Plasma Homocysteine Affects Fibrin Clot Permeability and Resistance to Lysis in Human Subjects

Anetta Undas, Jan Brożek, Miłosz Jankowski, Zbigniew Siudak, Andrew Szczeklik, Hieronim Jakubowski

Objective—Homocysteine (Hcy) is a risk factor for thrombosis. We investigated a hypothesis that the clot permeability and its resistance to fibrinolysis is associated with plasma total Hcy (tHcy) in human subjects.

Methods and Results—We studied healthy men not taking any medication (n=76), male patients with advanced coronary artery disease (CAD) taking low-dose aspirin (n=33), men with diabetes mellitus diagnosed recently (median hemoglobin A1c 7.65%; n=16), and patients with isolated hypercholesterolemia (>7.0 mmol/L; n=15). We assessed clot permeability and turbidimetric lysis time as the determinants of fibrin clot structure. In a regression model, including age and fibrinogen, plasma tHcy was an independent predictor of clot permeation and fibrinolysis time in healthy subjects ($R^2=0.88, P<0.0001$ and $R^2=0.54, P<0.0001$, respectively). In CAD patients, tHcy (tHcy) and fibrinogen were stronger predictors of the permeation coefficient ($R^2=0.84; P<0.0001$) than was fibrinogen alone ($R^2=0.66; P<0.0001$), whereas tHcy was the only predictor of lysis time ($R^2=0.69; P<0.0001$). Elevated tHcy levels observed after methionine load were not associated with any of the fibrin clot properties. In patients with diabetes or hypercholesterolemia, the influence of Hcy on permeation and, to a lesser extent, on the lysis time was obscured by dominant effects of glucose and cholesterol. In 20 asymptomatic men with hyperhomocysteinemia treated with folinic acid, reduction in tHcy levels resulted in increased clot permeability ($P=0.0002$) and shorter lysis time ($P<0.0001$).

Conclusions—Our results indicate that plasma tHcy predicts clot permeation and susceptibility to fibrinolysis in healthy men and CAD patients. Our data are consistent with a mechanism of thrombosis in hyperhomocysteinemia, which involves modification of fibrinogen by Hcy–thiolactone. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: homocysteine • fibrin • fibrinolysis • folate

Hyperhomocysteinemia is the consequence of a disturbed methionine metabolism attributable to genetic mutations, deficiencies of folic acid, vitamin B12 or B6, renal and other diseases, and several drugs (eg, trimethoprim, methotrexate, and fibrates). This metabolic disorder is implicated in the pathogenesis of atherosclerotic vascular disease, although it is still uncertain whether this association is causal.1 There is also evidence that elevated plasma total homocysteine (Hcy) levels are associated with thrombosis.2–5

Complex pathways of blood coagulation lead ultimately to the formation of a fibrin clot that is preceded by thrombin-mediated fibrinogen conversion to fibrin and fibrin cross-linking by activated factor XIII.6,7 The structure and function of the fibrin clot is modulated by a number of genetic and environmental factors that regulate the hemostatic system, especially plasma concentration and function of fibrinogen and thrombin.8 Clinical relevance of fibrin clot structure is unclear. Fibrin clots composed of dense fiber networks have been found in survivors of myocardial infarction <50 years of age and in their relatives.9,10 Altered fibrin clot architecture was also reported in dyslipidemia10 and in diabetes.11 Little is known about the effect of Hcy on fibrin formation and fibrin clot structure in humans. In hyperhomocysteinemic rabbits, fibrin clots are composed of thinner and more tightly packed fibers compared with normal animals.12 Furthermore, the clots formed from purified fibrinogen from homocysteinemic rabbits were lysed more slowly by plasmin than the clots from control fibrinogen.13 Whole blood thromboelastographic profiles obtained from rats fed with a folate-depleted diet showed that hyperhomocysteinemia is associated with increased maximum clot firmness.13 Clots formed from human plasma incubated in vitro with Hcy have also been reported to have a more compact structure, with shorter and more frequently branched fibers, than those formed in the absence of Hcy.14 However, it is unclear whether elevated Hcy levels can influence clot properties in human subjects.

