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PRELIMINARY REPORT

Protein Oxidation in Hemodialysis and Kidney Transplantation

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Oxidative damage of plasma proteins determined with the markers carbonyl group (CG) content and thiobarbituric acid–reactive substances (TBARS) was studied in 13 hemodialyzed and eight kidney-transplanted patients. The level of CGs was 38% higher in hemodialysis (HD) patients (1.49 ± 0.05 nmol/mg protein) than in the healthy subjects (1.08 ± 0.03 nmol/mg protein); the TBARS level was also higher in HD patients than in the control group (2.64 ± 0.15 *v* 1.81 ± 0.09 nmol/mL, $P < .001$). These data confirm that in end-stage renal failure, an increased oxidative stress is present and is able to induce protein damage. After transplantation, the CG content in protein was reduced (1.34 ± 0.08 nmol/mg protein), but it was not significantly different from the level in the HD group. The failure to return to the normal range suggests that an impaired redox status is maintained, resulting in a sustained elevation of CG. Conversely, the level of TBARS in transplanted patients (1.99 ± 0.22 nmol/mL) was not significantly different from that in the control group (1.81 ± 0.09), suggesting that lipoperoxidation may be inhibited. These results may be explained by the different turnover rates of the molecules and by the distinct origin of the two markers, resulting from the damage of proteins or lipids. Thus, lipoperoxidation would produce rapidly removable molecules, whereas protein oxidation damage would tend to accumulate. However, the significant correlation found between CGs and TBARS indicates that a common cause (oxidative stress) binds the two markers of damage.

REACTIVE OXYGEN SPECIES (ROS) produce injuries to cell membranes and to tissues in several pathological conditions.[1,2] Oxidative stress has been reported to be higher in subjects with chronic renal failure, and the substitutive treatment by hemodialysis (HD) is suspected to exacerbate the flux of free radicals, resulting in enhanced oxidative damage.[3-5]

This study was designed to evaluate oxidative damage in a group of hemodialyzed patients and kidney-transplanted patients.

SUBJECTS AND METHODS

Patients

Thirteen uremic patients (four women and nine men) undergoing HD (either bicarbonate HD or acetate-free biofiltration) three times weekly were studied. The mean age was 56 ± 4 years, and mean HD duration was 140 ± 28 weeks (35 ± 7 months). All patients were within 15% of their ideal body weight. The causes of chronic renal failure were as follows: hypertension or vascular disease (n = 4), polycystic kidney disease (n = 3), chronic glomerulonephritis (n = 1), nephrectomy for cancer (n = 1), and nonspecific chronic renal failure (n = 2). None of the patients were diabetic or affected by an acute illness; patients positive for hepatitis B or C virus were excluded.

All patients were receiving erythropoietin and calcium carbonate; some of them were on antihypertensive therapy (clonidine, β-blocker, or calcium antagonist), and two were on H_2-blocker therapy. Blood samples were drawn before and at the end of the hemodialysis session, collected in tubes with heparin for carbonyl group (CG) determination and in K_3-EDTA for thiobarbituric assay, immediately placed on ice, and centrifuged. The second group of patients (n = 8, four men and four women), aged 40 ± 4 years, had undergone a successful kidney transplant at least 6 months before the study; all were under immunosuppressive therapy (cyclosporin, azathioprine, and prednisone). The control group consisted of 29 healthy subjects (14 men and 15 women) from the hospital staff, with an age range (44 ± 2 years) comparable to that of the transplanted group.

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Methods

After an overnight fast, blood samples were collected in heparinized tubes and immediately centrifuged. Plasma proteins were precipitated with 20% trichloroacetic acid (1:1 vol/vol) and centrifuged at 11,000 × *g*. The pellet was then used for the 2,4-dinitrophenylhydrazine assay[6] as previously described[7] for CG content. Plasma protein content was determined by the bicinchoninic acid method (Pierce Chemical, Prodotti Gianni, Milan, Italy). Thiobarbituric acid–reactive substances (TBARS) were assayed by the method of Young and Trimble.[8] As a standard, pure malondialdehyde (monosodium trihydrate) was obtained from 1,1,3,3-tetraethoxypropane by hydrolysis, following the procedure of Nair et al.[9] The thiobarbituric reaction was initiated by mixing 750 μL phosphoric acid (0.44 mol/L) with 50 μL sample (plasma collected in K_3-EDTA and immediately separated from cellular component, or the standard). Two hundred fifty microliters of thiobarbituric acid solution (42 mmol/L) was added to the sample, and then high-performance liquid chromatography (HPLC)-grade distilled water was used to adjust the volume to 1.5 mL. Tubes were capped tightly and placed in a hot water bath (100°C) for 60 minutes. At the end of incubation, the samples were cooled in ice (until HPLC analysis was performed).

