The importance of serum proteins in the interpretation of total homocysteine

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RESUMEN

Objetivo: Se ha implicado a la circulación incrementada de homocisteina total (tHcy), como un factor de riesgo independiente para enfermedades ateroesclerótica y tromboembólica. Puesto que el 70% de la tHcy está ligada a proteínas, deseamos determinar si el análisis de la tHcy está influenciado por perfiles alterados de proteínas séricas, tales como las que son típicas del mieloma múltiple (MM).
Método: Con este propósito analizamos tHcy, albúmina, globulinas y creatinina en 46 pacientes con MM con paraproteinemia IgG para probar esta hipótesis y determinar la dependencia de la concentración de tHcy en el perfil de proteína en esta enfermedad.
Resultados: La medida de tHcy en individuos con MM fue de 15.9±9.9 μmol/L, significativamente más alto que en nuestra población de referencia (10.1±6.6 μmol/L, n = 711). Como era de esperarse la media de la IgG sérica se incrementó (22.5 ±16.6 g/L) y la albúmina sérica disminuyó moderadamente (39 ±6 g/L). Se observó correlación significativa (r=0.33, p<0.05) de tal modo que una disminución de 1g/L en albúmina fue asociada con disminución de 5.5% en tHcy. La creatinina sérica es moderadamente una covariante significativa de tHcy, puesto que el metabolismo de la homocisteina es dependiente de la función renal normal. Sin embargo no se observó correlación en nuestra cohorte de MM, de hecho algunos pacientes de MM con albúmina sérica baja tenía niveles de tHcy por debajo del límite de referencia (5 μmol/L), a pesar de creatininas séricas altas.
Conclusión: Nuestras observaciones enfatizan que los niveles de tHcy deben ser interpretados con cautela en cualquier paciente que tenga cualquier alteración que afecte al perfil de proteína sérica.

Palabras clave: homocisteína, albúmina sérica, proteinas séricas, creatinina, mieloma múltiple.

ABSTRACT

Objective: Increased circulating total homocysteine (tHcy) has been implicated as an independent risk factor for atherosclerosis and thromboembolic disease. Since 70% of tHcy is bound to proteins, we wished to determine whether tHcy assays are influenced by altered serum protein profiles, such as those typical of multiple myeloma (MM).
Method: We therefore assayed tHcy, albumin, globulins, and creatinine in 46 MM patients with IgG paraproteinemia to test this hypothesis and determine the dependence of the tHcy concentration on the protein profile in this disease.
Results: Mean tHcy in MM subjects was 15.9 ± 9.9 μmol/L, significantly higher than in our reference population (10.1 ± 6.6 μmol/L, n = 711). As expected, mean serum IgG was increased (22.5 ± 16.6 g/L), and serum albumin modestly decreased (39 ± 6 g/L). A significant correlation was observed (r = 0.33, p < 0.05), such that a 1 g/L decrease in albumin was associated with a 5.5% decrease in tHcy. Serum creatinine is normally a significant covariate of tHcy, since homocysteine metabolism is dependent on normal renal function. However, no correlation was seen in our MM cohort. In fact, some MM patients with low serum albumin had tHcy levels below the lower reference limit (5 μmol/L), despite high serum creatinines.
Conclusion: Our observations emphasize that tHcy levels should be interpreted with caution in any patients with any disorder affecting the serum protein profile.

Key words: homocysteine, serum albumin, serum proteins, creatinine, multiple myeloma.

INTRODUCTION

Increased total homocysteine (tHCy) has been implicated as an independent risk factor for atherosclerosis disease, including stroke, myocardial infarction, and peripheral vascular disease [1]. Homocysteine (hCySH) is a sulfur containing amino acid formed intracellularly by demethylation of dietary methionine and released by cells as a free thiol. In the extracellular space, it undergoes rapid disulfide exchange with a variety of thiol groups, especially free cysteine, glutathione, and albumin [2]. In healthy adult populations, the reference interval for tHcy is about 5 to 15 μmol/L, with a median concentration of 8 to 10 μmol/L [1,3]. The single free (cysteine) sulfhydryl of albumin is reactive with homocysteine [4,5], so it is not surprising that there is significant correlation between albumin and total homocysteine in healthy adults [6]. However, the covariation of tHcy with albumin has not been examined in any patient groups with large changes in serum protein profile.

