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Increase of erythrocyte resistance to hemolysis and modification of membrane lipids induced by hemodialysis

Evelyne Peuchant [1], Christine Salles [1], Catherine Vallot [2], Christian Wone [2] and Raymond Jensen [1]

[1] *Laboratory of Biochemistry and* [2] *Department of Nephrology, Hôpital Saint-André, Bordeaux (France)*

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Summary

Mechanical fragility, deformability and lipid composition of erythrocyte membranes were studied in 22 uremic dialysed patients before and after dialysis in comparison to controls. While deformability was not affected by a dialysis session, osmotic and saponin resistances to hemolysis were significantly increased after dialysis ($p < 0.001$). The lipid composition of erythrocyte membranes was also altered during dialysis, with a notable increase in cholesterol and a different phospholipid distribution, i.e. phosphatidylcholine and sphingomyelin significantly decreased ($p < 0.001$). Likewise, the fatty acid distribution showed large variations with an increase in polyunsaturated fatty acid. The acute effect of dialysis on the membrane lipid composition seems to be linked to erythrocyte alterations. Its significance is discussed in relation to the asymmetric transverse distribution of phospholipids previously described in human erythrocyte.

Introduction

Survival of erythrocytes is shortened in patients with renal failure [1,2] and in those treated with maintenance hemodialysis [3]. This is one of the factors implicated in the anemia associated with uremia [4].

Other functions characteristic of erythrocyte membranes may also be altered in these patients. For instance, the mechanical fragility of red cell membranes has been

Correspondence to: Raymond Jensen, Laboratory of Biochemistry, Hôpital Saint-André, Bordeaux, France.

found to increase in uremic hemodialysed patients in comparison to normal individuals [4–6], while the deformability of erythrocytes was impaired [6–8].

Cholesterol is known to play an important role in the flexibility of red cell membranes [9,10]. Modifications in lipid composition have been displayed in the erythrocytes of patients on hemodialysis, and these alterations might be related to changes in the rheological properties of red cells [11,12].

Our aim was to study the influence of a hemodialysis session on the properties of erythrocytes from uremic patients, such as hemolytic resistance and deformability, and on the composition and distribution of membrane lipids before and immediately after dialysis.

Materials and methods

Patients

Clinical details concerning the patients are listed in Table I.

Twenty-two uremic patients (both sexes, aged 21–79 yr) undergoing maintenance hemodialysis were studied. Primary renal diseases included: chronic glomerulonephritis (9 cases), polycystic kidney disease (6 cases), nephroangiosclerosis (4 cases),

TABLE I

Clinical and biological details of uremic dialysed patients

Patients	Years of dialysis	Value of hemoglobin (g/l)	Value of immunoreactive PTH (pg/ml)
1	11	105	200
2	7	85	640
3	10	98	2800
4	3	105	219
5	12	100	4000
6	6	75	355
7	2	120	90
8	14	89	4400
9	2	84	120
10	8	115	109
11	2	60	110
12	4	105	86
13	12	70	176
14	3	109	131
15	4	100	260
16	3	102	220
17	13	68	1225
18	3	95	130
19	5	92	119
20	4	77	127
21	11	97	154
22	4	82	130

diabetes (2 cases) and nephrocalcinosis (1 case). Hemoglobin values ranged from 60 to 120 g/l. All patients had been dialysed three times a week for an average of 6.5 yr (range 3–14 yr), each session lasting 4 h. Dialysis was carried out on plate and capillary dialysers with dialysis water containing 35 mmol/l of acetate. Blood was taken from the arterio-venous fistula before and immediately after dialysis. The control group consisted of 20 healthy volunteers aged 25–48 yr who had no history of hematologic or renal diseases. Blood samples were drawn from the cubital vein from fasting patients.

