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The effect of PMMA-based protein-leaking dialyzers on plasma homocysteine levels

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The effect of PMMA-based protein-leaking dialyzers on total plasma homocysteine levels.

Background. Hyperhomocysteinemia is a well-recognized independent risk factor for cardiovascular disease in end-stage renal disease (ESRD) patients. Since homocysteine (Hcy) largely binds to serum proteins (80 to 90%), in this study we investigated the possibility that polymethylmethacrylate (PMMA)-based protein-leaking dialyzers could reduce total plasma Hcy (tHcy) levels in ESRD patients.

Methods. Two matched groups of patients ($N = 13$) showing mild to intermediate hyperhomocysteinemia on standard hemodialysis (HD) with conventional non-protein-leaking dialyzers were included. In the control group membranes were maintained the same, while the study group was switched to protein-leaking dialyzers (BK-F series; Toray, Japan) and studied for 6 months. tHcy was measured by high performance liquid chromatography (HPLC) at baseline and after 1, 3, and 6 months. Proteins and Hcy were also measured in the spent dialysate.

Results. The pre-HD levels of tHcy in the control group remained close to baseline values (26.6 ± 5.0 μmol/L), while in the study group at 1, 3, and 6 months they decreased from a baseline value (in μmol/L) of 25.3 ± 5.9 to 21.5 ± 4.5, 16.9 ± 4.0, and 17.2 ± 4.2, respectively ($P < 0.01$ for values at 3 and 6 months vs. baseline). The intra-HD drop of tHcy (ΔHD_{Hcy}) slightly but progressively decreased during the 3 steps on protein-leaking dialyzers and a positive correlation was found between ΔHD_{Hcy} and pre-HD levels of tHcy. In spent dialysate samples from protein-leaking dialyzer–treated patients, the amount of protein-bound Hcy (bHcy) was approximately 10 times higher than in non-protein-leaking dialyzers, but the ΔHD_{Hcy} observed in non-protein-leaking dialyzers and protein-leaking dialyzers was comparable. Serum proteins and albumin were only slightly affected by protein-leaking dialyzers.

Conclusion. This study demonstrates that protein-leaking dialyzers used with a pure diffusive technique significantly lower pre-HD tHcy (approximately 33% of starting levels after 3 months of treatment) in ESRD patients. A possible underlying mechanism for this effect could be the removal of large molecular weight solutes responsible for a defective metabolism of the Hcy, as the removal of bHcy with protein-leaking dialyzers seems not sufficient, per se, to explain this steady reduction of tHcy levels in pre-HD.

[1]These authors contributed equally to this research work.

Key words: homocysteine, hyperhomocysteinemia, protein-leaking dialyzers, protein-bound toxins, proteins, uremia, hemodialysis.

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Homocysteine (Hcy) is a sulfur-containing amino acid formed during methionine metabolism [1]. Its accumulation is a well-recognized independent risk factor for cardiovascular diseases such as myocardial infarction, stroke, and thromboembolic disease in end-stage renal disease (ESRD) patients and the general population [2–5].

Under normal conditions, total plasma Hcy (tHcy) concentration is a function of its formation in tissues, elimination by renal clearance, and tissue uptake and transformation [6]. Only a minor percentage [i.e., the free form (fHcy)] is eliminated through the glomerular filter, while the majority is removed by metabolic processing, with the kidney playing an important role in that it eliminates up to 70% of the tHcy pool in the body [7]. When excessive amounts of Hcy are produced in tissues and/or the catabolic routes are defective, the accumulation of Hcy brings cells to export it in the plasma. Herein, Hcy is in equilibrium with the fHcy and the more prevalent protein-bound form (bHcy), which can represent up to 80% to 90% of the tHcy [8].

Hyperhomocysteinemia in ESRD patients appears to be the consequence of a decreased clearance and impaired metabolism of Hcy [9]. Genetic factors and an insufficient correction of the uremic toxicity can sustain hyperhomocysteinemia in some ESRD patients and can lead to unsuccessful therapy [10, 11]. The latter of these two is essentially based on supplementation protocols of folic acid, vitamin B_{12}, and B_6 [12], in that they are important cofactors for the enzymes of the remethylation and transsulfuration pathways in the Hcy metabolism [13, 14].

