Correlation between total homocysteine and cyclosporine concentrations in cardiac transplant recipients

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Increased circulating total homocysteine (tHcy) has been implicated as an independent risk factor for atherosclerotic disease. In cardiac transplant patients, accelerated coronary atherosclerosis is an important cause of late allograft failure; however, studies of tHcy in this at-risk group are limited. We sampled a cohort of 72 subjects 3.95 ± 3.14 (mean ± SD) years after transplantation and found that all had tHcy concentrations above our upper reference limit (15.0 μmol/L). The mean tHcy in the transplant group (25.4 ± 7.1 μmol/L) was significantly greater than in our reference group (9.0 ± 4.3 μmol/L; n = 457; P < 0.001). We also examined the effect of age, gender, time since transplant, serum folate and cobalamin, total protein, urate, creatinine, albumin, and trough whole blood cyclosporine concentrations. In a multiple linear regression model, only creatinine (mean 144 ± 52 μmol/L; P = 0.021) and trough cyclosporine concentrations (191 ± 163 μg/L; P = 0.015) were independent positive predictors of tHcy, whereas serum folate (8.35 ± 7.43 nmol/L; P = 0.018) and time since transplant (P = 0.049) were significant negative predictors. We conclude that hyperhomocysteinemia is a common characteristic of cardiac transplant recipients. Our analysis suggests that folate and renal glomerular dysfunction are important contributory factors; however, whole blood cyclosporine concentrations may also predict the degree of hyperhomocysteinemia in this population and therefore influence interpretation of any apparent response to treatment.

Materials and Methods

BLOOD COLLECTION AND SAMPLE PREPARATION

Transplant recipients were recruited consecutively from the Toronto Hospital Cardiac Transplant Clinic, and informed consent was obtained. All patients were receiving standard immunosuppressive therapy. No subject was...
prescribed a vitamin supplement, although some acknowledged taking over-the-counter vitamin supplements on their own. EDTA-anticoagulated blood was collected in the morning by routine venipuncture after the subjects were interviewed to confirm fasting status. The blood was placed on ice for a few minutes and transferred to a centrifuge, and the plasma was separated within 30 min of venipuncture. The reference group was unselected and drawn consecutively from a large pool of fasting, ambulatory outpatients. EDTA-anticoagulated blood was drawn on 457 adults (265 men, 57 ± 13 years of age, and 188 women, 55 ± 14 years of age) and separated within 2 h. All plasma samples were frozen at −70 °C within 24 h until analysis. This study protocol was approved by the ethics review board of the Toronto Hospital.

BIOCHEMICAL ASSAYS
tHcy and methionine (Met) were simultaneously assayed in three separate batches of plasma, using HPLC with electrochemical detection and pulsed integrated amperometry (13, 14). Within-run and between-run imprecision (CV) for tHcy assayed by this method is 3.1% and 3.8%, respectively (14). Urate was assayed by the uricase technique, albumin by brom cresol green binding, total protein by the Biuret reaction, and creatinine by the Jaffe method, all on the Olympus AU800. Serum folate and cobalamin were measured with a radioimmunoassay (Quantaphase II, Bio-Rad Laboratories, Inc.). Whole blood CsA was assayed with the Cyclo-Trac® (Incstar Corp.) specific monoclonal antibody kit (15, 16).

STATISTICAL ANALYSIS
Data were analyzed using the SPSS™ 7.5 software package (SPSS Inc.). Single variable data sets were examined for substantial departures from normality. As others have described for tHcy (17), the distribution in both reference and patient groups was moderately skewed to the right. One patient outlier with a tHcy concentration of 58 μmol/L was excluded from the regression models because of its disproportionate influence on the analysis. However, log transformation of tHcy concentrations did not change the subsequent regression analyses substantially, and untransformed tHcy data are used throughout.

The inverse relationship between tHcy and serum folate is not a linear one (18–21). Because two of our subjects had serum folates above the upper limit of the assay (>45 nmol/L) and the distribution of total folate data showed skewed distribution (coefficient of skewness ± SE, 3.64 ± 0.28), log-transformed serum folate data were used in the multivariate regression.

Refinement of the multiple linear regression model was conducted using backwards elimination, and the threshold for type I error (α statistic) was set to 0.05. The Bonferroni correction was implemented when multiple hypothesis testing was conducted.

