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Research Article

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Enzymatic Methyl Esterification of Erythrocyte Membrane Proteins Is Impaired in Chronic Renal Failure

Evidence for High Levels of the Natural Inhibitor S-Adenosylhomocysteine

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Abstract

The enzyme protein carboxyl methyltransferase type II has been recently shown to play a crucial role in the repair of damaged proteins. S-adenosylmethionine (AdoMet) is the methyl donor of the reaction, and its demethylated product, S-adenosylhomocysteine (AdoHcy), is the natural inhibitor of this reaction, as well as of most AdoMet-dependent methylations. We examined erythrocyte membrane protein methyl esterification in chronic renal failure (CRF) patients on conservative treatment or hemodialyzed to detect possible alterations of the methylation pattern, in a condition where a state of disrupted red blood cell function is present.

We observed a significant reduction in membrane protein methyl esterification in both groups, compared to control. The decrease was particularly evident for cytoskeletal component ankyrin, which is known to be involved in membrane stability and integrity. Moreover, we observed a severalfold rise in AdoHcy levels, while AdoMet concentration was comparable to that detected in the control, resulting in a lower [AdoMet]/[AdoHcy] ratio ($P < 0.001$).

Our findings show an impairment of this posttranslational modification of proteins, associated with high AdoHcy intracellular concentration in CRF. The data are consistent with the notion that, in CRF, structural damages accumulate in erythrocyte membrane proteins, and are not adequately repaired. (*J. Clin. Invest.* 1993. 91:2497–2503.) Key words: ankyrin • protein carboxyl methyltransferase • protein methyl esterification • repair • S-adenosylmethionine

Introduction

Several derangements of cell function present in chronic renal failure (CRF)[1] have been attributed to many factors, including various uremic toxins (1) and secondary hyperparathyroidism (2–4). The pathophysiological link between elevated blood concentrations of these compounds and alterations in cell function are not thoroughly elucidated at molecular level. Anemia, for example, is a common feature in CRF, along with inhibition of erythropoiesis and erythropoietin deficiency (5), but also a specific hemolytic component (6) contributes to its pathogenesis. The toxic origin of red blood cell (RBC) alterations is acknowledged, but how this interferes with RBC metabolism is not completely understood (7). Whatever is the underlying pathogenetic mechanism, anemia, increased hemolysis, and reduced deformability (8) monitor a state of deranged cell membrane function. This is expressed, for example, by a reduction in transport activity of the erythrocyte Cl^-/HCO_3^- anion exchanger (9), by reduced erythrocyte Na^+/K^+ pump activity (10), and by a defect in the activity of the $Na^+/K^+/2Cl^-$ cotransport (11, 12).

The protein carboxyl methyltransferase type II (type II MTase; D-aspartyl/L-isoaspartyl protein carboxyl methyltransferase; EC 2.1.1.77) is an enzyme class ubiquitously present in nature, with substrate specificity towards proteins containing abnormal L-isoaspartyl and D-aspartyl residues (13–15). These damaged residues, which can significantly alter protein structure and function, are generated from normal L-asparaginyl or L-aspartyl residues, through spontaneous deamidation and isomerization reactions (13–15). RBC possess an active type II MTase which has been purified and characterized (15, 16), its primary structure has recently been established (17, 18), and the in vivo substrates have been identified (19, 20). This enzyme has been found to be crucial in the repair of isoaspartyl-containing damaged proteins through the conversion of the isopeptide bond into a normal peptide bond (21–24). Type II MTase utilizes S-adenosylmethionine (AdoMet), as the methyl donor (25). The reaction generates S-adenosylhomocysteine (AdoHcy), which represents the natural inhibitor of this methyl transfer reaction (26, 27), as well as of most AdoMet-dependent methylations (28, 29).

Based on the established pattern of alterations in RBC membranes present in CRF, it is possible to hypothesize that changes in this protein methylation reaction, which is implicated in maintaining protein stability, may occur in CRF. This study was undertaken to examine RBC membrane protein methyl esterification in renal failure patients in conservative treatment, as well as in patients undergoing hemodialysis therapy.

Methods

Patients. Three groups were studied: (*a*) a control group of healthy subjects, that were age and sex matched with patients of groups *b* and *c*; (*b*) patients with varying degrees of renal failure, due to obstructive

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1. *Abbreviations used in this paper:* ACN, acetonitrile; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; BUN, blood urea nitrogen; CRF, chronic renal failure; GOT, glutamate oxaloacetate transaminase; Hb, hemoglobin; type II MTase, type II carboxyl methyltransferase.