Hemodialysis (HD) is not considered a therapy against

Table 1. Main characteristics of patients at baseline evaluation

	Control group ($N=13$)	Study group ($N=13$)
Sex	M = 7; F = 6	M = 7; F = 6
Age *years*	62.6 ± 12.0	67.6±14.8
Dialytic age *months*	49.3 ± 31.8	46.8±38.2
Membrane type	B1 = 4; CA = 5; CR = 4	B1 = 5; CA = 4; CR = 4
Kt/V *weekly*	3.9 ± 0.7	4.1 ± 0.7
Urea *mg/dL*	149.6 ± 43.4	153.4 ± 49.8
Creatinine *mg/dL*	9.8 ± 1.9	9.7 ± 2.3
Residual ClCl *ml/min*	<1	<1
Hematocrit %	33.0 ± 3.6	32.5 ± 4.1
Cholesterol *mg/dL*	195.4 ± 35.2	189.8 ± 55.7
Triglycerides *mg/dL*	175.5 ± 123.2	179.1 ± 137.0
Total proteins *g/dL*	6.7 ± 0.4	6.9 ± 0.5
Albumin *g/dL*	4.1 ± 0.3	4.0 ± 0.4
Folic acid *ng/mL*	22.9 ± 18.0	23.3 ± 19.5
Vitamin B_{12} *pg/mL*	375.9 ± 160.5	379.3 ± 169.5
tHcy *μmol/L*	26.6 ± 5.0	25.3 ± 5.9

hyperhomocysteinemia. Although the intra-HD decrease in tHcy could reach values ≥30% with different types of dialyzers [15–17], the inter-HD concentrations of tHcy remain above normal levels also after a so-called "adequate HD." This seems to be the consequence of an insufficient removal of Hcy, as current dialysis methods and membrane types only marginally affect the predominant form (i.e., the bHcy) and its equilibrium with the pool of Hcy in tissues.

Protein-leaking dialyzers have been recently proposed as a specific solution to decrease levels of protein-bound toxins [18, 19]. In particular, polymethylmethacrylate (PMMA)-based protein-leaking dialyzers (commercial name BK-F) have been the former example of membranes that, even when used in standard HD procedures, are able to remove a significant amount of proteins in a broad range of molecular weights [19]. Early studies showed that protein bound toxins and other high molecular weight toxins could be removed during dialysis with protein-leaking dialyzers; these solutes include advanced glycoxidation end products [abstract; Galli F et al, *J Am Soc Nephrol* 12:1831, 2001] [19], erythropoiesis inhibitors [20, 21], and the same Hcy [19, 22]. The direct clearance of bHcy could be counted as a mechanism through which protein-leaking dialyzers might reduce tHcy; alternatively, protein-leaking dialyzers might remove high molecular weight toxins able to inhibit the Hcy metabolism, as proposed by the basis of early studies on polysulfone (PS)-based protein-leaking dialyzers [22] and high-flux non-protein-leaking dialyzers [15, 16].

The present study investigated the protein leakage through PMMA-based protein-leaking dialyzers as a strategy to steadily decrease pre-HD levels of tHcy in ESRD patients. Intra- and inter-HD changes of tHcy and the amount of bHcy removed in the spent dialysate were assessed in detail.

METHODS

Patient selection and study design

The participants included in the study ($N = 26$; M=14, F=12, mean age, 66.1 ± 17.1 years) were patients on standard (three times/week) bicarbonate HD (mean dialysis age, 47.1 ± 40.8 months) showing mild to intermediate hyperhomocysteinemia (according to the current classification [12], the normal range of plasma tHcy is between 5 and 15 μmol/L; mild, intermediate, and severe hyperhomocysteinemia are defined as concentrations of 15 to 25, 25 to 50 and >50 μmol/L, respectively) and on folate therapy for at least 6 months (from 10 to 15 mg/wk). None of them received supplements of vitamin B_6 and B_{12}.

Exclusion criteria included acute and chronic infectious disease, malnutrition (serum albumin cut-off <3.3 g/dL), malignancy, and any other known metabolic defect and therapy that would affect Hcy metabolism. In particular, the presence of major folate deficiency was excluded on the basis of a preliminary test aimed to establish tHcy levels during the enrollment.

