

The modified proteins in erythrocytes and regulation of erythrocytes volume in patients with chronic kidney disease

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Abstract. – OBJECTIVE: The role of oxidatively modified proteins in progression of chronic kidney disease has been discussed. We have got the results demonstrating the alteration of band 3 protein activity in erythrocytes of patients with chronic kidney disease. We presumed that it might be associated with oxidative damage of intracellular proteins. The purpose of the research was to study the modified proteins (protein reactive carbonyl derivatives, membrane-bounded hemoglobin) in erythrocytes, as well as the regulation of erythrocyte volume in patients with chronic kidney disease.

PATIENTS AND METHODS: 132 patients with various stages of chronic kidney disease and degree of chronic renal failure were divided into four groups. We enrolled 32 healthy subjects. In erythrocytes modified proteins (protein reactive carbonyl derivatives, membrane-bounded hemoglobin) concentrations and activity of Cl⁻/HCO₃⁻-exchanger have been estimated.

RESULTS: The results demonstrated the strong disorder of Cl⁻/HCO₃⁻-exchanger activity in erythrocytes of patients. These data suggested the existence of erythrocytes subpopulations with different activity of Cl⁻/HCO₃⁻-exchangers in bloodstream of patients with chronic kidney disease depending on initial clinical form of the disease. In erythrocytes of all patients, the membrane-bounded hemoglobin concentration and reactive carbonyl derivatives of proteins were significantly higher than in control samples.

CONCLUSIONS: We have assumed that in erythrocytes oxidized hemoglobin interacts with band 3 protein present on erythrocyte membrane. The membrane-bounded hemoglobin increase leads to increased stiffness of the erythrocyte membranes and affects the volume of erythrocytes. We hypothesized that erythrocytes with changed ability to regulate their volume and high concentration of modified proteins contributed to chronic kidney disease progression.

Key words:

Chronic kidney disease, Cl⁻/HCO₃⁻ exchanger, Membrane-bounded hemoglobin, Reactive carbonyl derivatives of proteins.

Introduction

The identification of the oxidatively modified proteins and their possible participation in the development and progression of chronic kidney disease (CKD) has been discussed. As a result of their direct infringement by reactive oxygen species (ROS), the modified proteins have been formed, leading to the oxidation of the amino acid residues and the formation of cross-links. Indirect infringement of proteins led to formation of the wide range of modified proteins: reactive carbonyl derivatives, glycated derivatives, adducts with dialdehydes¹. Oxidation of tyrosines residues in proteins generated advanced oxidative protein products (AOPP)². Previously, we have noticed an increase in the protein reactive carbonyl derivatives concentration in plasma and erythrocytes of patients with chronic glomerulonephritis and chronic pyelonephritis associated with arterial hypertension³. The circulating AOPP detected in the plasma/serum in CKD patients have been regarded as mediators of inflammation because of the AOPP ability to induce oxidative burst in neutrophils and monocytes⁴. Possible mechanism of the AOPP detrimental impact was connected with their participation in kidney fibrosis development⁵. Protein-binding uremic toxins had an ability to induce prooxidant and proinflammatory answers and to inhibit endothelium repair⁶.

The AOPP accumulation in blood plasma has been regarded as a promotor of proteinuria and glomerulosclerosis progression. The AOPP may be able to induce intracellular generation of superoxide anions by NADPH oxidase activation, which leads to p53-Bax dependent apoptosis^{7,8}. However, not all researchers agree with the AOPP assessment as a key marker of oxidative stress and inflammation in CKD⁹.

Recently, we have got the results demonstrating the alteration of band 3 protein activity in erythrocytes in CKD patients¹⁰. We presumed that it might be associated with oxidative damage of intracellular proteins. To test this hypothesis, we studied the modified proteins concentration in red blood cells (RBCs) and the regulation of erythrocyte volume in CKD patients.

The purpose of the research was to study the modified proteins (protein reactive carbonyl derivatives, membrane-bounded hemoglobin) in erythrocytes, as well as the regulation of erythrocyte volume in patients with chronic kidney disease.

Patients and Methods

Several groups of patients with various stages of CKD and degrees of CRF (chronic renal failure) have been created. The first two groups included patients with CKD of 1,2 stages without CRF. The first group included 38 patients with chronic pyelonephritis and CKD of 1-2 stages in the absence of a renal failure (CRF 0). The second group consisted of 21 patients with chronic glomerulonephritis and CKD of 1-2 stages, also without CRF. The third and fourth groups included patients with an initial stage of CRF (CRF1) and the 3rd stage of CKD. The 3rd group (n=44) consisted of patients with chronic pyelonephritis which were on 3 stages of CKD and had CRF 1 stage. The 4th group (n=29) included patients with chronic glomerulonephritis and 3 stages of CKD; CRF of 1 stage has been also diagnosed. Blood of 32 healthy donors has been used for control testing.