Within 10 minutes before injection onto the column, all samples were neutralized with 0.5 mL methanol-NaOH solution (4.5 mL 1-mol/L NaOH plus 50 mL methanol HPLC-grade) for protein precipitation. Fifty microliters of clear supernatant was injected in a 3.9 × 300-mm C18 μBondapak column (Waters, Milan, Italy). The mobile phase contained 50% methanol and 50% 25-mmol/L phosphate buffer (pH 6.5); the flow rate was 0.8 mL/min. The detection system (Waters 470 fluorimeter) was set at 532 nm excitation and 553 nm emission with gain ×1,000 and attenuation at 32. TBARS were calculated on the basis of the malondialdehyde standard calibration curve.

Nonparametric ANOVA (Kruskal-Wallis) and a posttest test (Dunn) were used to evaluate statistical differences among the three groups. The Wilcoxon rank test was used for comparison of paired data. Linear regression analysis was performed by a nonparametric Spearman test. A probability level less than 5% ($P < .05$) was considered significant.

Age adjustment for TBARS was made to age 40 using the graph coordinates of the variable and the slope of the regression line for control subjects.

RESULTS

Protein CG levels in the plasma of HD patients were found to be higher than in the healthy control group (1.49 ± 0.05 *v* 1.08 ± 0.03 nmol/mg protein, $P < .01$), but were not significantly different from the levels in the group of transplanted patients (1.34 ± 0.08 nmol/mg protein; Fig 1b). However, the concentration observed in this group was higher than in controls ($P < .05$). CGs in plasma proteins were not significantly modified by the HD session (1.49 ± 0.05 *v* 1.54 ± 0.05 nmol/mg protein, $P = .34$; Fig 1a). Among the three groups, ANOVA revealed a significant difference ($H = 22.6, P < .001$).

Plasma TBARS were higher in HD patients than in healthy subjects (2.64 ± 0.15 and 1.81 ± 0.09 nmol/mL, $P < .001$); after a dialysis session, a slight but significant increase was observed (postdialysis, 2.99 ± 0.18 nmol/mL, $P < .05$). The group of transplanted patients showed a concentration of TBARS not significantly different from that in the control group (1.99 ± 0.22 nmol/mL), but significantly lower than the level in HD patients ($P < .05$). Finally, the TBARS level (all subjects) was significantly correlated with the CG content in protein ($r = .31, P < .03$, N = 50; Fig 2).

DISCUSSION

An excess of free ROS, not adequately restrained by the defense network, leads to compulsory oxidative cellular or structural damage. The difficulty lies in recognizing the specific alterations induced in the body by ROS. Several studies have reported that patients undergoing chronic HD are subjected to increased oxidative stress,[3-5] but the issue is still controversial[10]: data are usually obtained either by determination of lipoperoxidation by-products—such as conjugated diene fatty acids (CDFA) or TBARS—or by indirect measurement using the levels of active oxygen scavengers.[11,12] In this study, TBARS and protein CG content were evaluated in the plasma of HD and kidney-transplant patients.

The TBARS group of molecules are mainly represented

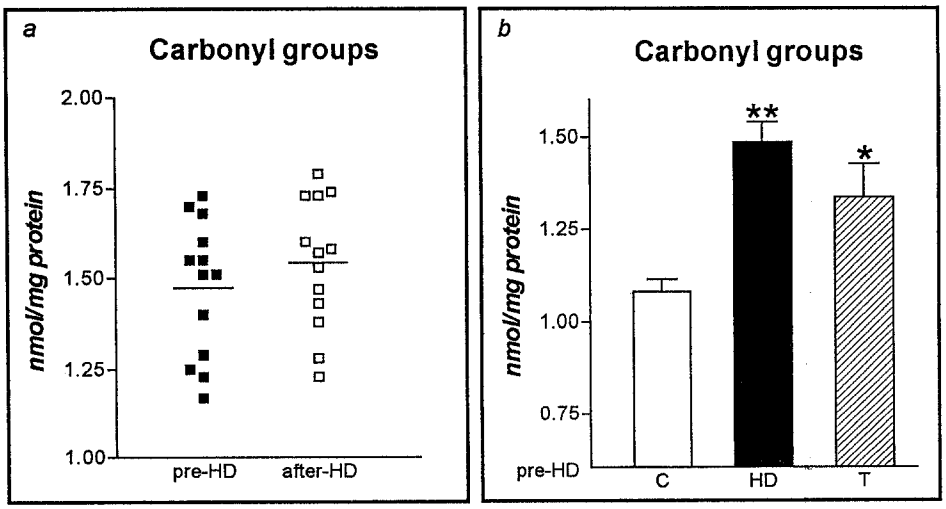

Fig 1. (a) Scatterplot of plasma CGS in 13 uremic patients before (■) and after (□) a HD session. (b) CG levels in controls (C, n = 29), HD patients (n = 13), and renal-transplant patients (T, n = 8). Values are mean ± SEM. *$P < .05$ *v* control group; **$P < .01$ *v* control group.