We have assessed the effects of such altered profiles on tHcy, in a group of multiple myeloma patients (MM) [7]. Multiple myeloma is characterized by neoplastic proliferation of a clonal plasma cell line, with marked overproduction of monoclonal immunoglobulins, usually IgG or IgA, and a decrease in serum

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albumin [8]. Since most of the tHcy pool is covalently bound to albumin, we wished to determine how important a covariate the serum albumin was in relation to other well-known determinants of tHcy, such as renal function [1].

MATERIALS AND METHODS

Blood collection and sample preparation

Overall, 46 MM patients were studied, 27 subjects from the Sunnybrook and Women's College Health Sciences Centre (SWCHC, Toronto ON) and 19 from Princess Margaret Hospital, University Health Network, Toronto ON. All patients were diagnosed by standard clinical and laboratory procedures as having multiple myeloma with IgG paraproteinemia. Serum concentrations of albumin, total protein, creatinine and IgG were assayed by routine methods. Globulin concentrations were calculated by subtracting the albumin from the total protein value. Five samples from subjects with evidence of substantial renal disease (serum creatinine >150 μmol/L – Reference intervals for serum creatinine: 60-110 μmol/L in males; 50-100μmol/L in females) were excluded from the correlation analyses. These studies received ethical approval from the institutional review board of Sunnybrook and Women's College Regional Health Centre.

Homocysteine assay

Total plasma homocysteine (tHcy) was measured using an HPLC method as previously described [9,10]. In brief, tHcy is obtained by converting all of the homocysteine to its free thiol form by reduction with tris(2-carboxyethyl) phosphine (TCEP). TCEP-reduced serum samples were assayed using the Dionex DX-500 Ion Chromatograph outfitted with two pumps in parallel, valves and 2 columns (OmniPac PCX-500, 4x50 mm pre-column and PCX-500, 4x250 mm analytical column) plumbed in serial to permit "heart-cut" trapping of tHcy. Detection was achieved using the ED40 electrochemical detector and pulsed integrated amperometry set to detect sulfhydryl groups. Detector output is analyzed with Peaknet (Dionex) software and this generated a display of the chromatograms, with homocysteine exiting the column and peaking at 8.1 min.

Statistical analysis

Graphical analysis was performed with Graphpad Prism 3.0 and other statistical analyses with the SPSS 10.0 software package (SPSS Inc., Chicago IL). Single variable data sets were examined for substantial departures from normality, and the data log-transformated, or subjected to non-parametric analyses as necessary. In comparisons with the reference group, the threshold for type I error (α statistic) was set at 0.05, and the difference in distributions assayed by F test for the respective variances. Biochemical data were also subjected to linear regression analysis and bivariate Pearson correlation coefficents computed for all variable pairs.

RESULTS

Circulating tHcy in MM subjects

Serum analyte values for all 46 subjects are shown in Table 1. Mean tHcy was 15.9 ± 9.9 (SD) μmol/L (range 3.4 to 41.5 mol/L). A histogram of the tHcy values for MM subjects, compared to a reference group of 711 healthy adults measured by the same technique [1], is shown in Figure 1. More than half of the MM subjects (24/46 or 52%) were hyperhomocysteinemic (tHcy > 15 μmol/L), in comparison to only 11% (83/711) of controls [3]. Significant skewing is evident in both groups and the dispersion of tHcy concentration values deviates significantly ($p < 0.001$) from a Gaussian distribution, by the Kalmogorov-Smirnov normality algorithm. However, comparison of the groups by non-parametric testing (Wilcoxon statistic) confirms a significantly higher ($p < 0.001$) median tHcy in MM subjects (15.3 μmol/L) than in the reference population (8.9 μmol/L). The tHcy dataset was log-transformed (log[tHcy]) for all subsequent analyses. In the transformed data, greater scatter of tHcy is still noted for the MM subjects. The distribution of the log[tHcy] in MM patients (SD 0.299) was significantly greater ($p = 0.00016$, F test) than for controls (SD 0.210).