Hemolytic resistance

To evaluate the hemolytic resistance of red blood cells (RBC), two tests were used: osmotic fragility and resistance to the hemolytic agent saponin. Heparinized blood was collected from healthy subjects and from the uremic patients on maintenance hemodialysis just before connection to and disconnection from the machine. The samples were centrifuged for 5 min at $3000 \times g$ and the plasma was discarded. The packed erythrocytes were washed once with a solution containing 0.153 mol/l NaCl and 10 μmol/l EDTA and twice with isotonic phosphate buffered saline solution: PBS (120 mmol/l NaCl–2.7 mmol/l KCl) in 10 mmol/l phosphate buffer, pH 7.4). The final washed RBC were suspended in PBS to obtain 10% hematocrit.

RBC osmotic fragility was evaluated by the method of Parpart et al. [13]. Twenty μl of the RBC suspension from each individual were incubated in different solutions containing NaCl concentrations ranging from 0.03 to 0.117 mol/l. Hemolysis was carried out for 30 min at room temperature. After centrifugation at $3000 \times g$ for 10 min at 4° C, the absorbance of the supernate was determined with a Seconam spectrophotometer (Labinter, Aix en Provence, France) at 540 nm, and the percentage of RBC lysed in each solution was calculated. The concentration of NaCl at which 50% of RBC were lysed was considered as the median osmotic fragility (MOF).

Hemolytic resistance to saponin (Sigma, St Louis, MO, USA) was determined by incubating 20 μl of each RBC suspension in different solutions containing saponin concentrations ranging from 0 to 30 mg/l. After incubation in a shaking water bath at 37° C for 30 min, the tubes were centrifuged at $3000 \times g$ for 10 min at 4° C and the absorption of the supernate was measured at 540 nm. The concentration of saponin at which 50% of RBC were lysed was determined as before.

Deformability

Erythrocyte deformability was measured according to the method of Kiesewetter et al. [14] using a cell transit time analysis (CTTA) device (Abx Snag-Duroc, Le Vallois, France) in which the transit time of a red blood cell passing through a 5 μm diameter pore was measured. The principle is as follows: a highly diluted red cell suspension passing through a few pores changes the electric conductance when a cell occupies a pore. The cell transit time is calculated from the signal derived from the conductance variation. Coincident cells are rejected. The transit time of up to 1000 cells can be obtained within one minute. The distribution of cell transit time is statistically analysed [15].

Erythrocyte membrane lipid analysis

Blood was collected into tubes containing 0.129 mol/l trisodium citrate (1 vol/9 vol blood) and RBC were washed three times with PBS buffer pH 7.4; the RBC were then suspended in 0.153 mol/l NaCl to provide a 50% hematocrit, and a sample was taken for red cell counting on the Coulter Counter model STKR (Coulter, Coultronics, Margency, France). Ghosts were obtained by putting 200 μl of the RBC suspension at -20° C for 2 h followed by 5 min at 37° C.

The ghosts were then treated with 5 ml of the solvent hexane/isopropanol (2/1, v/v) to extract lipids from the membranes. After centrifugation at $3000 \times g$ for 10 min at 4° C, a portion of the extract was used to determine cholesterol by an enzymatic method [16] using the reagents of BioMérieux (Charbonnière les Bains, France). Lipid phosphorus was measured by the technique of Ames [17]. Phospholipid classes were determined by thin-layer chromatography associated with flame detection ionization using a Iatroscan TH10 Mark II analyser (Iatroscan Labs., Tokyo, Japan) according to the method described by Tsuchiya and Sugai [18].

Peaks were integrated and identified by comparisons with pure standards (Sigma).

Fatty acid composition of phospholipids was determined from the lipid extract after obtaining isopropylic esters. For this, 2 ml of the lipid extract was treated with 50 μl of H_2SO_4 and then heated in a boiling water bath at 100° C for 1 h. After the addition of 2 ml of hexane and 3 ml of water, the contents were shaken and the newly formed fatty acid isopropylic esters were recuperated in the upper phase and directly chromatographed on a Carlo-Erba gas chromatograph model HRGC 5160. A Chrompack carbowax column (52 CB) was used with a temperature program ranging from 195 to 215° C, increasing by 2°5 per min and with pure nitrogen as the carrier gas. The temperature was 250° C for the injector and 240° C for the ionization detector. The fatty acids were identified by comparing the retention times with those of the various standards chromatographed under the same conditions.