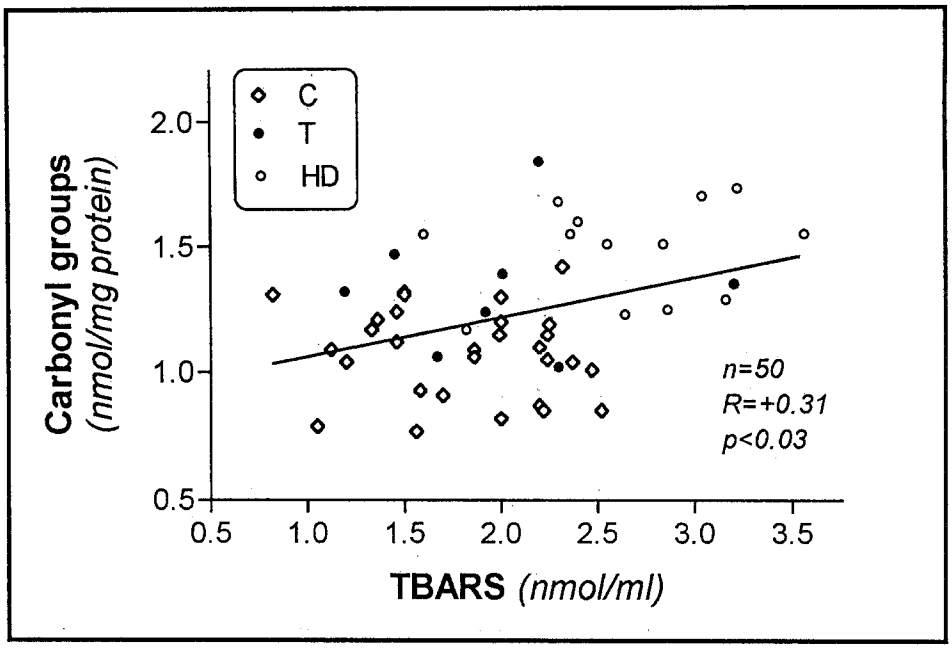

Fig 2. Correlation between TBARS and plasma protein CGs evaluated by Spearman analysis. C, control group (n = 29); T, renal-transplant patients (n = 8); HD, HD patients (n = 13).

by aldehydes and lipoperoxidation by-products, still highly reactive but with a short half-life.[13] The evaluation of CGs was proposed initially by Stadtman[14] as the most specific and sensitive marker of oxidative damage in cell and tissue proteins. Moreover, these steady groups represent the result of oxidative injuries throughout the protein lifetime. Smith et al[15] reported a higher level of protein CGs in the brain of old subjects than in young ones. Sohal et al[16] described a clear-cut pattern of an age-related increase of CG content in various animal tissue homogenates.

In this study, it was found that the TBARS mean level increased after the HD session. In transplanted patients, TBARS were close to the normal range.

In contrast, only a slight and statistically insignificant modification in CGs was observed after the HD session, and the mean level in transplanted patients was 28% higher than in controls.

The increase of TBARS and the insignificant change in CGs after HD might be explained by the higher susceptibility to oxidation of lipids than of proteins, so that the lipoperoxidation markers (CDFA and TBARS) were seen to increase readily after the HD session. However, the data in the literature are not homogeneous, and the debate on the topic is still open.[17-19]

The excess of oxidative damage in HD patients has been attributed to a low antioxidant status associated with a low level of selenium, zinc, or manganese[12] or to an increased consumption of antioxidant enzymes[20] or vitamins,[3] but mainly to a higher release of ROS, especially during the HD session and the filtration process.[21]

Our HD patients were receiving drugs for treatment of anemia and hypertension—their metabolites may interfere with the determination of protein CGs and might be a potential confounding factor. Although it seems unlikely to us, it has to be taken into account.

Another question may arise from the age of the patients undergoing HD, who were older than those in the two other groups. However, no age effect in plasma CGs has been found,[7] and TBARS, adjusted for age (40 years), remained significantly different in HD patients (2.47 ± 0.12 nmol/mL, $P < .05$ *v* controls and *v* transplanted group). Thus, the higher level was determined by the factors focused on in this discussion.

