OXIDATIVE STRESS AND TRACE ELEMENTS IN PROTEINURIC PATIENTS

PROTEİNÜRİK HASTALarda OKSIDATİF "STRESS" VE ESER ELEMENT SEVİYELERİ

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ÖZET

Proteinürik hastalarda eritrosit deformabilitesinin azaldığını gösterdiğimiz sonradan, buna sebep olabilecek etkenlerden biri serbest oksijen radikallerinin ve/veya antioksidan sistemin değişimleri olabileceğini düşündük. Eritrosit ve plazma malonildialdehid, eritrosit glutathione, eritrosit glutathione redüktaz aktivitesi, eritrosit glutathione peroksidaz ve eritrosit sodyum dismutaz aktiviteleri, oksidatif ve antioksidan sistem enzimleri olarak belirlendi. Antioksidanlar eser elementlere bağlı metalloenzimler olduklarından ötürü, onların değişimini etkileyebilecek plazma ve idrar çinko, bakır ve demir seviyeleri ölçüldü.

Eritrosit deformabilitesi intraselüler oksidasyon stresine ve azalmış antioksidan sisteme bağlı olarak bozulmuş olduğu kabul edildi. İdrarda aşırı eser element kaybı ise azalmış savunma mekanizmasının temelinde yattığı düşünüldü.

Anahtar Kelimeler: Lipid peroksidasyonu, malonildialdehid, sodyum dismutaz, eritrosit deformabilitesi, proteinüri, eser elementler

INTRODUCTION

Anemia, in different degrees of chronic renal failure is correlated directly with the extent of renal insufficiency. Within several factors, leading to anemia in renal failure, shortened red blood cell survival is always mentioned. Normal red cell deformability is the most important determinant for red cell life span in vivo and also for the microcirculation. The red cell deformability or the membrane stiffness is determined on one hand by the shape factor, that means the surface to volume ratio and on the other hand by the cellular contents (hemoglogin) and the membrane factor. To explain one of the possible mechanisms for the shortened survival of red blood cells, lipid-peroxidation in erythrocyte membrane structures, with accompanied increase of malondialdehyde (MDA) concentrations, as markers for oxidative stress, have been reported by several authors (1,2,3). That the membrane is less deformable can be shown by measurements of viscosity at different shear rates.(4,10,16).

It is shown that free radicals increase in renal failure (1,2,3). We also know that intracellular antioxidant levels decrease in renal failure, and this also favours lipid peroxidation (5,8). In healthy subjects, the antioxidant system works successfully against the different oxidants. For the hemodialysis population the metabolic situation is investigated by many authors, and the balance between the oxidant and antioxidant system

SUMMARY

After having shown decreased red cell deformability in proteinuric patients we planned this experimental study, in order to investigate a possible role of free oxygen radicals and the antioxidant system. Twelve proteinuric patients were compared with 20 age and sex-matched healthy controls. Erythrocyte and plasma malonyldialdehyde, erythrocyte glutathione, erythrocyte glutathione reductase, erythrocyte sodium-dismutase and erythrocyte peroxidase activities were measured.

Because of a possible effect of trace elements on the metalloenzyme activities zinc, copper and iron levels in blood and urine were determined. It is suggested that the red cell deformability was decreased because of increased malonyldialdehyde levels and decreased activity of the antioxidant system, may be due to high loss of urinary trace elements.

Key Words: Lipid peroxidation, malonyldialdehyde, sodium dismutase, red cell deformability, proteinurii, trace elements
is been found disturbed more from the uremic state than from the hemodialysis treatment (1,2,8).

The role of trace elements in the oxidative stress process was described by Halliwell and Gutteridge (6). They found that the presence of ferrous and ferric ions caused a series of radical reactions, among which the best known is the Fenton reaction, leading to the formation of the hydroxyl radical OH-. The hyperreactivity of this radical is directed against lipids and proteins, as well as nucleic acids (7), and the resulting structural modifications and fragmentations cause irreparable damage to the cell.