RESULTS

CLINICAL CORRELATES

In our 72 transplant patients (Fig. 1), mean tHcy (25.4 ± 7.1 μmol/L) was more than twice that of our reference group (9.0 ± 4.3 μmol/L; n = 457; P < 0.001). None of the transplant patients had tHcy concentrations within our reference interval of 5–15 μmol/L, the 6th and 95th centiles, respectively, of our reference group.

Our transplant subjects varied substantially in relation to time since transplantation, the range being 5 weeks to 10.7 years (mean ± SD, 3.95 ± 3.14 years). A modest but significant decrease in tHcy over time was evident in the bivariate regression (Fig. 2). Examination of tHcy trends in recipients <1 year posttransplantation failed to reveal a significant association with time since transplant (data not shown). No significant correlation was seen with age. Mean tHcy was only slightly less in the 8 female transplant patients (23.2 ± 2.2 μmol/L) than in the 64 male transplant patients (25.7 ± 0.90 μmol/L); the lack of

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**Fig. 1.** Relative frequency distributions of tHcy concentrations in the transplant (n = 72) and reference (n = 457) groups.

**Fig. 2.** Correlation between tHcy and time after transplantation. The line of best fit ( ) ; y = −0.66x + 28.0; F1,70 = 6.5; P = 0.013] and 95% confidence interval (– – – – – ) are shown for the bivariate linear regression.
positive correlation with serum folate (range (22.2 and 19.8) with concentrations above the upper limit of the health-related reference range. Moreover, mean tHcy in the folate-deficient group (<4.1 nmol/L) was significantly higher than in patients with higher serum folates (29.3 ± 1.9 vs 24.4 ± 0.9 μmol/L; \( t = -2.47; P = 0.016 \)). Plasma Met concentrations were also somewhat lower than reported for healthy adults (14). Plasma Met showed a significant negative correlation with tHcy (\( r = -0.284; P = 0.015 \)) but a positive correlation with serum folate (\( r = 0.247; P = 0.038 \)), suggesting that Hcy remethylation is reduced in a substantial proportion of the transplant population (23). The correlations are not linear but are well approximated by exponential curves (Fig. 3), emphasizing that almost all of the covariation is at serum folate concentrations that range from mild, subclinical insufficiency to frank deficiency. On the other hand, it should be noted that the two transplant recipients with markedly increased serum folate concentrations (>45 nmol/L) had tHcy concentrations above the upper limit of the health-related reference range (22.2 and 19.8 μmol/L).

A smaller proportion of subjects (7 of 72, or 9.7%) had serum cobalamin concentrations below reference values, indicating possible B12 deficiency; however, only one subject had a serum cobalamin <100 pmol/L. Comparison of mean tHcy in those with a serum cobalamin <162 pmol/L against those with higher concentrations (29.6 ± 5.6 vs 25.0 ± 0.88 μmol/L; \( t = -1.67; P = 0.099 \)) was only suggestive of a statistical difference, but the lack of significance may be attributable in part to the small numbers. We found no statistically significant correlation between tHcy and serum cobalamin. It might be expected that erythrocyte mean cell volume would reflect the presence of folate or B12 deficiency, but there was no significant correlation between this hematologic index and serum folate (\( r = 0.145; P = 0.23 \)), cobalamin (\( r = -0.026; P = 0.89 \)), or tHcy (\( r = -0.016; P = 0.83 \)). Moreover, mean tHcy in the 13 of 72 subjects with an mean cell volume >100 pL was not different from that in subjects with mean cell volumes in the health-related reference range (26.3 ± 3.2 vs 25.2 ± 0.75 μmol/L; \( t = 0.50; P = 0.61 \)).