Statistical evaluation

Data are presented as mean $\pm$ SD. Statistical analysis were determined by Student's *t*-test and the two-tailed Mann-Whitney test.

Results

Hemolytic resistance

The results of the osmotic and saponin resistances of the RBC of uremic patients before and immediatly after dialysis are presented in Figs. 1 and 2. In both cases, median fragility was altered when compared with controls.

As for osmotic hemolysis before dialysis, 21 of the 22 patients had above normal values of MOF (0.082 ± 0.003), causing the RBC to be more susceptible to hypotonic lysis when compared to controls (0.078 ± 0.002, $p < 0.001$). However, the values calculated after dialysis were significantly lower (0.079 ± 0.003, $p < 0.001$) and reached the control mean value, thus indicating an increase of erythrocyte osmotic resistance.

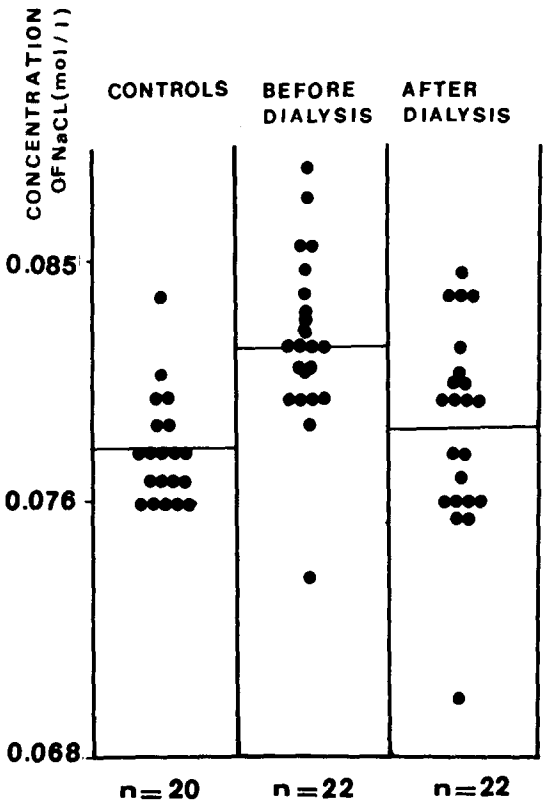

Fig. 1. Comparison of the NaCl concentrations required to obtain the MOF values in RBC of controls and uremic patients before and after dialysis.

Before dialysis, most patients required higher concentrations of saponin (10.00 ± 0.69 mg/l) than controls (9.29 ± 0.61 mg/l, $p < 0.01$) to hemolyse 50% of RBC, indicating a slight initial resistance of the erythrocytes of these patients. After dialysis, the resistance of RBC was significantly increased, since the concentration of saponin required to hemolyse 50% of RBC (12.03 ± 1.19 mg/l) was significantly higher than before dialysis (10.00 ± 0.69, $p < 0.001$).

Deformability

The deformability of the RBC (Fig. 3) in uremic patients before dialysis was impaired when compared to controls. The proportion of cells having a transit time greater than 2 ms was more important in patients before dialysis, giving a high mean cell transit time. After dialysis no difference was noted in the cell distribution as well as in the mean cell transit time of the patients.

Lipid analysis

The cholesterol content (Table II) of RBC from uremic patients before dialysis was significantly higher (43.07 ± 5.30 μmol/10^{11} cells) than RBC controls ($38.32 \pm$

