)	13 (33)	18 (33)	15 (38)	NS
EPO, %	12 (8)	0	1 (3)	3 (6)	8 (20)	0.009
Hb, g/dl	13.2 ± 1.6	13.8 ± 1.2	14.2 ± 1.1	13.2 ± 1.7	12 ± 1.3	<0.001
Total protein, g/dl	7.3 ± 0.5	7.4 ± 0.4	7.3 ± 0.4	7.4 ± 0.5	7.2 ± 0.5	NS
Albumin, g/dl	4.2 (4.1–4.4)	4.3 (4.1–4.4)	4.2 (4.0–4.5)	4.2 (4.1–4.4)	4.2 (4.0–4.4)	NS
Urine protein, mg/24 h	260 (130–920)	176 (108–3,640)	145 (99–212)	251 (130–497)	960 (418–2,135)	<0.001
Ca ²⁺ × PO ₄ [−] , mg ² /dl ²	32.3 ± 6.2	31.2 ± 4.9	30.9 ± 5.4	31.7 ± 6.6	35.2 ± 6.3	0.008
iPTH, pg/ml	71 (47–115)	47 (32–55)	53 (37–90)	63 (47–112)	124 (99–190)	<0.001
Cholesterol, mg/dl	212 ± 44	201 ± 30	215 ± 39	214 ± 45	212 ± 54	NS
Triglycerides, mg/dl	154 ± 77	132 ± 63	141 ± 63	157 ± 88	175 ± 74	NS
Uric acid, mg/dl	6.5 ± 1.8	5.5 ± 1.5	5.5 ± 1.4	7.2 ± 1.9	7 ± 1.6	<0.001

ined included gender, age, smoking, BMI, mean BP, pulse pressure, BP control, DM and history of CVD, hemoglobin (Hb), serum total protein, serum albumin, 24-hour urine protein, uric acid, calcium-phosphate product, iPTH, total cholesterol, triglycerides, CRP, IL-6, treatment with agents blocking the renin-angiotensin axis (RAA blockers), statins and EPO. Factors with $p < 0.05$ in univariate analyses (for online suppl. table see www.karger.com/doi/000345352) entered a multivariate linear regression analysis, while outcome parameters [Bcl-2 monocytes median (Bcl-2MMed), Bcl-2 lymphocytes median (Bcl-2LMed), sFas, TNF- α , fibrinogen] were log-transformed before entering the model. The significance of interactions between variables was tested as well. A $p < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, Ill., USA) statistical software.

Results

The distribution of patients in the 4 CKD stages, eGFR, demographic and somatometric characteristics, comorbidity 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-2MMed 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-2LMed expression. sFas levels were significantly altered in CKD stages

Table 2. Apoptosis and inflammation markers for all patients and across CKD stages

	All patients	CKD 1	CKD 2	CKD 3	CKD 4	p
Bcl-2LMed, molecules/cell	1,579 ± 44	1,801 ± 115	1,799 ± 97	1,508 ± 59	1,356 ± 81	<0.001
Bcl-2MMed, molecules/cell	682 (608–800)	780 (721–900)	769 (635–892)	659 (607–732)	624 (531–709)	<0.04
sFas, pg/ml	10,900 ± 275	9,176 ± 746	8,542 ± 369	11,423 ± 403	13,462 ± 461	<0.001
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)	<0.003
Fibrinogen, mg/dl	377 (301–480)	300 (278–323)	365 (291–433)	385 (294–481)	461 (377–630)	<0.001
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)	NS
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)	NS
sFasL, pg/ml	79 ± 32	72 ± 23	79 ± 31	80 ± 34	81 ± 35	NS
Annexin V-FITC+/PI–, %	12.6 ± 8.6	12.5 ± 6.8	14.9 ± 9.1	12.2 ± 8.3	10.7 ± 8.9	NS

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

rather than reduced renal clearance is a primary cause of rising inflammatory cytokines including TNF- α [18]. Moreover, we found a significant positive correlation between TNF- α and IL-6 serum levels, preserved after adjustment, which could possibly be explained by the reported 'cytokine network' in CKD [4].