The study was designed as a 9-month prospective two-step controlled trial. At the basal step, patients were enrolled and maintained for 3 months on treatment with non-protein-leaking dialysers including high-flux PMMA (B1 series; Toray, Japan), low-flux cellulose acetate (CA) (Cobe, Japan), and cuprammonium rayon (CR) (Terumo, Japan). In the second step, patients were randomly assigned to two subgroups, the main characteristics of which are shown in Table 1. The first subgroup that served as control was maintained on treatment with the same type of dialyzers used at the inclusion, while in the second subgroup (study group), the patients were assigned to the treatment with protein-leaking dialyzers (BK-F series; Toray, Japan) for a period of 6 months. The main physical characteristics of this membrane series are de-

Table 2. Physical characteristics of protein-leaking dialyzer series membranes

Pore size *Å*	100
Porosity %	62
Nominal cut-off *kDa*	70
Inside diameter *μm*	200
Wall thickness *μm*	30
Effective surface area m^2	1.6
UFR in vitro *mL/hr at 100 mm Hg*	2000
Sieving coefficient of albumin	ca. 0.03

scribed in Table 2. Criteria for computerized randomization included the presence of clinical and dialysis variables comparable in the study and control group; major parameters scored were tHcy levels at the enrollment, comorbidity as severe hypertension, diabetes, and hyperlipidemia, age and sex, plasma proteins, vitamin supplements, and pharmacologic therapy. Major dialysis parameters scored were dialysis age and schedule (frequency and duration), membrane surface area, blood and dialysate flows, and Kt/V. All of these parameters did not change during the study in the two groups of patients.

A group of 15 sex- and age-matched healthy controls was also included in the study. Informed consent was obtained from each subject.

Sample collection

Blood sampling was carried out in the midweek dialysis of the last week at baseline (step 1) and after 1, 3, and 6 months in the step 2. Five mL of blood were drawn by vacutainer technique into heparinized green-top tubes just before and after HD. Plasma samples were obtained by centrifugation immediately after collection and preserved at −80°C until assayed.

Twenty liters of dialysis fluid (from the 10th to the 50th minute of HD) were collected in the study group in the last dialysis of the step 1 and at the first dialysis in the step 2. Samples were then concentrated to 5 mL by ultrafiltration with a 10 kD cut-off membrane (Pall Gelman Laboratory, Vernon Hills, IL, USA) and stored at −80°C.

As a preliminary evaluation, we measured the protein concentration in the dialysate collected during HD with non-protein-leaking dialyzer and protein-leaking dialyzer membranes to evaluate the removal of plasma proteins.

Hematochemical analyses

The following parameters were monitored by routine analysis of blood samples: vitamin B_{12}, folic acid, total proteins and albumin, lipids, creatinine, urea, and hematocrit. Kt/V values were calculated using the single-pool method.

Total homocysteine analysis

Chemicals. D,L-homocysteine, L-cysteine, and cysteinylglycine were obtained from Sigma (Milan, Italy); these compounds were used as standards and dissolved in 0.05 mol/L hydrochloric acid to make a stock solution with a concentration of 2.5 mmol/L and stored at 4°C; the working standard solutions were made up freshly by dilution of the stock solution with 0.01 mol/L hydrochloric acid. Analytic grade trichloroacetic acid, ortophosphoric acid, potassium dihydrogen phosphate, and acetonitrile were purchased from Sigma (Milan, Italy). Tri-n-butylphosphine was obtained from Fluka (Milan, Italy) and used as 10% (vol/vol) solution in dimethylformammide. 7-Fluoro-2-oxa-1,3-diazole-4-sulfonate (SBD-F) was obtained from Sigma and used as a 1 mg/mL solution in 0.125 mol/L borate buffer, pH 8.0, containing 4 mmol/L ethylene diaminetetraacetic acid (EDTA).

Plasma and dialysate processing

Samples were treated accordingly with the procedure described by Ubbink [23] with some minor changes. Briefly, 0.1 mL aliquot of plasma or protein concentrates from dialysate samples were treated with 10 μL of 10% (vol/vol) tri-n-butylphosphine for 30 minutes at 4°C. The solution was then mixed with 0.1 mL of 10% (weight/vol) trichloroacetic acid cold solution containing 1 mmol/L Na_2 EDTA under vigorous mixing, followed by centrifugation at 10,000*g* for 5 minutes. To a 0.1 mL aliquot of the clear supernatant were added 0.02 mL of 1.55 mol/L sodium hydroxide solution, 0.25 mL of 0.125 mol/L potassium borate buffer, pH 8.0, containing 4 mmol/L Na_2 EDTA, and 0.02 mL of 1 mg/mL of SBD-F reagent; the derivatization was carried out at 70°C for 10 minutes. The solution was then cooled in crushed ice, centrifuged at 2000*g* for 5 minutes, and an aliquot of 0.05 mL was subjected to high performance liquid chromatography (HPLC) analysis. This procedure allows measurement of the tHyc in the samples (protein-bound and free Hcy).