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nephropathy, chronic glomerulonephritis, or polycystic renal disease, and with a creatinine clearance between 10 and 40 ml/min, on conservative treatment, age range 12–68 yr (mean 45±7 yr); (*c*) patients on standard hemodialysis therapy thrice weekly, clinically stable at the time of investigation, age range 44–64 yr (mean 52±3 yr), and treated with erythropoietin intravenously to reach an hematocrit of about 30%. The causes of renal failure were the same as in group *b*. Blood pressure and phosphate levels were maintained under therapeutic control. Patients with systemic pathologies (hypertension, diabetes, vasculitis, heart disease), as well as patients treated with inhibitors of cyclic nucleotide phosphodiesterases (30), were excluded from the study. All patients gave informed consent.

On the day of the study, routine biochemical tests (Hitachi model 717 automatic analyzer) and blood cell profile, plus PTH levels (immunoradiometric assay), were performed. In the dialysis group, blood was drawn immediately before the dialysis session. Blood samples were collected by venipuncture, using EDTA (1 mg/ml of blood).

L-[*methyl*-^{3}H]Methionine (sp act 83.3 Ci/mmol), S-adenosyl-L-[*methyl*-^{14}C]methionine (sp act 60 mCi/mmol), and S-adenosyl-L-[*methyl*-^{3}H]methionine (sp act 80 Ci/mmol) were from Amersham International, Amersham, UK.

Methyl esterification of membrane proteins in intact erythrocytes. Whole blood was processed and deprived of leukocytes and platelets, as described by Galletti et al. (31), with further modifications (32). Erythrocytes were then washed several times with isotonic saline solution. Protein methyl esterification was then performed on intact red cells by incubating the cells with tritiated methionine, the in vivo precursor of AdoMet according to Kim et al. (19), as further modified in Galletti et al. (32). 250 μl of packed erythrocytes was resuspended in an equal volume of 5 mM Tris/HCl buffer (pH 7.4), containing 160 mM NaCl, 0.96 mM $MgCl_2$, 2.8 mM glucose. A 5-μl aliquot was saved for cell counting in a Bürker chamber after dilution. 0.93 nmol of L-[*methyl*-^{3}H]methionine (15 μCi) was added. The mixture was incubated at 37°C for 60 min. At the end of incubation, cells were hemolyzed, and then membranes were isolated by rapid addition of 20 ml of ice-cold 5 mM sodium phosphate buffer pH 8.0, containing 25 μM PMSF, and centrifuged at 10,000 *g* for 30 min. The membrane pellet was subsequently washed twice with the same ipotonic solution at pH 7.2 and 6.8, respectively. Radioactivity in the supernatant was measured, to insure thoroughness in the washing procedures. Extent of methyl labeling was determined after solubilizing 40 μl of the membrane preparation in 0.5 ml of 10 mM acetic acid, 2.5% SDS, and counted for radioactivity with a liquid scintillation spectrometer (model LS 7800, Beckman Instruments, Inc., Palo Alto, CA) (19). Measurement of base stable radioactivity was also carried out, as described by Kim et al. (19), with minor modifications, to determine the percentage of methyl esters over total protein labeling. Briefly, 40 μl of membranes, plus 1 ml of borate buffer, was incubated for 5 min at 100°C; then 5 mg of γ-globulins as carrier proteins and 1 ml of TCA 30%, were added. The mixture was centrifuged for 15 min at 3,000 *g*. The pellet was subsequently washed with 1 ml of 15% TCA solution, then with 1 ml of ethanol, and finally counted for radioactivity. Base labile radioactivity, due to methyl incorporation, was finally determined according to reference 19. Proteins were determined by the method of Bradford (33).

SDS/PAGE of methylated membranes. SDS/PAGE of methylated membranes was performed according to the general procedure of Fairbanks et al. (34) with some modifications. 1.5-mm-thick gels, containing acrylamide 5.6% (wt/vol) and bisacrylamide 0.2% (wt/vol), in the presence of 1% SDS, at pH 7.4 were used. On each day, one control sample and one patient sample were run. Samples containing 22 μg of proteins were diluted 1:4 with SDS reducing buffer (0.5 mM Tris, pH 6.8, 1.0 ml; glycerol, 0.8 ml; 10% wt/vol SDS, 1.6 ml; β-mercaptoethanol, 0.4 ml; H_2O, 4.0 ml; 0.05% Bromophenol blue) and incubated at 37°C for 15 min. Samples were run in duplicate on the same gel, under constant voltage mode (150 V), using a gel electrophoresis apparatus (model GE-2/4 LS, Pharmacia, Uppsala, Sweden). Gels were then cut into halves. One half was stained with Coomassie Brilliant Blue for proteins and scanned with a Pharmacia/LKB UltroScan XL laser densitometer, for area quantification. The second half, loaded with duplicate samples run in parallel, was sliced into 2-mm fractions, and incorporated radioactivity was determined after elution of proteins from each slice, using 0.4 ml of 50 mM sodium acetate buffer, 0.25% Triton X-100, pH 5.2, incubated for 24 h at room temperature, with constant shaking (32). Samples were then counted for radioactivity. Background radioactivity was subtracted. Data are expressed as cpm/band area.