The informed consent was received from all the patients before the blood sampling.

Blood samples were taken by venipuncture in the morning. For biochemical researches, blood has been stabilized by heparin. All blood tests have been conducted not later than one hour after the blood collection. Plasma has been separated from erythrocytes by centrifugation. Cl-/HCO₃⁻-exchanger activity has been detected using the

protocol of Mindukshev et al¹¹. Washed erythrocytes were placed in isotonic solution in which sodium ions have been replaced by ammonium ions (140 μM NH₄Cl, 5 μM KCl, 5 μM glucose, 1 μM CaCl₂). Under these conditions, the alkalization of intracellular pH by the penetration of NH₄⁺ led to the activation of surveyed exchanger, regulating entrance of chloride anions, which led to swelling of the cells. The kinetics of cell volume changes (Mean Corpuscular Volume, MCV), in the presence of ammonium load, have been recorded on a hematology analyzer BC-3200 (Mindray, Shenzhen, China) for 5 minutes and expressed in fL. We have also included two ratios: the MCV change (ΔV) and the velocity of ΔV change (vΔV) from 0 to 5 minutes of erythrocytes incubation with ammonium ions. The concentration of intracellular reactive carbonyl derivatives has been detected following the protocol of Levine et al¹². The concentration of membrane-bounded hemoglobin has been detected following the protocol of Toktamysova and Birzhanova¹³.

Statistical Analysis

Comparison of the results obtained has been performed using non-parametric Mann-Whitney U-test (for independent variables) and Wilcoxon matched pairs $p < 0.05$ was considered as statistically significant.

Results

The data demonstrated that prior to ammonium load the MCV erythrocytes in 1, 2 and 3 groups of CKD patients were the same as the MCV erythrocytes in the control patients group. The MCV decrease was observed in the 4th group of patients (84.10±0.70 fL versus 88.39±1.15 of the control ones, $p < 0.05$). After 5 minutes of ammonium load, the MCV (1, 2 and 3 groups) tendency to decrease has been observed. Significant increase of MCV was observed in 4th group of CKD patients (92.25±1.75 fL versus 77.94±3.99 of the control ones, $p < 0.05$).

The ΔV and vΔV analysis showed significant differences within groups and between groups of patients (Table I). Based on the results obtained, the patients of the 1 and 2 groups divided into 2 subgroups based on positive or negative ΔV ratios. We surmised the positive ΔV ratio meant that in 5 minutes after ammonium load, erythrocytes did not reach the maximum volume. The negative ΔV ratio of erythrocytes

Table I. The alteration of ΔV and $v\Delta V$ ratios of erythrocytes in 5 minutes after ammonium load ($M \pm m$).

Groups	ΔV		$v\Delta V$	Membrane-bounded hemoglobin (%)	Protein reactive carbonyl derivatives (nmoles/ml)
	Subgroup 1	Subgroup 2			
Group N 1. Patients with CKD 1,2 (CRF0), (chronic glomerulonephritis as initial clinical form of the disease)	$8.17 \pm 0.70^*$	$-13.60 \pm 1.69^*$	6.42 ± 2.03	$9.86 \pm 1.15^*$	$15.19 \pm 1.53^*$
Group N 2. Patients with CKD 1,2 (CRF0) (chronic pyelonephritis as initial clinical form of the disease)	$8.38 \pm 1.78^*$	$-10.40 \pm 2.27^*$	$4.79 \pm 1.13^*$	$8.19 \pm 1.02^*$	$16.51 \pm 1.53^*$
Group N 3. Patients with CKD 3 (CRF1), (chronic pyelonephritis as initial clinical form of the disease)	$-7.23 \pm 2.16^*$		6.61 ± 1.46	$7.14 \pm 2.25^*$	$18.62 \pm 2.15^*$
Group N 4. Patients with CKD 3 (CRF1) (chronic glomerulonephritis as initial clinical form of the disease)	$8.15 \pm 1.05^*$		$1.40 \pm 0.60^*$	$12.10 \pm 1.05^*$	$19.07 \pm 2.58^*$
Control subjects	-20.31 ± 2.79		7.66 ± 1.47	5.2 ± 0.02	9.62 ± 2.22

*in comparison to control subjects ($p \leq 0.05$)

in 5 minutes after ammonium load showed the tendency to decrease erythrocyte volume. Group differences in relative ΔV ratios have been observed for all the tests subjects. Different changes in ΔV ratio in patients of the 3 and 4 groups have been noted.