Table 1. Clinical and biochemical data

	Mean ± SD	Range	Reference Interval
Total homocysteine (μmol/L)	15.9 ± 9.9	3.4 - 41.5	5.0 - 15.0
Creatinine (μmol/L)	87 ± 22	53 - 148	50-100 (women) 60 –110 (men)
Albumin (g/L)	39 ± 6	23 - 52	38 - 50
Total protein (g/L)	79 ± 14	51 - 115	65 - 80
Globulin (g/L)	40 ± 16	18 - 64	15 - 30
IgG (g/L)	22.5 ± 16.6	2 - 63	4.6 - 13.3
IgA (g/L)	2.4 ± 5.1	0.07 - 27.0	0.6 - 3.2
IgM (g/L)	4.0 ± 11.0	0.17 - 62.5	0.2 - 2.2

tHcy and serum proteins

Mean total protein was 79 ± 14 g/L (Table 1), at the high end of the reference interval (65 - 80 g/L). Although serum IgG was increased (22.5 ± 16.6 g/L), there was a wide range of values, and 15 of 46 subjects (32%) had IgG levels were within the normal reference interval. This is due in part to treatment, but total levels of IgG also vary widely in untreated multiple myeloma, depending on the cross-reactivity of the monoclonal IgG species and the extent to which intact immunoglobulin species are secreted by the clonal neoplasm. Similar variations in measured IgA and IgM (Table 1) were also observed.

As expected, mean serum albumin (39 ± 6 g/L) was decreased and the calculated globulin fraction (40 ± 16 g/L) increased in MM. As Figure 2 shows, the inverse correlation was highly significant ($p < 0.0001$) and the slope of the regression (- 0.219 ± 0.051 SE) consistent with the approximate 5-to-1 ratio of molecular weights for the two protein groups, given the expectation of oncotic pressure homeostasis.

Further analysis of the correlation matrix (Table 2) shows strong and significant direct correlations between total globulin and IgG, along with significant inverse correlations of either fraction with albumin. However, a partial correlation coefficient for log[tHcy] and albumin, correcting for serum creatinine ($p = 0.325$), was also significant ($p = 0.041$).

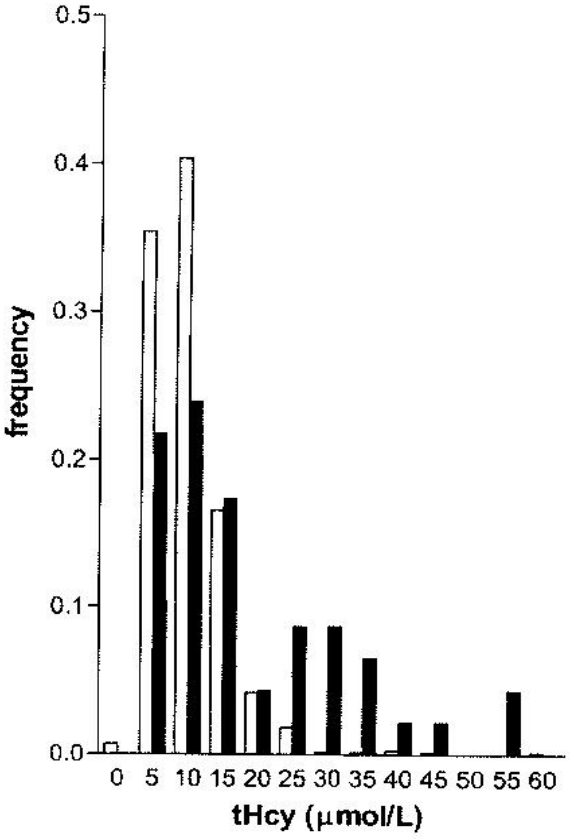

Figure 1. Distribution histogram of tHcy. Closed bars are relative frequencies for the MM subjects. Open bars are comparative frequencies for a reference outpatient population of 711 adults aged 58 ± 14 yr (range: 18 - 90 yr), with mean tHcy of 10.1 ± 6.6 μmol/L.