HPLC determination of intracellular AdoHcy and AdoMet. In preparation for HPLC analysis, frozen packed red cells were thawed, and 400 μl was treated with 100 μl of 20% perchloric acid, and centrifuged at 10,000 *g* for 3 min. The supernatant was filtered through a 0.2-μm pore filter, and AdoHcy and AdoMet concentrations were determined by HPLC, according to Cools et al. (35). Briefly, 100 μl was injected into a System Gold Solvent Module (model 126, Beckman Instruments, Inc.) equipped with a 25-cm × 4-mm Zorbax C8 reverse-phase column (Du Pont-New England Nuclear, Boston, MA), and an UV detector module model 167 (set at 254 nm). The entire system was controlled, and data handling was carried out by a System Gold software package, running on an IBM PS_2 personal computer. The column was equilibrated with buffer A (50 mM NaH_2PO_4/10 mM heptane sulfonic acid buffer, pH 3.2), containing 4% acetonitrile (ACN). Nucleosides were eluted, with a 15-min linear gradient from 4–20% ACN, followed by a 10-min linear gradient from 20–25% ACN, at a 1 ml/min flow rate. Retention times were 15.1 min for AdoHcy, and 17.2 min for AdoMet, respectively. Recovery was calculated for the two compounds of interest, by adding trace amounts of S-adenosyl-[*methyl*-^{3}H]-methionine and S-adenosyl-[^{14}C]homocysteine to different cell samples, processed in parallel, and assessed to be 40% for AdoHcy, and 50% for AdoMet. Micromolar concentrations of the indicated compounds are referred to 1 liter of packed erythrocytes.

Type II MTase assay. Erythrocyte cytosol was prepared by rapidly diluting erythrocyte samples 1:9 with a stabilizing solution, consisting of 2.7 mM EDTA, pH 7.0, and 0.7 mM β-mercaptoethanol, followed by rapid freeze-thawing, according to Beutler et al. (36). Membranes were removed by centrifugation at 10,000 *g* for 30 min. Type II MTase specific activity of erythrocyte cytosol was determined in vitro, in the presence of saturating concentrations of both ovalbumin as the methyl accepting protein and AdoMet, by using the vapor diffusion assay procedure of Macfarlane (30). The assay mixture contained, in 40 μl final volume, 1.6 mg of ovalbumin and ~ 2.8 μg of cytosolic proteins, in the presence of 0.1 M sodium citrate buffer, pH 6.0, and S-adenosyl-L-[*methyl*-^{14}C]methionine, at a 30 μM final concentration. After 10-min incubation at 37°C, the assay was quenched by adding an equal volume of 0.2 N NaOH/1% SDS solution. 60 μl was spotted on filter paper, which was placed in the neck of a 20-ml scintillation vial, prefilled with 10 ml of Ready Gel scintillation cocktail (Beckman Instruments, Inc.). Vials were rapidly tightly capped, and allowed to stand for 2 h. After this interval, filters were removed, and vials counted for radioactivity. Results are expressed as units per milligram Hemoglobin (Hb). One Unit of enzyme activity is defined as one pmol of methyl groups transferred/min. Hb concentration was determined by measuring ultraviolet absorbance at 280 nm wavelength, as reported by Gilbert et al. (16).

Glutamate-oxaloacetate transaminase (GOT) activity. GOT (EC 2.6.1.1) specific activity on the cytosol was measured at 37°C, on diluted hemolyzed samples, obtained as described in the subsection "Type II MTase assay," except that membranes were not preliminarily pelletted. The assay method described by Beutler et al. (36) was employed and the results expressed as units per milligram Hb (micromoles of substrate converted per minute).

Statistical analysis. Statistical analysis was performed employing Student's unpaired *t* test. Linear regression analysis was done to assess the independent effects of the different variables (37). All calculations were performed using the software package StatWorks (Cricket Software, Inc., Philadelphia, PA), running on an Apple Macintosh IIfx personal computer. All results are presented as the means±SE.