SOD (sodium-dismutase) is one of the antiradical activity enzymes. It is shown to be diminished in the hemodialysis population (8). This enzyme is known to depend on Zn and Cu concentrations. Forman and Fridovich (25) described the effects of Zn and Cu in the structure of this enzyme. Its catalytic activity depends on the presence of a prosthetic group containing Cu. Zn stabilizes the apoenzyme in the native configuration. This enzyme would be rendered fragile by the low Zn levels in renal failure patients.

MDA (as a marker for lipid peroxidation) and GSH (as a marker for defense mechanism for oxidative stress) are inversely affected in the hemodialysis population and in the patients with severe chronic renal failure (serum creatinine of about 4 mg/dl) (9).

According to this background, we wondered if in a patient group with moderately or even no loss of renal function (glomerular filtration rate (GFR) mean 70±38ml/min) but significant proteinuria, the red cell membrane deformability is decreased and if the oxidant/antioxidant system is altered. In a patient group with different degrees of renal failure and proteinuria, the levels of erythrocyte and plasma malodialdehyde (MDA), as markers for intra-and extracellular lipid peroxidation, and erythrocyte glutathione (E-GSH) and plasma-SOD level, as antioxidants were measured. Because of their well-known role in the oxidative process, the plasma and urine levels of three trace elements copper (Cu), iron (Fe), and zinc (Zn) were determined.

MATERIAL AND METHOD

Seven female and five male patients with an average age of 30±17 (range 17-65), a mean glomerular filtration rate of 70±38ml/min and a mean proteinuria of 6.5±5.0 g/day were enrolled in the study. Two patients had membranoproliferative glomerulonephritis, four patients had membranous glomerulonephritis, two had amyloidosis secondary to now inactive tuberculosis, one patient had chronic pyelonephritis and two patients had diffuse proliferative glomerulonephritis. This group was compared with an age and sex matched healthy control group. (Patient characteristics are shown in Table 1).

Table 1: Characteristics of patients and control group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Patients</th>
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<tbody>
<tr>
<td>N</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>Age (years)</td>
<td>33±10</td>
<td>30±17</td>
</tr>
<tr>
<td>Male/female</td>
<td>10/10</td>
<td>5/7</td>
</tr>
<tr>
<td>Arterial pressure (systolic/diastolic mm Hg)</td>
<td>105±10/67±12</td>
<td>123±17/80±13</td>
</tr>
<tr>
<td>Blood hematocrit %</td>
<td>41 ±4.5</td>
<td>33±5.4</td>
</tr>
<tr>
<td>Pl. urea(mg/dl)</td>
<td>30.5±15</td>
<td>53.5+28</td>
</tr>
<tr>
<td>Pl. creatinine (mg/dl)</td>
<td>0.81 ±0.24</td>
<td>1.4±0.95</td>
</tr>
<tr>
<td>Proteinuria (g/day)</td>
<td>0.001 1 ±0.02</td>
<td>6.53±5.05</td>
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20 healthy volunteers of the Biophysics Department with an average age of 33+10 and male to female ratio 1:1 with no clinical signs, and no pathologic biochemical findings of metabolic disease and without medical treatment, were chosen as control group.

Biochemical evaluation and blood counts were performed with a multianalyzer Technicon in the biochemistry laboratory of the internal medicine department.

Patients with diabetes mellitus, end-stage renal failure, chronic respiratory disease and those treated with iron preparation were excluded. None of the patients was smoking. Proteinuria was significant in all patients and each value was corrected by correlating to body surface. Also glomerular filtration rate was corrected for different body surfaces. For accurate blood pressure measurement all patients were measured three times on different days and the day when the blood sample was obtained. Blood samples were taken fasting and 24 hours collected urine was taken for trace element evaluation, GFR and Proteinuria determination. All patients were asked to give their informed written consents.