HPLC analysis

Analyses were performed using an HPLC apparatus equipped with a model 880 PU Jasco pump (Jasco Europe, s.r.l., Cremerla, Italy) and a Rheodyne 7125 injection valve fit with a 100 μL sample loop (Rheodyne Europe GmbH, Benshoim, Germany). The separation of derivatized thiol compounds in plasma was performed at room temperature on an analytical column named Spherisorb ODS2 (150 × 4 mm I.D., 5 μ particle size) and protected by an ODS2 guard cartridge (30 × 2 mm) (Wahrs Spa, Milan, Italy).

The fluorescence of derivatized analytes was measured at 515 nm with excitation at 385 nm, using a model FP 920 Jasco spectrofluorimeter equipped with a 12 μL flow cell. The detector signal was recorded and the peak quantified with a Model 3394 Hewlett Packard integrator (Geneva, Switzerland).

Chromatographic elution was carried out isocratically at 1.0 mL/minute flow rate, using as the mobile phase a mixture of 0.05 mol/L potassium dihydrogen phosphate

Table 3. Pre- and post-HD levels and intra-HD decrease (ΔHD_{Hcy}) of tHcy in patients treated with non-protein-leaking dialyzers and protein-leaking dialyzers (BK-F)

	Patients on non-protein-leaking dialyzers (control group)			Patients on protein-leaking dialyzers (study group)				
	Plasma tHcy *μmol/L*			Plasma tHcy *μmol/L*		Mean % decrease vs. baseline levels		
	Pre-HD	Post-HD	Absolute ΔHD_{Hcy}	Pre-HD	Post-HD	Pre-HD	Post-HD	Absolute ΔHD_{Hcy}
Baseline	26.6 ± 5.0	17.7 ± 6.9	33.5 ± 7.4% (8.9 ± 3.4 *μmol/L*)	25.3 ± 5.9	16.9 ± 4.9	—	—	33.7 ± 7.0% (8.4 ± 2.1 *μmol/L*)
1 month	24.2 ± 7.3	16.9 ± 5.5	30.3 ± 8.6% (7.3 ± 2.8 *μmol/L*)	21.5 ± 4.5[a]	14.3 ± 3.3[a]	15.0 ± 5.1	15.4 ± 19.7	32.9 ± 4.9% (7.2 ± 3.0 *μmol/L*)
3 months	23.9 ± 8.8	15.8 ± 8.1	33.9 ± 10.6% (8.1 ± 3.3 *μmol/L*)	16.9 ± 4.0[b,d]	12.1 ± 3.8[b]	33.2 ± 12.9	28.4 ± 19.0	29.1 ± 5.2%[a,c] (4.8 ± 1.8 *μmol/L*)
6 months	25.9 ± 7.3	16.3 ± 6.6	37.1 ± 13.6% (9.6 ± 3.9 *μmol/L*)	17.2 ± 4.2[b,d]	12.4 ± 6.1[b,c]	32.0 ± 16.7	26.6 ± 19.9	27.9 ± 6.8%[b,d] (4.8 ± 1.9 *μmol/L*)

Absolute ΔHD_{Hcy} values were calculated as described in **Methods**. The mean level of tHcy in healthy controls was 10.5 ± 2.6 μmol/L.
[a] $P < 0.05$ and [b] $P < 0.01$ vs. baseline levels; [c] $P < 0.05$ and [d] $P < 0.01$ vs. control group values

in water and acetonitrile (90:10). The pH of this solution was adjusted to 2.9 with concentrated phosphoric acid.

Hcy and other thiol compounds (cysteine, cysteinylglycine, glutathione) were used to calibrate the method throughout different steps of the sample preparation procedure and quantification of tHcy levels. Two different solutions of the Hcy derivative prepared daily were used as external standards and assessed in duplicate to make appropriate standard curves.

The absolute decrease of tHcy during dialysis was calculated using mean plasma levels of tHcy before (pre) and after (post) HD according to the following equation:

$$\Delta HD_{Hcy} = ([Hcy]_{pre} - [Hcy]_{post}) \times 100/[Hcy]_{pre}$$

In some experiments the amount of albumin that bound Hcy in the spent dialysate proteins was measured. After extensive dialysis against a solution of phosphate-buffered saline (2 changes × 5 hrs at 4°C using a 10 kD cut-off nylon membrane), the albumin fraction was separated by Cybacron Blue affinity chromatography (Affiland S.A., Ans-Lige, Belgium) and Hcy was analyzed (see above) after reduction of the albumin-Hcy disulfide under denaturating conditions with an excess of mercaptoethanol. A molar ratio of 1/1 was used to calculate the amount of albumin to which Hcy is bound.