**Table 1. Patient characteristics and multivariate regression analysis for tHcy.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Complete model* Coefficient ± SE</th>
<th>t</th>
<th>( P )</th>
<th>Final model* Coefficient ± SE</th>
<th>t</th>
<th>( P )</th>
</tr>
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<tbody>
<tr>
<td><strong>Clinical characteristic</strong></td>
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<tr>
<td>Gender, M/F</td>
<td>64/8</td>
<td>2.06 ± 2.23</td>
<td>0.92</td>
<td>0.36</td>
<td></td>
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<tr>
<td>Age, years</td>
<td>52.4 ± 10.0</td>
<td>-0.016 ± 0.069</td>
<td>-0.23</td>
<td>0.82</td>
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<tr>
<td>Time after transplant, years</td>
<td>3.95 ± 3.14</td>
<td>-0.346 ± 0.225</td>
<td>1.53</td>
<td>0.13</td>
<td>-0.401 ± 0.200</td>
<td>-2.005</td>
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<tr>
<td><strong>Blood analyte</strong></td>
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<tr>
<td>Erythrocyte mean cell volume, pL</td>
<td>93.6 ± 7.4</td>
<td>-0.088 ± 0.095</td>
<td>-0.93</td>
<td>0.36</td>
<td></td>
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<tr>
<td>Plasma Me, μmol/L</td>
<td>14.1 ± 3.3</td>
<td>-0.326 ± 0.220</td>
<td>-1.48</td>
<td>0.15</td>
<td></td>
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<tr>
<td>Serum creatinine, μmol/L</td>
<td>144 ± 52</td>
<td>0.030 ± 0.014</td>
<td>2.24</td>
<td>0.03</td>
<td></td>
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<tr>
<td>Serum cobalamin, pmol/L</td>
<td>317 ± 134</td>
<td>-0.0023 ± 0.006</td>
<td>-0.4</td>
<td>0.69</td>
<td>0.030 ± 0.013</td>
<td>2.359</td>
</tr>
<tr>
<td>Serum folate, nmol/L</td>
<td>8.35 ± 7.43</td>
<td>-2.02 ± 1.24</td>
<td>-1.63</td>
<td>0.11</td>
<td>-2.62 ± 1.08*</td>
<td>-2.425</td>
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<tr>
<td>Serum urate, μmol/L</td>
<td>495 ± 128</td>
<td>&lt;0.0001</td>
<td>0</td>
<td>0.99</td>
<td></td>
<td></td>
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<tr>
<td>Serum albumin, g/L</td>
<td>45.5 ± 3.6</td>
<td>0.223 ± 0.197</td>
<td>1.13</td>
<td>0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA, μg/L</td>
<td>191 ± 163</td>
<td>0.0091 ± 0.004</td>
<td>2.16</td>
<td>0.04</td>
<td>0.010 ± 0.004</td>
<td>2.504</td>
</tr>
</tbody>
</table>

* The complete model includes all variables entered into the regression; the final model includes only the four significant (\( P < 0.05 \)) variables.

* * In the final model, the regression is based on log-transformed serum folate data.
between albumin and tHcy (12). However, we were unable to identify any significant codependence in our transplant patients \( (r = 0.13; P = 0.13) \), nor did we observe any correlation with serum urate \( (r = 0.05; P = 0.35) \). In contrast, serum creatinine \( (r = 0.19; P = 0.05) \) and whole blood CsA \( (r = 0.27; P = 0.01) \) were significantly correlated. Bivariate regression analysis showed a significant linear trend between CsA and tHcy concentrations (Fig. 4) such that an increase of 100 \( \mu g/L \) in CsA was associated with an increase of 1.75 \( \mu mol/L \) in tHcy.

**REGRESSION ANALYSIS**

When backwards elimination was applied to an inclusive multivariate linear regression model (Table 1), with tHcy as the dependent variable, only four independent variables remained in the final model \( (F_{4,65} = 5.98; P < 0.0001) \): time since transplant \( (P = 0.049) \), serum creatinine \( (P = 0.021) \), log serum folate \( (P = 0.018) \), and CsA \( (P = 0.015) \). In this model, more than 50% of the variation in tHcy \( (r = 0.518) \) is explained by the four variables. The explanatory power of CsA was the greatest \( (\beta = 0.01 \pm 0.004; t = 2.50; P = 0.015) \) of the four predictive variables and appeared independent of the significant correlation between tHcy and creatinine \( (\beta = 0.030 \pm 0.013; t = 2.36; P = 0.021) \), serum folate, or time since transplantation.