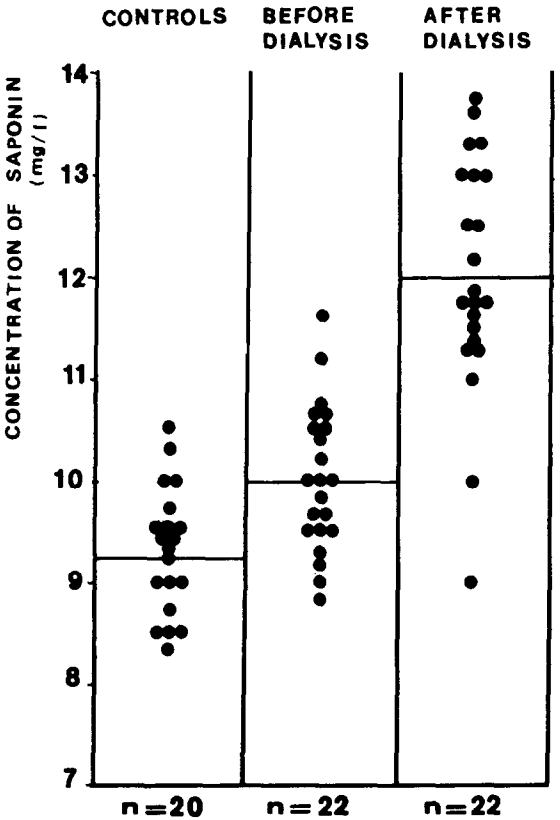

Fig. 2. Saponin concentrations required to hemolysate 50% of RBC in controls and uremic patients before and after dialysis.

TABLE II

Erythrocyte lipids in hemodialysed patients

	Controls	Before dialysis	p value versus controls	After dialysis	p value versus before
Cholesterol	38.32 ± 3.19 *	43.07 ± 5.30	$p < 0.01$	46.93 ± 5.63	< 0.001
Phospholipids	37.25 ± 3.10	35.67 ± 5.00	NS	36.55 ± 5.19	NS
Cholesterol/ phospholipids (molar ratio)	0.98 ± 0.13	1.24 ± 0.23	$p < 0.01$	1.30 ± 0.30	NS

Results are expressed as $\mu mol/10^{11}$ red cells.

* Mean $\pm$ SD; p = probability of error; NS = not significant.

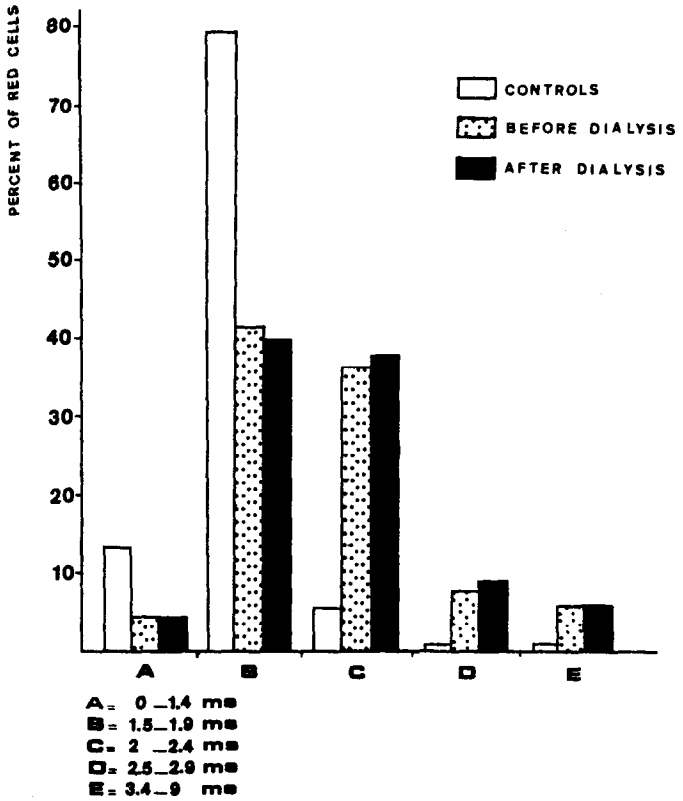

Fig. 3. Distribution of transit times of RBC in controls and uremic patients before and after dialysis.

3.19, $p < 0.01$). After dialysis, the cholesterol content was significantly higher than the values before dialysis, $p < 0.001$.