Results

Erythrocyte membrane protein methyl esterification in CRF. Membrane protein methyl esterification was evaluated in intact RBC after incubation with tritiated methionine, as described above. Data were normalized both per cell number and per milligram of protein to highlight any interference owing to protein content or modification in cell number between groups.

Total protein carboxyl methyl esterification levels are shown in Table I. A significant reduction in total carboxyl methyl esterification levels was observed in both CRF groups, compared with controls. Results are highly consistent either by expressing methylation levels as cpm/mg protein, or as cpm/10^6 cells.

Protein methyl esterification is quantitatively the most important AdoMet-consuming reaction in human erythrocytes, accounting for the bulk of the utilization of this thioether (25). In our study, we were able to confirm that methyl esters, measured as base-labile methyl groups, accounted for the large majority of total radioactivity incorporated into membranes in all samples. Background, base-stable radioactivity was found to be not significantly different between the groups: control 1,600±400 cpm/mg protein, $n = 6$; nondialysis patients 1,300±600 cpm/mg protein, $n = 6$, P NS vs. control; and dialysis patients 1,800±700 cpm/mg protein, $n = 6$, P NS vs. control. This result is highly consistent with that reported from erythrocytes of normal subjects (20) and shows that, also in erythrocyte membranes from CRF patients, the majority of methyl groups are incorporated as labile methyl esters, while contributions from both residual protein synthesis and other transmethylation reactions are in fact negligible.

It has been reported that membrane protein methyl esterification increases as erythrocytes age in circulation (32, 38), owing to the progressive accumulation of altered aspartyl residues (39). In order to check if the decrease in carboxyl methylation has to be referred to a shorter life span of uremic red cells, we measured GOT activity in the erythrocyte lysates, as a marker of cell age (36). GOT specific activity was expressed as units per milligram Hb. Values were not significantly different between the groups: control 7.03±0.5, $n = 9$; nondialysis 6.57±0.6, $n = 6$, P NS vs. control; dialysis 7.56±1.76, $n = 7$, P NS vs. control. GOT activity of each sample never correlated with the corresponding values of membrane protein methyl incorporation (data not shown). These results indicate that the decrease in membrane protein methylation, in these patients is not due to a reduced RBC age.

Characterization of methyl accepting proteins in erythrocyte membranes in CRF. A remarkable amount of information on enzymatic methyl esterification has been obtained in the human erythrocyte model system (13, 14). In mature erythrocytes, de novo protein synthesis is not operative, and this makes it possible to utilize labeled methionine as the AdoMet precursor, with no interference due to the incorporation of methionine itself in protein backbone. Furthermore, erythrocyte membrane proteins have been identified in sufficient detail, thus allowing the characterization of methylated protein species (15, 19, 20). In the present study, methyl accepting proteins in erythrocyte membranes have been identified by means of SDS-PAGE, according to the procedure detailed under Methods. The distribution pattern of methyl labeling, among the various protein species, is showed in Table II. The major methyl acceptors, in all patient groups, were cytoskeletal component bands 2.1 (ankyrin), 4.1, and 4.2, and the integral membrane protein of band 3, corresponding to the anion transporter (Table II). From the quantification of incorporated methyl esters normalized per band area, a significant decrease

Table I. Total Protein Carboxyl Methyl Esterification Levels

	Methylation levels	
	cpm/10^6 cells	*cpm/mg protein*
Control ($n = 24$)	34.21±1.91	84445±5348
Nondialysis ($n = 12$)	28.58±1.46*	70151±4261§
Dialysis ($n = 12$)	24.03±2.51‡	55289±4899‖

Subjects under study include: (*a*) a control group of healthy subjects age and sex matched to patients of groups *b* and *c*; (*b*) patients with varying degrees of renal failure, creatinine clearance between 10 and 40 ml/min, on conservative treatment, age range 12–68 yr (mean 45±7); (*c*) patients on hemodialysis therapy thrice weekly, clinically stable at the time of investigation, age range 44–64 yr (mean 52±3), treated with erythropoietin intravenously to reach an hematocrit of ~30%. 250 μl of packed erythrocytes, prepared from each subject, was incubated for 1 h at 37°C, in an equal volume of 5 mM Tris/HCl (pH 7.4), containing 160 mM NaCl, 0.96 mM $MgCl_2$, 2.8 mM glucose, in the presence of L-[*methyl*-^{3}H]methionine (15 μCi/0.93 nmol), precursor of the methyl donor AdoMet. Cells were then lysed and membranes washed repeatedly and isolated. Membranes were then counted for radioactivity. Background owing to nonmethyl ester radioactive incorporation was subtracted in all cases.