The bivariate correlation between log[tHcy] and albumin was statistically significant ($r = 0.33$, $p < 0.02$). Regression analysis, conducted with log[tHcy] as the dependent variable, showed a significant non-zero relationship (slope: + 0.019 ± 0.007 SE, $p < 0.02$). The value of y-intercept (0.36 ± 0.28 SE), representing the theoretical log[tHcy] without albumin present, is not significantly different from zero, in keeping with the small fraction of unbound homocysteine found in normal serum [2]. The line of best fit (Figure 3) predicts a 5.5% increase in tHcy for a 1 g/L increase of serum albumin (e.g., 0.72 μmol/L tHcy increase with a serum albumin increase from 40 to 41 g/L). There is considerable scatter in tHcy concentrations, consistent with the heterogeneous nature of this unstratified MM group of patients, but the variance explained by the linear relationship between log[tHcy] and albumin was 11% of the total ($R^2 = 0.110$).

tHcy AND CREATININE

In MM subjects, mean serum creatinine was 87 ± 22 μmol/L (Table 1). However, no significant correlation was observed between serum creatinine and log[tHcy], or any of the protein measures. (Table 2). For three MM subjects, the mean tHcy (4.12 ± 0.38 μmol/L) was less than the lower limit of the reference range, despite a mean serum creatinine of 111 ± 20 μmol/L. On the other hand, the five MM subjects with frank renal disease (mean serum creatinine 260 ± 41 μmol/L) that were excluded from correlation analysis, had markedly elevated tHcy (38.9 ± 9.6 μmol/L), despite lower serum albumin (33.0 ± 2.3 g/L).

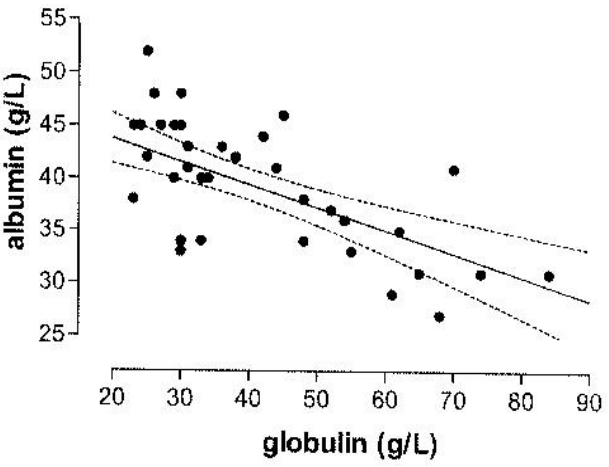

Figure 2. Inverse correlation between albumin and globulin fractions in MM subjects. Also shown is the line of best fit (y = - 0.219x + 48.2, $F_{1,38} = 23.2$, $p < 0.0001$) and the 95% confidence interval of the regression (hatched lines).

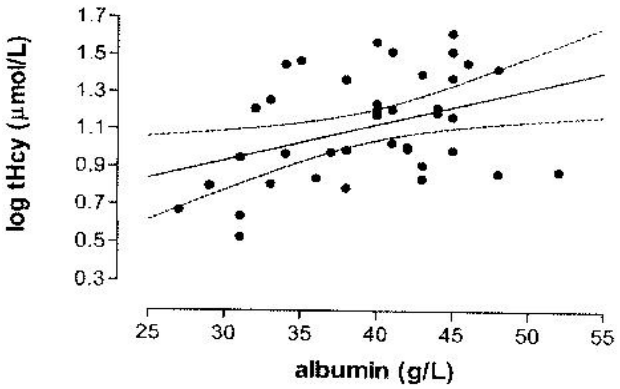

Figure 3. Linear regression of albumin and log-transformed tHcy. The line of best fit (y = - 0.019x + 0.36) was significantly different from zero ($F_{1,38} = 7.3$, $p = 0.01$).

DISCUSSION

Total homocysteine is now a widely accepted marker of cardiovascular risk, although the pathogenetic mechanisms relating the two are still being delineated [1]. Despite the fact that

Table 2. Correlation matrix

	albumin	globulin	IgG	creatinine
log [tHcy]	**0.332 ***	**- 0.393 ***	- 0.092	- 0.11
creatinine	- 0.104	- 0.067	- 0.032	
IgG	**- 0.408 ****	**0.824 *****		
globulin	**- 0.547 *****			