Protein assay

Plasma and dialysate proteins were measured with the method of Bradford using the Bio-Rad Protein Assay kit (Bio-Rad, Milan, Italy). The blue comassie chromogen binds proteins to form a complex with a maximum absorbance at 595 nm. This versatile assay showed a good linearity and reproducibility. However, depending on different sources of proteins and amino acid composition, this method can lead to different results and preliminary experiments showed that protein levels could be underestimated by approximately 15% when compared to other procedures, such as the method of Lowry. This limit of the assay did not affect the aims of the research, which were to compare in a prospective evaluation the concentration of total proteins in plasma and dialysate.

Statistics and data processing

The results are expressed as mean ± 1 standard deviation (SD) or as median (range). The Student-Newman-Keuls (SNK) test for multiple comparisons was applied to evaluate differences among groups of patients. The Student *t* test for paired data and the nonparametric one-way analysis of variance (ANOVA) were used when appropriate to compare data in the prospective study. Regression analyses and data plotting were carried out using the software Microcal™ Origin V.6 (Microcal Software, Inc., Northampton, MA, USA). Probability values of <0.05 were accepted, and values ≤ 0.01 were considered highly significant.

RESULTS

Effect of protein-leaking dialyzers on hematochemical parameters

In the study group, hematochemical and dialysis parameters (Table 1) did not change significantly during the treatment with protein-leaking dialyzers (not shown), with the exception of hematocrit and total plasma protein and albumin levels. Hematocrit levels increased progressively from the baseline value of 32.5 ± 4.1% to 37.1 ± 3.8% at the end of the study (+14.2%, $P < 0.01$), and total proteins and albumin decreased to the lower part of the control range (6.0 to 8.4 g/dL and 3.5 to 5.0 g/dL, respectively) during the first month of treatment with protein-leaking dialyzers, but were restored as compared to baseline evaluation in the remaining steps of the study. In detail, total proteins (g/dL) were 6.9 ± 0.5 at baseline evaluation and 6.3 ± 1.0 (−8.7%), 6.7 ± 0.7 (−2.9%), and 6.8 ± 0.6 (−1.4%) after 1, 3, and 6 months of treatment with protein-leaking dialyzers. Albumin levels (g/dL) were 4.0 ± 0.4 and 3.7 ± 0.6 (−7.5%), 3.8 ± 0.3 (−5%) and 3.8 ± 0.4 (−5%).

Regarding the effect of protein-leaking dialyzers on plasma levels of folic acid and vitamin B_{12}, we did not

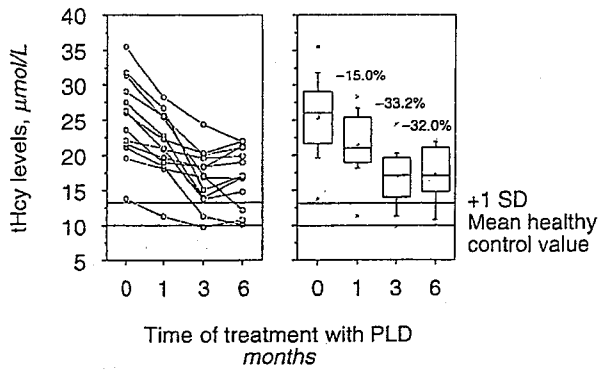

Fig. 1. Individual and average pre-HD tHcy levels in patients on treatment with protein-leaking dialyzer membranes (BK-F) over a period of time of 6 months. The mean decrease of tHcy observed after 1, 3, and 6 months of protein-leaking dialyzers is statistically significant with respect to baseline evaluation ($P < 0.01$).

find significant changes during the prospective study. In the study group, folic acid levels (ng/mL) were 23.3 ± 19.5 at baseline and 20.8 ± 20.2 after 6 months of treatment with protein-leaking dialyzers; vitamin B_{12} (pg/mL) levels were 379.3 ± 169.5 at baseline and 370.6 ± 203.7 after 6 months of treatment with protein-leaking dialyzers.

In the control group, all these parameters were not significantly modified at the different steps during the 6 months of the study (not shown).