Six patients were on no medication, from the other six, two patients received amloidipin 5 mg/day, two were taking dipyriramole 75 mg/twice a day and two other patients were set on Furosemide 40 mg/day. The patients examined had not undergone any hypolipidemic drug therapy nor any iron, zinc or
copper or antioxidant drug therapy at least for two months before examination. In this stage of renal failure there was no need for treatment of secondary hyperparathyroidism.

**Hemorheological Methods**

**Whole blood viscosity** determination was made with help of a differential speed rotational viscometer, the Wellis-Brookfield viscometer, Model CV-DV III, Brookfield laboratories, Stoughton, Massachusetts, incorporating a cone-plate type spindle arrangement and circulating constant temperature both at 37 degrees C. The first reading for each specimen was taken at 7.5/sec, after one minute of rotation, and subsequent reading were made at one minute intervals with decreasing shear rates. The shear rates used varied from 600 to 225/second. The shear rate was calculated directly from the cone angle (0.8 deg), the conversion factor relating shear stress to scale reading was determined using viscosity standard calibrating oils.

**Erythrocyte Deformability**

**Red cell deformability** was determined by a stroboscopic recording centrifuge method (23). Blood samples hematocrit values were fixed to 40%. The samples were taken in to microhematocrit tubes, placed on the hematocrit centrifuge plate containing a millimetric scale. Erythrocyte column length which was separated from plasma was read at 3,000 rpm using a stroboscope and recorded at 10th, 15th, 30th, 45th, 60th, 90th, 120th and 240th steps. Initial slope of the trend which was obtained from hematocrit changes time dependently gave us erythrocyte membrane stiffness as hematocrit/min. Using this method requires the minimum quantity of whole blood and results can be obtained within several minutes. Attempts to correlate using the filtration technique have not been satisfactory. With the filtration technique the red cells are hardened by various physico-chemical methods and this causes difficulties to obtain reproducible results (24). Also sucking cells into pipettes require large number of measurementst to obtain representative samples.

**Hematocrit**

In the Nephrology department hematocrit value was determined with a multianalyzer from a venous blood sample.

At the Biophysics department hematocrits of the same persons were measured again with the microhematocrit centrifuge method.

**Method for determination of erythrocyte and plasma malondialdehyde**

(E-MDA, P-MDA)

Plasma and erythrocyte malonyldialdehyde (PMUA, EMDA) levels were measured by the method of Buege ane Aust (11). One volume of plasma or erythrocytes was mixed with two volumes of a stock solution of 15% (w/v) trichloracetic acid, 0,375% (w/v) thiobarbituric acid and 0.25 mol/l (w/v) hydrochloric acid. The mixture was heated for 30 minutes in boiling water bath. The absorbance of the clear supernatant is determined at 535 nm and concentration was calculated using a molar absorption coefficient of 1,56x105 M^-1cm^-1. The intra-inter assay coefficients of variation for MDA were 4.7% and 4.9% respectively.

**Erythrocyte Glutathione (E.GSH)** concentration were measured by Beutlers' method (12). After proteins were deproteinized using metophosphoric acid, the colour developed in the presence of dithio-bis-2-nitrobenzic acid in sodiumphosphate buffer(pH:7.8). The absorbance at 412nm was measured. Results were expressed in imol/gHb. The intra and inter assay coefficient of GSH were 4.4% and 4.8% respectively.

**Erythrocyte GSH-Peroxidase(E.GSH-Px)** activity was determined by the method of Paglia and Valentine (13). Enzyme activity was determined from the oxidation of NADPH in the presence of H2O2 and monitored spectrophotometrically at 340 nm. Results were expressed in terms of U/gHb.

**Erythrocyte GSH Reductase (E.GSH-Red)** activity was determined by following the rate of NADPH oxidation in the presence of oxidized GSH (10d). One unit enzyme activity was defined as 1 imol NADPH oxidised per minute. Activity was expressed as U/gHb.