* $P < 0.02$; ‡ $P < 0.005$; § $P < 0.05$; ‖ $P < 0.001$ vs. control.

Table II. Methyl Esterification Pattern of Erythrocyte Membrane Proteins

	Band			
	2.1	3	4.1	4.2
	cpm/band area			
Control ($n = 18$)	1,334.0±182.0	129.6±16.5	326.8±77.0	310.8±56.8
Nondialysis ($n = 8$)	609.2±87.6*	106.3±15.4	282.5±68.1	243.6±63.9
Dialysis ($n = 8$)	700.4±123.0‡	82.8±15.9	262.4±73.5	229.1±60.9

Membrane proteins were methyl esterified in intact erythrocytes from the three groups of subjects, as in Table I. Duplicate samples, each corresponding to 22 μg of methyl-labeled membrane proteins and prepared as described in Table I, were analyzed by SDS/PAGE, according to Fairbanks et al. (34). 1.5-mm-thick slab gels, containing acrylamide 5.6% (wt/vol) and bisacrylamide 0.2% (wt/vol), in the presence of 1% SDS at pH 7.4, were used. Gels were cut into halves, which were further processed separately. One half was stained with Coomassie Blue and densitometrically scanned for band quantification. The other half was cut into 2-mm sections, subjected to protein extraction, and finally counted for radioactivity. Radioactivity recovered from gels was, in all study groups, 75% of the amount loaded at start. This value was comparable to what we have previously observed by using gel systems under acidic separating conditions, such as the one described in reference 20.

* $P < 0.005$; ‡ $P < 0.01$.

in methyl esterification of band 2.1 (ankyrin) was observed in both patient groups, compared with that of control. Differences were not significant for the other methyl esterified protein bands. Interestingly, we could not detect any significant modification in protein composition of erythrocyte membranes, owing to CRF with or without need of hemodialysis therapy, as evaluated densitometrically among the three study populations.

Determination of type II MTase activity. As a potential cause for the observed decrease in membrane protein methyl esterification, we examined the possible occurrence of an altered erythrocyte type II MTase activity, as a result of CRF. To check this hypothesis, the in vitro specific activity of type II MTase in the RBC cytosol was measured in the three study groups.

The data are shown in Table III and clearly indicate that type II MTase specific activity did not vary significantly among the groups, allowing us to rule out any contribution of this factor to the reduced methyl incorporation observed in RBC membrane proteins.

AdoMet and AdoHcy intracellular concentrations. AdoHcy is the natural by-product of the methyl transfer reaction from AdoMet to methyl accepting proteins, and also a potent inhibitor of AdoMet-dependent reactions (26–29).

We therefore measured the intracellular concentrations of both AdoHcy and AdoMet in RBC under our experimental conditions. The results are presented in Table III and show that the concentration of AdoHcy is increased almost 10 times in the dialysis group, while in the group on conservative treatment the increase was fourfold with respect to control. With regard to AdoMet levels, concentrations between the groups were not statistically different.

Because the AdoHcy inhibitory mechanism is competitive with respect to AdoMet, changes in relative concentrations of the two compounds can actually determine the extent of inhibition (26–29). As an indicator, we therefore calculated the [AdoMet]/[AdoHcy] ratio, as shown in Fig. 1. Ratios relative to each group were the mean±SE of ratios derived from each individual experiment. It can be observed that the decrease in the dialysis and in nondialysis patients is highly significant.

Table III. Type II MTase Specific Activity and AdoMet and AdoHcy Concentration in Erythrocyte Cytosol

	Type II MTase sp act	AdoHcy	AdoMet
	U/mg Hb	*μM*	*μM*
Control (n = 7)	4.66±0.19 (n = 9)	0.77±0.05	2.56±0.46
Nondialysis (n = 6)	5.23±0.22	3.01±0.87*	1.76±0.26
Dialysis (n = 6)	5.11±0.20	6.87±1.51‡	2.10±0.54

Type II MTase specific activity was measured in vitro by radiochemical enzyme assay in the presence of saturating concentrations of both the methyl donor [*methyl*-^{14}C]AdoMet and a methyl-accepting protein substrate. The vapor diffusion technique of Macfarlane et al. (30) was employed. Results, expressed as type II MTase units, have been normalized per milligram Hb (1 enzyme unit = 1 pmol methyl groups transferred/min). AdoMet and AdoHcy intracellular concentrations were HPLC-determined according to Cools et al. (35), in the TCA-soluble fraction prepared from erythrocyte lysates. Micromolar concentrations of the two compounds are referred to 1 liter of packed cells.

* $P < 0.05$ vs. control; ‡ $P < 0.005$ vs. control.