Effect of protein-leaking dialyzers on tHcy levels

Baseline levels of tHcy in plasma were 26.6 ± 5.0 and 25.3 ± 5.9 μmol/L, respectively, in the control and study group (P = NS) (Table 3). These levels are significantly higher compared with levels of tHcy measured in healthy subjects (10.5 ± 2.6 μmol/L, $P < 0.001$). After 6 months, in the control group mean tHcy levels in pre-HD were not significantly modified (27.4 ± 5.9 μmol/L), while in the protein-leaking dialyzer group a significant decrease was observed (17.2 ± 4.2 μmol/L, $P < 0.01$ vs. control group) (Fig. 1). Although this decrease kept mean levels of tHcy significantly higher as compared to healthy controls ($P < 0.01$), 3 out of 12 patients in the protein-leaking dialyzer group showed pre-HD levels of tHcy below the arbitrary cut-off line represented from the mean healthy control value +1 SD (13.1 μmol/L). One patient showed levels below the cut-off limit during the entire time of the study on protein-leaking dialyzers.

In all the patients, tHcy concentration before HD was positively correlated with tHcy assessed after HD (R = 0.642; $P < 0.001$) and with plasma albumin levels (R = 0.323, $P < 0.020$).

In both groups a significant difference between pre- and post-HD tHcy levels was observed at every experimental time ($P < 0.01$). As shown in Table 3, pre- and post-HD levels of tHcy steadily and progressively lowered in the study group in a similar way during the different steps in the study, but remained the same in the control group. The mean decrease of tHcy with respect to baseline data improved during the steps of the treatment with protein-leaking dialyzers both before and after HD, with a mean value of decrease in pre-HD of approximately 15.0% at the first month and ≥32% after 3 and 6 months ($P < 0.01$). In the control group the change of pre- and post-HD tHcy during the different steps of the study compared to baseline data was negligible in each step.

In the control group, the baseline intra-HD decrease of tHcy (ΔHD_{Hcy}) was 33.5 ± 7.4% and remained steadily above the value of 30% during the study. No significant differences were observed for the ΔHD_{Hcy} of subgroups of patients on different non-protein-leaking dialyzers (not shown).

In the study group, the baseline ΔHD_{Hcy} was 33.7 ± 7.0%, and after 1, 3, and 6 months, it decreased to 32.9 ± 4.9% (P = NS), 29.1 ± 5.2% ($P < 0.05$), and 27.9 ± 6.8% ($P < 0.01$). A positive correlation between ΔHD_{Hcy} and pre-HD tHcy levels in all the patients at different time-points was observed ($R = 0.666$, $P < 0.02$).

Hcy and protein content in spent dialysate samples

The total amount of proteins removed with standard dialyzers was calculated to be <0.5 g/dialysis session (median, 0.26, range, 0.20 to 0.32), while in the first dialysis session with protein-leaking dialyzers it ranged from 1.28 and 4.13 g (median, 2.86 g). In the spent dialysate obtained from patients on protein-leaking dialyzers, albumin was observed to be 75% to 90% (85%) of the total protein, and the quota of albumin that bound Hcy ranged from 33% to 86% (68%). From these data it can be roughly calculated that the amount of bHcy removed during a single dialysis session with protein-leaking dialyzers and non-protein-leaking dialyzers is 25.0 and 2.3 μmol, respectively.

DISCUSSION

Specific Hcy-lowering therapy and current dialysis procedures can provide only a limited correction of hyperhomocysteinemia in a large portion of ESRD patients [11, 15–17]. The main reason for this fruitless battle is the sustained generation of Hcy by the impaired metabolic and excretive functions and its accumulation in the uremic blood mainly as bHcy (≥80%) [8]. The bHcy form may represent a sort of reservoir in equilibrium with the fHcy and tissue Hcy. The kinetics of exchange between the free and bound forms and the continuous refueling of Hcy from tissues could then keep the form dialyzable with non-protein-leaking dialyzers (i.e., the fHcy) too low compared to the bound one, which would

limit the capability to affect the total pool of Hcy in the body enormously.

In the last years, a new generation of dialyzers able to remove proteins (protein-leaking dialyzers) has been introduced in the HD practice. The first example in this context was that of a PMMA-based membrane [18]. We first characterized the performance of these dialyzers as concerns protein leakage and removal of some classes of high molecular weight solutes such as advanced glycoxidation end products and erythropoiesis inhibitors [abstract; Galli F et al, *J Am Soc Nephrol* 12:1831, 2001] [19, 21]. Recently, protein leakage by BK-F protein-leaking dialyzers has been compared with that of other high-flux membranes and protein-leaking dialyzers [24]. This comparison showed that the BK-F series provides nearly five times greater protein leakage than PS-based protein-leaking dialyzers (Superflux series from Fresenius Medical Care Ag, Bad Homburg, Germany) and also immunoglobulin G (IgG) could be detected in filtrate samples.