Another finding of the present study was the gradual increment of plasma fibrinogen levels with eGFR <60 ml/min/1.73 m² being the threshold. Several studies have resulted in similar findings, while increased production has been suggested as the main pathophysiologic mechanism [19, 20]. Moreover, recent epidemiological data have linked fibrinogen with incident CKD [21], and clinical evidence has suggested that fibrinogen is a risk factor for CKD progression to end stage possibly through the development of atherosclerosis and glomerulosclerosis [22].

In our study, the deterioration of CKD stage was associated with a marginal rise in serum IL-6 and no change in CRP level, while other investigators have found strong negative correlation between IL-6 and CRP levels with eGFR [23, 24]. The discrepancy between the results might suggest that there is diversity in the expression of the inflammatory markers in the CKD population. Another possible explanation could be that CRP and IL-6 levels fluctuate over time, being mainly influenced by comorbidity and subclinical infections; thus, continuous monitoring of these inflammatory markers is recommended as it provides more precise information on the 'real' inflammatory state [25].

Some limitations of this study should be considered. First, we did not include healthy controls as several previous studies have documented that apoptosis and inflammation are significantly higher in CKD patients compared to healthy controls. Second, our study was cross-sectional in nature, which might be the most important limitation. However, the sufficient size of our non-dialysis study population, along with its distribution in all 4 CKD stages, ascribes to the study an advantage in comparison to previous similar studies.

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

CKD stage	Bcl-2MMed					Bcl-2LMed					sFas					Fibrinogen					TNF- α				
	mean difference/ beta	95% lower CI	95% upper CI	p		mean difference/ beta	95% lower CI	95% upper CI	p		mean difference/ beta	95% lower CI	95% upper CI	p		mean difference/ beta	95% lower CI	95% upper CI	p		mean difference/ beta	95% lower CI	95% upper CI	p	
1 vs. 2	1.82	-160.4	164.12	0.98		2.23	-266.2	270.70	0.98		-0.73	-1.51	0.05	0.067		-70.9	-176.9	35.2	0.454		634.54	-1,382.4	2,651.5	1.000	
1 vs. 3	107.29	-47.5	262.03	0.17		282.08	24.83	539.32	0.03		-0.88	-1.63	-0.13	0.021		-105.1	-206.8	-3.2	0.039		-2,247.7	-4,170.7	-324.7	0.012	
1 vs. 4	176.47	14.83	338.10	0.001		455.92	186.30	725.55	0.000		-1.47	-2.25	-0.69	0.000		-209.3	-315.8	-102.8	0.000		-4,286.4	-6,295.1	-2,277.7	0.000	
2 vs. 3	105.47	-16.4	227.37	0.09		279.84	76.57	483.12	0.007		-0.148	-0.736	0.44	0.618		-34.1	-112.9	44.73	1.000		-2,882.4	-4,397.2	-1,367.3	0.000	
2 vs. 4	174.65	44.1	305.19	0.000		453.69	234.96	672.42	0.000		-0.74	-1.37	-0.11	0.001		-138.4	-223.3	-53.5	0.000		-4,920.9	-6,543.3	-3,298.6	0.000	
3 vs. 4	69.18	-51.83	190.19	0.15		173.85	40.95	378.64	0.02		-0.59	-1.17	-0.010	0.016		-104.3	-183.7	-24.8	0.004		-2,038.7	-3,542.6	-534.7	0.002	
sAlb						-538.1	-875.2	-200.9	0.001							-131.8	-210.2	-53.6	0.001						
CRP						-419.4	-712.9	-125.9	0.004							15.2	8.65	21.4	0.000						
Statin no vs. yes											-1,166.8	-2,065.9	-267.69	0.001							0.12	0.03	0.21	0.001	

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Disclosure Statement

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