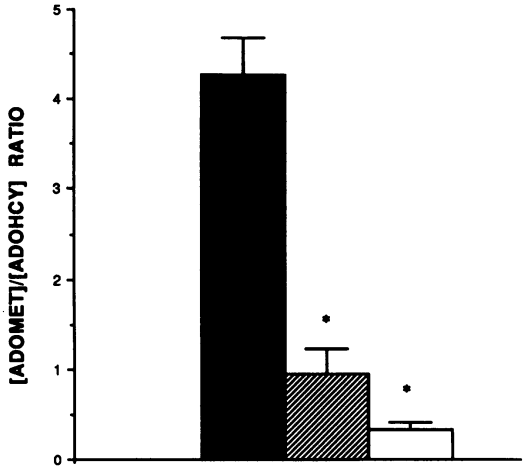

Figure 1. [AdoMet]/[AdoHcy] ratio in erythrocyte cytosol. In preparation for HPLC analysis, frozen packed red cells were thawed, treated with perchloric acid, and centrifuged. The supernatant was then filtered. AdoHcy and AdoMet concentrations were determined according to the method of Cools et al. (35). 100 μl was injected into a Beckman System Gold Solvent Module, equipped with a 25-cm × 4-mm Zorbax C8 reverse-phase column, and an UV detector set at 254 nm. The column was equilibrated with buffer A (50 mM NaH_2PO_4/10 mM heptane sulfonic acid buffer, pH 3.2), containing 4% ACN. Nucleosides were eluted with a 15-min linear gradient from 4–20% ACN, followed by a 10-min linear gradient from 20–25% ACN, at a 1 ml/min flow rate. Retention times were 15.1 min for AdoHcy and 17.2 min for AdoMet, respectively. Recovery was assessed to be 40% for AdoHcy, and 50% for AdoMet. Ratios were derived from the mean of ratios relative to each individual experiment. [AdoMet]/[AdoHcy] ratio of control (*solid bar*), nondialysis (*crossed hatched bar*), and dialysis patients (*clear bar*) are shown. * $P < 0.001$ vs. control. An altered intracellular concentration of AdoHcy, as derived from HPLC analysis, brings about a significantly reduced ratio, signifying that an environment not suitable with normal methyl group transfer is present in these cells (41).

Interestingly, among all the variables we considered for any degree of correlation (Hb, creatinine, PTH, etc.) with AdoHcy concentration (or methylation levels), one strong correlation that we were able to document was between AdoHcy levels and blood urea nitrogen (BUN) concentration, mmol/liter (Fig. 2), a positive simple correlation with a correlation coefficient $r = 0.76$, $P < 0.001$.

Discussion

The inherent instability of protein molecules can cause the spontaneous formation of less active or potentially toxic derivatives (39). Major structural alterations are represented by the spontaneous occurrence of D-aspartyl and L-isoaspartyl residues, which can be selectively recognized and modified by the enzyme type II MTase (13, 21–24). Independent research groups have demonstrated that this enzyme plays a crucial role in the repair of proteins affected by these specific molecular damages. In particular, it has been shown that isoaspartyl methylation would serve as the enzymatic step in a mechanism for quantitative conversion of isopeptide bonds into normal peptide bonds in proteins (21–23). Convincing evidence of type II

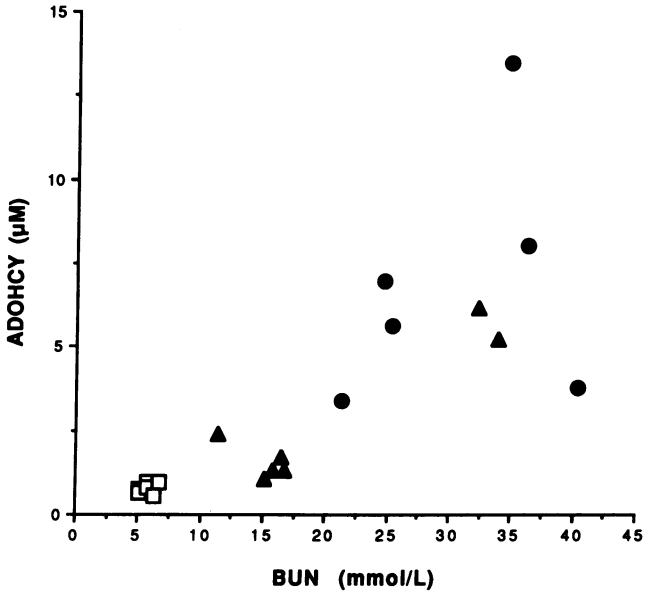

Figure 2. Correlation between BUN and intracellular AdoHcy. BUN was determined from serum samples of subjects under study (normal range 1.7–8.3 mmol/liter). AdoHcy intracellular concentration was also measured in erythrocyte samples from the same patients, as reported in Table III. Scattered plot of BUN values (mmol/liter) on the abscissa, relative to AdoHcy levels (μM) on the ordinate, is shown. (□) Control values, (▲) nondialysis patients, (●) dialysis patients. A positive simple correlation between AdoHcy and BUN concentrations, $r = 0.76$, $P < 0.001$, is documented. L, liter.