In agreement with our early findings on PMMA-based protein-leaking dialyzers [19], van Tellingen et al [22] recently showed that PS-based protein-leaking dialyzers could steadily reduce tHcy in blood of chronic HD patients. The underlying mechanism of this effect of protein-leaking dialyzers remains to be clarified and could be of relevance in understanding the role of protein-leaking dialyzers in the correction of other high-molecular-weight solutes. Hcy is only one of the several protein-bound toxins that, together with a number of still unknown high-molecular-weight uremic solutes, could play an important pathogenic role in different uremic comorbidities, such as uremic anemia, vascular damage, chronic inflammation, and immune dysfunction [19, 21, 25, 26].

The present study first investigates in detail the effect of PMMA-based protein-leaking dialyzers on tHcy levels of folate-supplemented ESRD patients showing mild-intermediate hyperhomocysteinemia. The results demonstrate that pre-HD values of tHcy are steadily reduced by PMMA-based protein-leaking dialyzers (BK-F). A significant decrease was achieved already after 4 weeks of treatment (15.0%), and reached a maximum at 3 months (33.2%). Importantly, after 6 months of protein-leaking dialyzers, individual values of tHcy in 3 out of 12 patients fell below the arbitrary cut-off line represented from the mean healthy control value + 1 SD. This decrease of pre-HD tHcy in the study group largely exceeds even the best results achieved here and in other studies using high-flux membranes on patients showing mild hyperhomocysteinemia [16]. This result was observed in the presence of plasma folate levels and vitamin therapy kept steady during the study. Since our patients showed lower starting folate levels as compared with supplemented patients in other studies, a better effect of protein-leaking dialyzers on tHcy correction in the presence of higher doses of this cofactor cannot be ruled out and will be the subject of further investigation.

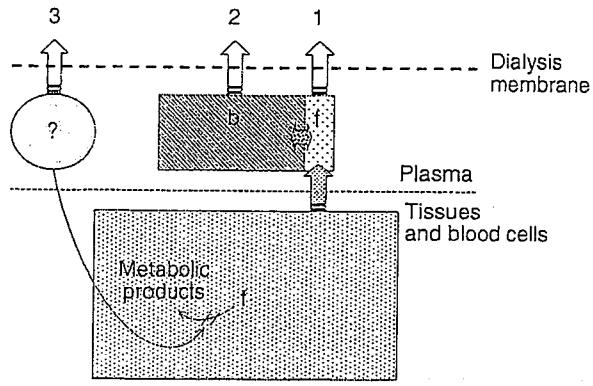

Fig. 2. Components that may participate in lowering plasma tHcy during dialysis with protein-leaking dialyzers and non-protein-leaking dialyzers. The fHcy and bHcy fractions in plasma are in equilibrium with them and with the tissue pool of fHcy. During pure diffusive dialysis with conventional non-protein-leaking dialyzers, tHcy levels in plasma can be reduced almost exclusively through the removal of fHcy (arrow 1). This leads to an intra-HD drop in tHcy (ΔHD_{Hcy}) of approximately 30%, which is promptly restored in the first hours after HD by the tissue reservoir. The same dialysis method carried out with protein-leaking dialyzers can act on two other aspects that contribute to steadily lower pre-HD tHcy. The first one is the removal of albumin-bound Hcy or bHcy (arrow 2). In the present study this component of the effect of protein-leaking dialyzers on plasma tHcy was shown to be important but largely insufficient to explain the observed lowering of tHcy in pre-HD. This aspect, in fact, does not explain why protein-leaking dialyzers provide a similar ΔHD_{Hcy} as non-protein-leaking dialyzers, while removing 10 times greater amounts of bHcy. The second aspect (arrow 3) could serve to explain this apparent paradox. As previously suggested [22], protein-leaking dialyzers could remove some still yet unidentified large molecular weight solutes able to impair metabolic pathways responsible of the tissue recycling of Hcy. Abbreviations are: b, bHcy; f, fHcy; ?, possible large molecular weight inhibitors of Hcy metabolism

In the study group, a direct relationship was observed between the intra-HD drop of tHcy (ΔHD_{Hcy}) and pre-HD tHcy. Accordingly, the ΔHD_{Hcy} showed a slight but progressive decrease during the treatment with protein-leaking dialyzers. However, as shown in Table 3, at different time points in the protocol, protein-leaking dialyzers used with a pure diffusive method maintain a good efficacy to clear blood Hcy, but this is obviously a self-limiting process regulated from pre-HD tHcy levels, with a value of approximately 33% as the highest attainable after 3 months of treatment.