**Erythrocyte Cu-Zn-SOD (E.SOD)** activity was determined by the method of Sun et al (15). The assay involves inhibition of nitroblue tetrazolium (NBT) reduction with xanthine-xanthine oxidase used as superoxide generator. One unit of SOD is defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%.

**Plasma zinc (Zn), copper (Cu) and iron (Fe)** levels were measured by atomic absorption spectrophotometry (AAS-Shimadzu AA 680 Japan). AA680 was calibrated with standard solutions of Zn as 0.5 and 1.0 ig/ml, Cu as 0.5 and 1.0 ig/ml and Fe as 1.0 and 2.0 ig/ml. Plasma samples were directly aspirated. Results were expressed as ig/ml. Spectral absorption photometer analysis from a sample taken of a 24 hour urine collection was done for evaluation of 24 hour urinary loss of Zn, Cu and Fe in ig/dl.

Serum urea, creatinine and protein concentrations were measured by commercial enzymatic kits (Sigma Chem Co. USA)

**Statistical evaluation**

Results were expressed in terms of means and standard deviations. Statistical evaluation was done by using Student-T test and regression and correlation analysis of Windows statistical programm.
p values less than 0.05 were rendered to be significant.

RESULTS

Mean arterial pressure was 101±13.5 mm Hg, mean systolic pressure was 123±17 mm Hg and mean diastolic pressure was 80±13 mm Hg in the patient group. Plasma total protein concentration revealed a value of 4.7±1.04 g/l, with an albumin fraction of 2.2±0.8 g/l. Daily proteinuria was 6.53±5.05 g/day with a range of 1.8-15 g/day. Blood urea and creatinine 53.5±28 mg/dl and 1.4±0.95 mg/dl respectively. (see table 1)

Hematocrit in the patient group was 33±5.4 % and 41±4.5 % in the control group. Against our expectations the hematocrit of the patient group was found low for the grade of renal insufficiency. In the patient group two patients had hematocrit values which were 20 and 24%. The female patient (17 years) with the hematocrit value of 20% had a plasma creatinine of 2.1 mg% and a proliferative glomerulonephritis with acute exacerbation while taking part in the study. The female patient (63 years) with the hematocrit value of 24% had a membranous glomerulonephritis with an underlying Lupus erythematodes which was diagnosed very late.

The mean values and standard deviations for plasma zinc, copper and iron in plasma and urine can be seen in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Patient group</th>
<th>Control group</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>P.MDA (nmol/ml)</td>
<td>5.9±1.15</td>
<td>2.9±0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E.MDA (nmol/ml)</td>
<td>231.5±43.1</td>
<td>150±50</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E.GSH (nmol/gHb)</td>
<td>6.5±1.48</td>
<td>11.8±0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E.GSH-Red (U/gHb)</td>
<td>5.8±1.8</td>
<td>8.5±1.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E.SOD (U/gHb)</td>
<td>875.5±69.7</td>
<td>1140.5±47.5</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Blood viscosity was decreased at all shear rates that means, in all caliber vessels from capillaries to arterioles. At 3 rpm (adequate to 22.5/sec) 4.4±1.4 in the patient group but 9.6±2.8 in the control group (p<0.001). At 15 rpm (adequate to 112.5/sec) to 3.5±0.8 versus 6.6±1.9 in the control group (p<0.001) and at 30 rpm (adequate to 225/sec) blood viscosity was 3.25±0.65 in the patient group versus 4.15±0.7 in controls (p<0.001). Erythrocyte membrane stiffness was high in the patient group with 17.55±4.0 versus 11.9±3.5 in the healthy control group (p<0.001). (see table 4). This means that the deformability of red cells was decreased, the suffer the membrane, the lesser the deformability!