MTase ability to restore protein function loss due to isoaspartyl formation is that deamidated calmodulin, deficient in the activation of calmodulin-dependent protein kinase, regains through methyl esterification an activity approaching that of native calmodulin (24).

This ubiquitous enzymatic posttranslational modification has been examined in detail in human RBC, where some critical membrane protein components are substrates of type II MTase (15, 19, 20). This represents, by far, the most prominent methyltransferase reaction that takes place in red cells (20). Thus, RBC represent an ideal model system to study this reaction (15). It has been demonstrated that specific erythrocyte membrane proteins, among which, in particular, cytoskeletal components bands 2.1 (ankyrin) and 4.1 (the same proteins that provide some rigidity to the membrane) are substrates for the methyltransferase (20, 32, 38).

Anemia is a frequent complication in renal insufficiency, and, although this condition has been commonly related to erythropoietin deficiency, hemolysis represents an additional factor (5, 6). It has been proposed that in CRF toxic effects can be exerted by PTH, among other proposed compounds, but how these lead to RBC damage is not clear (7). Nevertheless, the final effects on erythrocytes are a decline in metabolic function and membrane stability (6, 8–12).

Data presented here indicate that erythrocyte membrane protein methyl esterification is significantly decreased in CRF, and that this is not modified in patients requiring maintenance dialysis therapy (Table I). The reduction has been found to be significant for band 2.1 (Table II), a cytoskeletal component involved in membrane stability and integrity (20). These findings are therefore consistent with the notion that damaged membrane proteins accumulate in CRF, and are not adequately repaired.

Our data cannot be explained by a reduced activity of type II MTase itself, in that we found that type II MTase specific activity was not different from control (Table III).

Previous studies (32, 38) demonstrated that protein methyl esterification of all major methyl-accepting membrane proteins, namely bands 2.1, 3, 4.1, and 4.5, increases with RBC aging, band 4.1 being the most dramatically affected by these changes (32). In all these studies, variations in protein methylation always correlated with the corresponding alterations in red cell age markers. This progressive increment of protein methylation was explained by the age-dependent accumulation of molecular damages, leading to the formation of new methyl-accepting sites (26, 32, 38). In the present study, we found that reduction in methylation does not correlate with GOT levels (an established marker of the age of circulating red cell population), so it does not appear to be related to the decrease in RBC life span, frequently seen in CRF (6). Moreover, we found that methylation of band 2.1 (ankyrin) solely is significantly reduced among erythrocyte membrane proteins in CRF patients, whereas bands 3 and 4.1 both appear to be poorly affected (Table II).

As an alternative explanation for the decrease in methyl esterification, we probed for the presence of an enzyme inhibitor, and in particular we focused our attention on AdoHcy (Fig. 3). An important role in the regulation of AdoMet-dependent methylation rates applies to AdoHcy. This demethylated AdoMet derivative is in fact a potent competitive inhibitor of all AdoMet-dependent methylation reactions (26–29). The K_i of the reaction, particularly in the case of type II MTase, is in the same order of magnitude of the K_m for AdoMet (26, 29). The intracellular accumulation of AdoHcy is normally prevented by its hydrolysis to adenosine and homocysteine,