These data on the reduction of tHcy levels in pre-HD by BK-F protein-leaking dialyzers are in agreement with the data obtained using PS-based protein-leaking dialyzers [22]. However, that study did not investigate the removal of proteins and Hcy with the dialysate and the intra-HD reduction of tHcy, leading to the unsupported hypothesis that protein-leaking dialyzers might reduce tHcy by removing toxins that negatively influence the metabolic processing of Hcy.

In the present study, the analysis of spent dialysate samples at the first dialysis with protein-leaking dialyzers demonstrated that this membrane removes a total amount of proteins per HD session more than 10 times the values observed with non-protein-leaking dialyzers (median values, 2.86 vs. 0.26 g/dialysis session). Moreover, it was observed that in the dialysate, on average, 68% of the molecules of albumin-bound Hcy with a molar ration of 1/1, and albumin is 85% of total plasma proteins in the spent dialysate. These data led to rough estimates that each dialysis session with protein-leaking dialyzers could remove up to 25.0 μmol of bHcy, while only 2.3 μmol are removed with non-protein-leaking dialyzers. In a simplified monocompartment model in which the tHcy would be distributed into 2.5 L of plasma, the bHcy found in the spent dialysate corresponds to decreases of tHcy of 10 and 0.9 μmol/L, respectively, in the presence of baseline levels of tHcy of approximately 25 μmol/L. Of course, these simplifications make the calculations to overestimate the contribution that the removal of bHcy might provide to lower plasma tHcy. In fact, the pool of fHcy and its metabolic processing in the tissues, as well as the changes of blood tHcy consequent to the treatment with protein-leaking dialyzers treatment, can affect these calculations. However, they give a hint of the relevance of subtracting bHcy with BK-F protein-leaking dialyzers in the context of the intra-HD drop of tHcy (ΔHD_{Hcy}) observed after 1 month (7.4 μmol/L) and 3 months (4.4 μmol/L) of treatment.

However, the finding that non-protein-leaking dialyzers leads to a ΔHD_{Hcy} similar to that of the protein-leaking dialyzers at different time points in the study (30% to 35% and 28% and 34%, respectively), but different (more than 10 times greater) removal of bHcy, suggests that the progressive and steady decline of pre-HD tHcy is a consequence of the rapid restoration during dialysis of the equilibrium of concentration between the free and bound form of Hcy. This influences the tissue output of Hcy and its clearance during HD, ultimately representing a boundary in the correction of tHcy when pure diffusive dialysis methods are used.

The data in this study seem to confirm the hypothesis that protein-leaking dialyzers improve hyperhomocysteinemia mainly by removing large molecular weight solutes able to affect the Hcy metabolism [22] and the consequent refill of blood tHcy in the intra and inter-HD intervals by the tissue pool, while the direct removal of bHcy could play a less important role (Fig. 2). This hypothesis is currently under further investigation in our lab. Preliminary data confirm that at least a component of this inhibitory activity of the uremic plasma shows a proteinaceous nature while other factors may be low molecular weight solutes that can interfere with folate function and can be removed/inactivated during dialysis with standard non protein-leaking dialyzers [Franceschini M, Galli F, Floridi A, unpublished observation, 2003].

In regards to the widespread concern about the chronic loss of grams of proteins (mainly albumin) in patients already at risk for malnutrition and hypoalbuminemia and its possible contribution to the incidence of cardiovascular morbidity and overall mortality [27], this study demonstrated that the protein leakage achieved during a standard dialysis with protein-leaking dialyzers (up to 4 g/dialysis session) is largely compensated in patients who would show a good metabolic and nutritional status. Moreover, preliminary data on patients on treatment for more than 2 years with BK-F protein-leaking dialyzers show good nutritional status and overall clinical conditions (unpublished data).

CONCLUSION

In patients with mild to intermediate hyperhomocysteinemia supplemented with folates, the treatment with protein-leaking dialyzers is effective in achieving a steady, even if partial, correction in pre-HD levels of tHcy (30% of decrease). The results leave further space in the hypothesis that an accumulation of large molecular weight inhibitors of the Hcy metabolism could be a major underlying mechanism of hyperhomocysteinemia in HD patients.

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