The phospholipid values before and after dialysis were not significantly different from the controls. There was a notable increase of the molar ratio cholesterol/

TABLE III

Erythrocyte phospholipids in hemodialysed patients (relative contents expressed in percent: 100% = total phospholipids)

	Controls	Before dialysis	p value versus controls	After dialysis	p value versus before
PE	36.66 ± 2.32	37.55 ± 5.65	NS	41.60 ± 6.05	< 0.05
PS	15.22 ± 1.54	14.75 ± 4.50	NS	15.82 ± 4.98	NS
PC	24.64 ± 2.70	23.64 ± 3.13	NS	20.98 ± 4.41	< 0.001
SM	22.57 ± 2.71	22.18 ± 3.70	NS	20.41 ± 4.01	< 0.001

PE = phosphatidylethanolamine; PS = phosphatidylserine; PC = phosphatidylcholine; SM = sphingomyelin.

TABLE IV

Fatty acid composition in uremic patients before and after dialysis (expressed as percent: 100% = total fatty acids)

	Controls	Before dialysis	p value versus controls	After dialysis	p value versus before
C14:0	0.66 ± 0.17	0.78 ± 0.34	NS	0.98 ± 0.42	NS
C15:0	0.61 ± 0.17	0.78 ± 0.39	< 0.01	1.43 ± 0.44	< 0.001
C16:0	31.14 ± 2.82	29.36 ± 3.23	NS	26.57 ± 2.85	< 0.001
C17:0	1.52 ± 0.26	1.88 ± 0.59	< 0.05	2.34 ± 0.75	NS
C18:0	21.85 ± 2.07	23.02 ± 2.16	NS	19.34 ± 1.99	< 0.001
C18:1	14.57 ± 1.07	13.44 ± 1.26	NS	14.23 ± 1.57	NS
C18:2 (n-6)	7.78 ± 1.20	7.88 ± 1.72	NS	9.26 ± 1.35	< 0.01
C20:2 (n-6)	0.36 ± 0.05	0.25 ± 0.11	NS	0.24 ± 0.11	NS
C20:3 (n-6)	1.14 ± 0.22	1.10 ± 0.34	NS	1.11 ± 0.37	NS
C20:4 (n-6)	10.07 ± 1.74	11.70 ± 2.34	NS	13.47 ± 2.32	< 0.01
C20:5 (n-3)	0.46 ± 0.22	0.54 ± 0.25	NS	0.67 ± 0.37	NS
C22:4 (n-6)	1.74 ± 0.18	2.12 ± 0.32	NS	2.85 ± 0.35	< 0.01
C22:5 (n-6)	0.80 ± 0.12	1.58 ± 0.27	< 0.01	2.05 ± 0.56	< 0.05
C22:6 (n-3)	2.48 ± 0.22	3.21 ± 0.46	< 0.05	3.54 ± 0.43	NS

phospholipids (CL/PL) (1.24 ± 0.23) before dialysis in comparison to controls (0.98 ± 0.13, $p < 0.01$), but there was only a slight change in this ratio during dialysis.

The phospholipid distribution (Table III) showed a slight increase in phosphatidylethanolamine (PE) and a slight decrease in phosphatidylcholine (PC). Following dialysis there was a significant increase in PE from 37.55 ± 5.65% to 41.60 ± 6.05% ($p < 0.05$) and a decrease in PC from 23.64 ± 3.13% to 20.98 ± 4.41% ($p < 0.001$) and of sphingomyelin from 22.18 ± 3.70% to 20.41 ± 4.01% ($p < 0.001$).

The fatty acid composition (Table IV) before dialysis was similar to the controls. However, after dialysis the C16 and C18 were significantly decreased ($p < 0.001$) and C18:2 (n-6), C20:4 (n-6), C22:4 (n-6) and C22:5 (n-6) were significantly increased.