**Discussion**

Cardiac transplantation is a recognized treatment for end-stage heart failure, with 90% survival after 1 year and 70% after 5 years (24). Despite continued improvement in survival rates, the major complications have changed little. Among the most complex and problematic is the occurrence of progressive obliterative vascular disease affecting large, medium, and small arteries, termed “cardiac allograft vasculopathy” (7). This condition can lead to myocardial infarction, congestive heart failure, ventricular arrhythmias, or sudden death. Human cytomegalovirus has been cited as a potential etiologic agent; however, frequency of rejection is important, and other significant nonimmunologic factors include donor age, presence of diabetes or hyperlipidemia, insulin resistance, smoking, hypertension, and use of prednisone and CsA (6, 7, 25, 26).

Recent reports summarizing large collaborative studies show that increased tHcy is an important, independent risk factor in the development of cardiovascular disease (1–3) and in the mortality risks for those with established coronary arterial disease (27). A smaller literature in renal transplantation also points to a substantial risk of hyperhomocysteinemia contributing to the increased risk of early cardiovascular disease (11, 28–31).

To date, there have been three studies examining tHcy in cardiac transplant populations (8–10). In 44 patients, Berger et al. (9) reported a 70% increase in tHcy 3 months after cardiac transplantation \( (13 \pm 4 \text{ to } 21 \pm 13 \mu mol/L); P < 0.002) \). This short-term change is different from our long-term cross-sectional observations, in which tHcy is decreased in those who have been transplanted for a longer time. However, our study may be biased by inclusion of a greater proportion of healthier transplant recipients, who are the ones more likely to live beyond the first few years after transplantation.

We also observed a higher mean tHcy in our subjects than Ambrosi et al. (8), who reported on 27 cardiac transplant recipients 14–63 months after transplantation. Comparison of our data with either of these two studies may be difficult because of differences in tHcy assay methodology. For example, tHcy concentrations in the controls reported by Ambrosi et al. (8) \( (6.5 \pm 4 \mu mol/L); n = 17; P < 0.01) \) were considerably lower than most others have found (17).

In a much larger study of 189 cardiac transplants, Gupta et al. (10) found that 68% of subjects had tHcy concentrations >14.6 \( \mu mol/L \), the 90th centile for their controls. Age was not a significant predictive factor, but serum creatinine, folate, cobalamin, and pyridoxine were. No trends were observed between tHcy and time since transplantation or CsA usage; however, CsA concentrations were not reported (10).

Arnadottir et al. (11) first documented the association between CsA treatment and increased tHcy in a renal transplantation group; ours, however, is the first description to our knowledge, of a significant correlation between CsA and tHcy concentrations in the same sample. We cannot exclude the alternative possibility that CsA concentrations are covariant with some other therapeutic intervention, because most subjects receive multiple immunosuppressants, such as prednisone or azothioprine, which could also affect Hcy metabolism. However, no patient was receiving methotrexate, an agent that is known to cause hyperhomocysteinemia through its folate-antagonizing actions (32).

We interpret the statistical significance of the indepen-
dent correlation between CsA and tHcy concentrations as an indication that the effect of CsA is not entirely the result of impaired renal glomerular function (33), which Arnadottir et al. (11) also suspected in their renal transplant group. The possibility that postglomerular vasoconstriction and consequent reduction in renal blood flow (34) caused by CsA plays a role in the generation of hyperhomocysteinemia is one we could not address in this study. However, CsA-mediated decreases in tHcy delivery to and uptake at the antiluminal surface of the renal tubular epithelium, which appear crucial for renal tHcy catabolism (35,36), offer a biologically plausible explanation for the tHcy correlation with CsA that would be independent of serum creatinine. Arnadottir et al. (11) also suggested that CsA may directly impair the remethylation of Hcy to Met, although the major route of Hcy clearance in the rat kidney occurs through the transsulfuration pathway (35–37).

In summary, whole blood CsA concentrations at the time of blood sampling may be an important determinant of tHcy in cardiac transplant recipients. More detailed studies to identify mechanisms underlying this effect are justified in view of the emerging relationship between tHcy and accelerated allograft vasculopathy in these patients (36,38). A practical consequence of our observation is that serial tHcy measurements in the individual transplant patient cannot be compared with one another without taking into account the differences in immunosuppressant regimen, as well as those in vitamin and renal status, at the time the blood samples are drawn.

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References

8. Wilcken DEL, Gupta VJ, Betts AK. Homocysteine in the plasma of