The patients who underwent renal transplant showed a normal concentration of TBARS, but the plasma protein CG concentration was higher than that of the control group. The difference seems negligible, but it is important because 2 nmol CG/mg protein is calculated to represent a 10% damage to total cellular protein in vivo.[14] The failure to return to the normal range even 6 months after transplantation might be attributed either to the relatively short period elapsing after transplant or to the yet unsatisfactory antioxidant status. Further study is required to address specific causes and establish the correct supportive therapy.

The weak but statistically significant correlation between the elevation in TBARS and CG suggests that they have a common etiology (eg, oxidative stress). Their different origins and turnover rates[1,13] are likely to be the principal cause of discrepancies in TBARS and CG levels in different disease states.

Moreover, our results are in agreement with the data of Berkelhammer et al[22] reporting that enzymatic protein oxidation is activated in patients undergoing chronic HD. The increased protein oxidation rate has been attributed to endocrine disturbances and to metabolic acidosis. It is conceivable that nonenzymatic oxidative damage of proteins leads to premature aging and acts as an activating step for enzymatic degradation that results in a faster removal of damaged protein.[14]

Thus, the incapability to renew all the damaged protein might be the final cause of the loss of functions in uremia.

REFERENCES

1. Halliwell B, Gutteridge JMC, Cross CE: Free radicals, antioxidants, and human disease: Where are we now? J Lab Clin Med 119:598-620, 1992

2. Ames BN, Shigenaga MK, Hagen TM: Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 90:7915-7922, 1993

3. Peuchant E, Carbonneau MA, Dubourg L, et al: Lipoperoxidation in plasma and red blood cells of patients undergoing haemodialysis: Vitamins A, E and iron status. Free Radic Biol Med 16:339-346, 1994

4. Lucchi L, Banni S, Botti B, et al: Conjugated diene fatty acids in patients with chronic renal failure: Evidence of increased lipid peroxidation? Nephron 65:401-409, 1993

5. Loughrey CM, Young IS, Lightbody JH, et al: Oxidative stress in haemodialysis. Q J Med 87:679-683, 1994

6. Levine RL, Garland D, Oliver CN, et al: Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 186:464-479, 1990

7. Garibaldi S, Aragno I, Odetti P, et al: Relationships between protein carbonyls, retinol and tocopherol level in human plasma. Biochem Mol Biol Int 34:729-736, 1994

8. Young IS, Trimble ER: Measurement of malondialdehyde in plasma by high performance liquid chromatography with fluorimetric detection. Ann Clin Biochem 28:504-508, 1991

9. Nair V, Vietti DE, Cooper CS: Degenerative chemistry of malondialdehyde. Structure, stereochemistry, and kinetics of formation of enaminals from reaction with amino acids. J Am Chem Soc 103:3030-3036, 1981

10. Schettler V, Wieland E, Verwiebe R, et al: Plasma lipids are not oxidized during hemodialysis. Nephron 67:42-47, 1994

11. Paul J-L, Sall N-D, Soni T, et al: Lipid peroxidation abnormalities in hemodialyzed patients. Nephron 64:106-109, 1993

12. Richard MJ, Arnaud J, Jurkovitz C, et al: Trace elements and lipid peroxidation abnormalities in patients with chronic renal failure. Nephron 57:10-15, 1991

13. Esterbauer H, Schaur RJ, Zollner H: Chemistry and biochemistry of 4-hydroxynonenal and malondialdehyde and related aldehydes. Free Radic Biol Med 11:81-128, 1991

14. Stadtman ER: Protein oxidation and aging. Science 257:1220-1224, 1992

15. Smith CD, Carney JM, Starke-Reed PE, et al: Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. Proc Natl Acad Sci USA 88:10540-10543, 1991

16. Sohal RS, Ku H, Agarwal S, et al: Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. Mech Ageing Dev 74:121-133, 1994

17. Halliwell B, Gutteridge JMC: Diene conjugation, in Free Radicals in Biology and Medicine. Oxford, UK, Clarendon, 1989, pp 220-223

18. Janero DR: Malondialdehyde and thiobarbituric acid–reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 9:515-540, 1990

19. Bird RP, Draper HH: Comparative studies on different methods of malondialdehyde determination. Methods Enzymol 105:299-305, 1984

20. Toborek M, Tomasz W, Drozdz M, et al: Effect of haemodialysis on lipid peroxidation and antioxidant system in patients with chronic renal failure. Metabolism 41:1229-1232, 1992

21. Dasgupta A, Hussain S, Ahmad S: Increased lipid peroxidation in patients on maintenance haemodialysis. Nephron 60:56-59, 1992

22. Berkelhammer CH, Baker JP, Leiter LA, et al: Whole-body protein turnover in adult hemodialysis patients as measured by ^{13}C-leucine. Am J Clin Nutr 46:778-783, 1987