In all patients of 1-4 groups the velocity of erythrocyte volume regulation ($v\Delta V$ ratios) was lower than in controls samples, especially those related to the group 4 patients ($p < 0.05$).

The results obtained demonstrated the strong disorder of $\text{Cl}^-/\text{HCO}_3^-$ - exchanger activity in erythrocytes of CKD patients depending on the initial clinical form of the disease. These data suggest the existence of erythrocytes subpopulations with different activity of $\text{Cl}^-/\text{HCO}_3^-$ -exchangers in bloodstream of CKD patients. The disorder of $\text{Cl}^-/\text{HCO}_3^-$ -exchanger activity might be determined by the virtue of its modifications, including oxidative damage, or by means of structural changes in erythrocyte membranes that affect the activity of that integral protein complex.

Reduction/extension of the time period ΔMCV depended on the prevalence or low resistant or highly resistant subpopulations of red blood cells in circulation.

We surmised that it also might be determined by alteration as a result of association with membrane-bounded hemoglobin (Table I). In all pa-

tients of 1-4 groups, the membrane-bounded hemoglobin concentration and reactive carbonyl derivatives of proteins were significantly higher than in controls samples ($p < 0.05$). The data show synchronous increase of modified proteins and alteration of the erythrocyte volume regulation in CKD patients.

Discussion

The results obtained have demonstrated significant increase in reactive carbonyl derivatives of proteins and membrane-bounded hemoglobin in CKD patients. These biochemical changes were associated with alteration of activity of $\text{Cl}^-/\text{HCO}_3^-$ -exchangers in RBC in CKD patients. We have surmised that the hemoglobin and cytoskeletal proteins may be the most likely targets for oxidative damage. The origin of membrane-bounded hemoglobin is not clear yet. It is known that membrane-bounded hemoglobin has high ability to generate H_2O_2 ¹⁴. It could support high prooxidant potential into red blood cells.

We have assumed that in erythrocytes of CKD patients oxidized hemoglobin interacts with band 3 protein present on RBC membrane. This interaction induces the cascade of different metabolic

alteration including ion imbalances (increased calcium leakage into the RBC, leakage of potassium out of the RBC). Ions imbalance causes cell shrinkage, impaired deformability¹⁴ and may affect hemoglobin level¹⁵.

The membrane-bounded hemoglobin increase leads to increased stiffness of the RBC membrane. Oxidative stress can induce the damage of spectrin and other cytoskeletal components¹⁶. Under oxidative stress spectrin can be aggregated with hemoglobin and other intracellular proteins, inducing alteration of RBCs metabolism¹⁷.

Oxidative modification of erythrocytes affects the shape, volume and deformability of red blood cells. This is supported by our data on the dysregulation of cell volume due to decrease in the activity of Cl/HCO₃⁻ exchangers. RBCs with reduced ability to regulate their volume undergo rapid elimination from the circulation. Oxidative stress contributes to the uptake of erythrocytes by macrophages, which plays a major role in the removal of RBCs from circulation¹⁸. Together, all those molecular events contribute to development of anemia in CKD patients.

We suggested that red blood cells with reduced ability to regulate its volume would be less resistant to shear stress and may easily undergo hemolysis and/or may be involved in the formation of microparticles. The role of microparticles in blood derived from platelets, white blood cells and endothelium in pathogenesis and progression of CKD has been discussed¹⁹⁻²². It is likely that RBC in CKD patients may release microparticles, which have deleterious effects by promoting coagulation and inflammation or by modifying endothelial function. Camus et al²³ have assumed that microparticles in bloodstream derived from RBCs can induce renal vascular occlusion and that microparticles are derived from RBCs to promote apoptosis of endothelial cells through the generation of ROS and adhesion of erythrocytes to the endothelium.

Conclusions

Our results demonstrate the significant molecular changes occurring in erythrocytes from CKD patients. The circulation of erythrocytes with altered physico-chemical properties and impaired metabolic status contribute significantly to the progression of chronic kidney disease and must be regarded as additional pathogenic factor.

Conflict of interest

The Authors declare that they have no conflict of interests.

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