Plasma -MDA in the patient group was elevated with 5.9±1.15 nmol/ml in comparison to 2.9±0.4 nmol/ml in the control group on the p<0.001 level, (see table 2)

Erythrocyte -MDA level in the patient group was elevated with 231.5±43.1 nmol/g Hb in comparison to the control group (150±50 nmol/g Hb) on the p<0.001 level, (see table 2)

Erythrocyte glutathione reductase level was 5.8±1.8 in the patients and 8.5±1.5 U/gHb(p<0.001). (see table 2)

Erythrocyte SOD level in the control group was 1140±47.5 and 875.5±69.7 U/gHb(p<0.001). (see table 2)

Plasma zinc level was decreased in the patient group 69.13±26.28 ug/dl compared to the level of 77.08±14.56 ug/dl in the control group but this was not significant (p>0.05). (see table 3)

Plasma iron level was elevated non-significantly in the patient group (p>0.05) but plasma copper (100.81 + 17.24 control - 73.45±42.77 patients) was significantly diminished compared to the control group (p<0.05). (see table 3)

Urinary Fe was 814±750 ig/dl in patients, but 40±15 in the healthy control group. Urinary Zn was 872±507 ig/dl in the patients and 512±100 ig/dl in control. Urine copper was 399±182 ig/dl and 55±12 ig/dl in the control group. All three were high
concentrations in regular hemodialysis patients, could contribute to slow down SOD activity. We could show the decrease only for copper. On the other hand, SOD activity may be directly inactivated by the production of superoxide ions and hydrogen peroxide which could be enhanced in both patient groups (22).

Richard et al. stated in their investigation about trace elements and lipid peroxidation abnormalities (8) that the altered status of Zn besides Se is associated with hyperlipoperoxidation and that this situation is existing before dialysis. In our study differences in the plasma levels of zinc or iron could not be shown, but urinary losses were significantly high in the patient group. The link to proteinuric patients can be over the extracellular antioxidant system, which includes besides plasma tocopherol and urate, also transferrin, haptoglobin and ceruloplasmin. All proteinuric patients lost daily at least 18 up to 15 g nonselective protein. The small proteins like haptoglobin, ceruloplasmin and transferrin were surely lost in great amounts. The deficient antioxidant activity in our patient group might be partly related to this fact. The role of trace elements in the oxidative stress process was described by Halliwell and Gutteridge (6). They found that the presence of ferrous and ferric ions caused a series of radical reactions, among which the best known is the Fenton reaction, leading to the formation of the hydroxyl radical OH-. The hyperreactivity of this radical is directed against lipids and proteins, as well as nucleic acids (7), and the resulting structural modifications and fragmentations cause irreparable damage to the cell. In the present study, we did not find a significant rise of Fe in the patient group. This can be due to the small number of patients.

SOD (sodium-dismutase) is one of the antiradical activity enzymes. It is shown to be diminished in the hemodialysis population (8) This enzyme is known to depend on Zn and Cu concentrations. Forman and Fridovich (25) described the effects of Zn and Cu in the structure of this enzyme. Its catalytic activity depends on the presence of a prosthetic group containing Cu. Zn stabilizes the apoenzyme in the native configuration. This enzyme would be rendered fragile by the low Zn levels in renal failure patients. In our study Zn levels were not significantly low, but Cu concentration was (p<0.05). Any disturbance in the nutritional balance of trace elements thus reduces the efficacy of antiradical defence mechanisms and increases the susceptibility of the organism to damage caused by radicals. Urinary losses and may be high turnover of the-trace elements could have lead to disturbances in our patient group. Similar disturbances of trace elements have been described especially for Zn, Cu and Selenium (Sc) in the dialysis population (8).
CONCLUSION

The present study confirms the existence of a sustained oxidizing phenomenon, intra- and extracellular, in proteinuric patients with no to moderate renal failure. Our results also confirm a disturbance in the enzyme systems which detoxify free radicals in these patients, i.e. antioxidant system. The red cell deformability is diminished, probably due to intracellular oxidizing stress and defective antioxidant activity. The high loss of trace elements in urine could contribute to the demonstrated decreased defence mechanisms. Further studies, with more patients and determination of parameters like haptoglobin, coeruloplasmin and transferrin may help to elucidate the altered oxidant/antioxidant system in proteinuria.

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