Discussion

It is known that uremic state produces many alterations in various important erythrocyte membrane functions such as osmotic fragility and deformability [5,6,8,19]. Our results show that, before a dialysis session, the RBC of uremic patients are more sensitive to osmotic hemolysis than controls, which is in good agreement with various literature data. This increased osmotic fragility has been related to the high levels of parathyroid hormone (PTH) frequently observed in dialysed uremic subjects [20–23]. However, as shown in Table I, the patients studied in our experiment had very different blood levels of PTH measured by radioimmuno assay using a C-terminal antiserum: 12 had normal or subnormal values while

2 had very high levels of PTH (40 times the normal) and 8 had levels 2 to 20 times above normal). All patients were on long-term dialysis treatment (Table I) and no correlations were found between the values of the MOF of RBC patient and PTH blood levels ($r = 0.31$). Our results corroborate those of Docci et al. [6,24,25], indicating that hyperparathyroidism is not a major factor influencing osmotic fragility in hemodialysed patients.

The saponin test indicated a resistance of the erythrocytes to hemolysis before dialysis in comparison to controls. Saponin is known to form a complex with membrane cholesterol [26] to the detriment of phospholipid–cholesterol interaction, and leads to RBC lysis [27]. Our results on the erythrocyte lipid composition before dialysis indicate a significant increase in cholesterol in comparison to controls; this could explain the higher resistance of RBC to saponin hemolysis in these subjects.

The cell transit time was decreased before dialysis in comparison to controls, indicating impaired deformability of RBC. As pre- and post-dialysis cell transit times were very similar, it appears that dialysis alone is not responsible for the impairment of deformability.

Since other authors have attributed the alterations of specific properties of RBC in uremic patients to modifications of the membrane lipid composition, we also investigated the RBC lipids before and after dialysis. The major lipid components involved in the alterations of RBC functions have been reported as follows: molar ratio (CL/PL), phospholipid distribution, length and degree of unsaturation of phospholipid acyl chains [28,29]. In our study, we observed before dialysis, a significant increase in the molar ratio CL/PL between controls and patients, which was mainly due to a high level of membrane cholesterol.

As for the results obtained after dialysis, it can be seen that cholesterol and phosphatidylethanolamine increased while phosphatidylcholine and sphingomyelin decreased. High levels of polyunsaturated fatty acids and decreased levels of saturated fatty acids were also found. Likewise, we observed some modifications of erythrocyte properties after dialysis, especially a significant increase in RBC resistance to osmotic and saponin hemolysis.

It is known that the structural order of lipids in membranes is essentially determined by the cholesterol and sphingomyelin content and by the degree of saturation of the phospholipid acyl chains [30–35]. Moreover, sphingomyelin, which is enriched in longer saturated acyl chains compared to other phospholipids [36], not only plays a very important role by exerting a rigidifying effect in biomembranes, but also preferentially interacts with cholesterol [34,37,38]. This might amplify the ordering effect of lipid membranes [31,39]. The high levels of cholesterol and of the CL/PL ratio that we found during dialysis should lead to decreased deformability [32]. Yet on the one hand, the ordering effect of cholesterol levels off when $CL/PL > 1$ [40], and on the other hand, the rigidifying effect of sphingomyelin should be impaired, owing to the decrease of this phospholipid during dialysis. These two lipid effects may be related to the non-significant alteration of deformability that we observed during dialysis.

It should also be noted that the lipid modifications of RBC membranes obtained after dialysis seem to affect those lipids preferentially located in the outer layer.

Previous studies have shown that not only sphingomyelin and phosphatidylcholine are the principal phospholipids present in this outer layer [41–43] but that loading cholesterol enters this layer preferentially [44]. Therefore, the modifications of the lipid composition of the outer layer could be responsible for the alterations of the RBC osmotic resistance observed during dialysis. Such an asymmetric modification of RBC lipids during dialysis strongly suggests the possible involvement of lipid exchanges between red cells and plasma proteins. Indeed, these two compartments have been shown to exchange free cholesterol as well as intact phospholipid molecules [45–47]. Although the present study did not emphasize the possible role of plasma lipoproteins, preliminary experiments revealed that plasma cholesterol is increased by 20% during dialysis, owing to a process of hemoconcentration. Further studies should indicate whether RBC lipoprotein interactions are modified during dialysis.

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