Pearson correlation coefficients are shown for 41 samples with serum creatinine less than 150 μmol/L. Those in bold are statistically significant (* $p < .05$; ** $p < .01$; *** $p < .001$)

most of the homocysteine in the circulation is covalently bound to albumin, it is not known whether the tHcy concentration is a surrogate marker for the free homocysteine fraction, or whether the homocysteine fraction linked by disulfide bridge to protein and other thiols also correlate with this risk. While there has been considerable interest in the kinetics of exchange between free and bound pools of Hcy [2,4,5,11-13], there has been less attention focussed on the ligands themselves. In a study of 584 healthy adults, Lussier-Cacan and coworkers showed a significant correlation between tHcy and serum albumin [6], independent of other significant covariates, including creatinine. A multivariate linear regression analysis suggested that albumin could account for up to 2.74% of the total and 18% of the explained variation. Rasmussen et al.[14] found that the differences in tHcy concentrations induced by altering phlebotomy conditions (supine posture, duration of tourniquet application) were correlated with the associated physiological shifts in serum albumin concentration. Similarly, the acute hemoconcentrating effect of heavy exercise that causes a rise and fall in serum albumin has been suggested as the likely explanation for a parallel rise and fall in tHcy [15].

In patients with systemic disease accompanied by impaired renal function, however, glomerular filtration rate is usually the most important determinant of tHcy [1]. In multiple myeloma, we hypothesized that disease-based changes in the serum protein profile might be of equal significance in modulating tHcy. Serum albumin in MM patients is correlated with the level of IgG, the hemoglobin concentration, the clinical stage of disease and the estimated tumor burden [8]. Given our *a priori* assumption that there would be an increased probability of associated renal disease in MM subjects (tending to raise tHcy levels), but also an increased likelihood of hypoalbuminemia (tending to lower tHcy), it was not clear what the expected distribution should be. What we observed was a significant increase in mean and median concentrations, along with a substantial increase in scatter. The statistically significant correlation, however, was the inverse one with serum albumin. Regression analysis suggests that the proportion of the overall variation (11%) due to albumin is larger than the intra-individual variation in healthy adults [14], making it relevant in future clinical analyses of tHcy in this disease.

There was also a significant correlation between serum IgG levels and tHcy, matching the inverse correlation with serum albumin. Nevertheless, evidence that the lone albumin thiol at Cys-38 is the site of covalent linkage for the majority of protein-bound Hcy [5,16], makes it more likely that albumin, not IgG, is the primary determinant of Hcy. However, it is not known to what extent other proteins can bind homocysteine under pathophysiologic conditions, and it is within the realm of possibility that Hcy may be bound, in some MM subjects, to the clonal immunoglobulins themselves, particularly if somatic mutation has introduced free cysteine thiol residues in the hypervariable domains. Similar arguments have been advanced for the preferential homocysteinylation of specific class I HLA antigens, which invokes yet another pathogenetic mechanism by which homocysteine may modulate human disease [17,18].

A somewhat unexpected finding was the absence of significant correlation between tHcy and creatinine. This was true for simple bivariate comparisons as well as a separate multivariate regression analysis (data not shown). There are three possible reasons for this finding. First, serum creatinine may be a poor indicator of renal function in MM patients, since rates of creatinine formation may change with tumor burden and nutritional status [8]. Second, effects of hypoalbuminemia and altered homocysteine metabolism in MM may have obscured any underlying relationship between renal function and circulating tHcy. Third, the sample size (n=41) is small for multivariate analysis and the likelihood of a Type II error is not trivial. Whatever the reason, it is clear that homocysteine cannot be interpreted in MM patients without consideration of the albumin effect. It is also clear, as evidenced by our group of 5 MM patients with frank renal failure and marked hyperhomocysteinemia, that renal function can also be a strong co-determinant.

In summary, mean tHcy concentrations are higher in multiple myeloma, although low tHcy may occur. Strong correlation is observed with immunoglobulin burden and the accompanying hypoalbuminemia. Whether tHcy may prove to be of some clinical importance in MM diagnosis or follow-up would require a detailed longitudinal study with proper disease staging, stratification, and consideration of treatment protocols. Our observations in a defined patient group demonstrate the importance of serum proteins as determinants of tHcy. Other disorders that induce hypoalbuminemia, such as nephrotic syndrome, are likely to show a similar relationship. The importance of serum albumin should be borne in mind by investigators intent on exploring associations between renal disease, hyperhomocysteinemia, and the increased prevalence of associated cardiovascular complications.

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