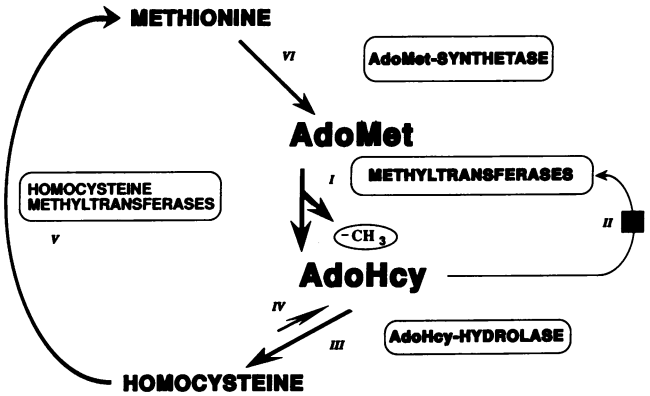

Figure 3. Hypothesis on the mechanism for the increased intracellular concentration of methylation inhibitor AdoHcy. AdoMet-dependent methyl transfer reactions yield AdoHcy as the demethylated product (*step I*). AdoHcy is the competitive inhibitor of all AdoMet-dependent methyltransferases, including type II MTase (*step II*). Under normal conditions, the intracellular concentration of AdoHcy is maintained low through its hydrolysis, yielding homocysteine (*step III*). AdoHcy-hydrolase is a bidirectional enzyme, in that AdoHcy buildup is thermodynamically favored, in the presence of excess homocysteine (*step IV*). The latter compound can also be utilized for the endogenous synthesis of methionine (*step V*), which is the intracellular precursor in the ATP-dependent AdoMet synthesis, catalyzed by AdoMet-synthetase (*step VI*).

through the action of AdoHcy-hydrolase (28, 40). Consequently, under normal conditions, the rate of transmethylations is not affected by AdoHcy (28, 40). However, in consideration of the remarkably potent inhibitory effect of AdoHcy on type II MTase activity, and the close numeric values of the kinetic constants, any decrease of [AdoMet]/[AdoHcy] ratio can potentially slow down the transmethylation rate. All enzymatically mediated transfers of methyl groups from AdoMet, either to proteins or to nucleic acids, phospholipids, etc., can be affected, in a competitive fashion, by a conspicuous increase of intracellular AdoHcy, and/or a reduction of the [AdoMet]/[AdoHcy] ratio (26–29, 40, 41).

Our data show a significant increase in AdoHcy RBC intracellular concentration in both renal failure groups. AdoMet levels are not reduced, and [AdoMet]/[AdoHcy] ratios are therefore significantly reduced severalfold in both patient groups. Moreover, our finding that AdoHcy levels could be correlated in a significant manner to BUN concentration, and poorly to other independent variables, may indicate an association between sulfur and nitrogen metabolism.

We explain the reduction in methyl esterification levels via the inhibitory effect of an altered [AdoMet]/[AdoHcy] ratio. Elevation of AdoHcy levels to the range of values observed by us induced a decrease of methyl esterification of about 30% in the set of experiments performed by Barber and Clarke (26) in normal RBC, a finding which is highly consistent with our results. Thus, it can be concluded that the extent of the reported reduction can be expected and even predicted.

Various investigators have described that high plasma levels of homocysteine are present in CRF patients, treated or not with dialysis, while this compound is almost undetectable in normal subjects (42–44). This was thought to be a consequence of decreased renal excretion, decreased metabolism to cystathionine, and/or remethylation to methionine (due to a postulated deficiency of folic acid) and linked to the increased cardiovascular disease risk present in uremia (45). It was not connected, until now, to its parent compound, AdoHcy, to which homocysteine can be converted, if in excess, through the action of AdoHcy-hydrolase (Fig. 3). This enzyme catalyzes the equilibrium reaction between AdoHcy and its hydrolysis products homocysteine and adenosine (26). It has been shown that the shift to hydrolysis in vivo depends on the removal of the products, while in vitro thermodynamics favors synthesis of AdoHcy (28, 40).

We therefore suggest that increased homocysteine, which easily crosses RBC membrane and leads to accumulation of AdoHcy (26), could significantly slow down AdoHcy hydrolysis in CRF. The resulting chronic accumulation of this compound would induce an inhibition of methyl esterification reactions. Our data indicate that this situation is not significantly modified in dialysis patients. This interpretation is consistent with the finding by Laidlaw et al. (43), that elevated blood homocysteine can only be partially lowered by hemodialysis.

Our findings, demonstrating an impairment of membrane protein methyl esterification in RBC accompanied by high intracellular AdoHcy, may not be limited to RBC alone. AdoMet is in fact involved in a great number of important reactions, ranging from small-molecule to lipid, DNA, and protein methylations. As an example, DNA methylation represents a strong modulator of cell growth and oncogene expression (46–48). Methylation inhibitors consequently display several metabolic effects (49–52). It is worthwhile noting that, according to Cantoni et al. (41), an inhibitory effect on a number of methyl transfer reactions, ranging from 20% to 80% of maximal activity, can be calculated when the [AdoMet]/[AdoHcy] ratio drops to 1.6.

These considerations suggest the possibility that elevated AdoHcy, if present in other cells as well, could affect the function of other methyltransferases. If indeed such alterations occur, this could provide at least partial explanation for some of the several cell dysfunctions present in uremia. Such studies in other cells are beyond the scope of our investigation and require additional exploration.

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