The aim of this study was to investigate the clot permeability and its resistance to fibrinolysis in relation to plasma

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total Hcy (tHcy) occurring in vivo. Our data suggest that plasma tHcy levels affect fibrin clot structure assessed by clot porosity and susceptibility to lysis, which might contribute to prothrombotic tendency observed in hyperhomocysteinemia.

Methods

Patients
We studied 4 groups of male patients 35 to 65 years of age. The healthy controls comprised 76 apparently healthy subjects with total cholesterol levels <6.5 mmol/L (250 mg/dL) and triglycerides <3.0 mmol/L (260 mg/dL) who did not take any medication for ≥ 6 weeks before the enrollment. The advanced coronary artery disease (CAD) patients group included 33 subjects with a history of myocardial infarction or hospitalization for unstable angina ≥ 6 months before enrollment (average 16 months). Patients with personal or family history of thromboembolism or bleeding disorders, diabetes (fasting plasma glucose >7.0 mmol/L), renal insufficiency, acute vascular event within the preceding 6 months, and severe comorbidity (eg, cancer, or chronic infection) were excluded. All patients took low-dose aspirin (75 mg per day) for ≥ 6 weeks before the enrollment, 7 subjects were treated with β-blockers, and none used lipid-lowering agents.

To determine a possible role of diabetes and hypercholesterolemia (HCH) in Hcy-associated changes in clot structure,10,11 we studied 2 additional groups of men.

The patients with diabetes mellitus (DM) group consisted of 16 subjects with newly diagnosed type 2 DM, fasting glucose levels >7.0 mmol/L, and elevated glycosylated hemoglobin (HbA1c) >7.0%. None of the patients had a history of cardiovascular disease or thromboembolism, renal insufficiency, or took any medication.

The patients with HCH group comprised 15 subjects with markedly increased total cholesterol level (>7.0 mmol/L [270 mg/dL]) and triglycerides <3.0 mmol/L (260 mg/dL) not previously treated with any lipid-lowering agent or aspirin. Subjects with diabetes, CAD, renal insufficiency, or other serious diseases were excluded.

The study was approved by the university ethics board, and all patients provided written, informed consent.

Blood Collection
Venous blood samples were drawn between 8 and 10 AM after a 14-hour overnight fast. Blood was taken into 0.13 mol/L trisodium citrate tubes (Becton Dickinson) and centrifuged within 30 minutes at 2500g for 20 minutes. Platelet-poor plasma was frozen and stored at −80°C. Blood samples for Hcy determination were collected into EDTA tubes and immediately placed on ice until the plasma was separated.

Biochemical Tests
Lipid profiles, glucose, and creatinine concentration were determined by routine laboratory methods. Fibrinogen concentration was assessed by nephelometry (Dade Behring). Fasting plasma Hcy levels were measured using an IMX System immunoassay (Abbott Diagnostics). Serum folate levels were determined with an IMMULITE analyzer (Diagnostic Products Corp.), and the reference range was 3 to 17 ng/mL.

Fibrin Permeation Analysis
Fibrin clot permeation was determined as described previously.15 A total of 20 mmol/L calcium chloride and 1 U/mL human thrombin (Sigma) were added to 120 μL citrated plasma. The mixture was placed in plastic tubes and allowed to clot. After incubation for 120 minutes in a wet chamber, tubes were connected with a reservoir of a buffer (0.05 mol/L Tris HCl, 0.15 mol/L NaCl, pH 7.5), and its flow rate through the gel was measured. The permeation coefficient (Darcy constant [Ks]), which represents the surface of the clot that allows flow through a fibrin network, was calculated from the equation 

\[ K_s = \frac{Q \cdot \eta}{(\frac{L}{A}) \cdot \Delta \rho} \]

where Q is the volume of the buffer flowing through the gel in time t, L is the length of the fibrin gel (13 mm), η is the viscosity of buffer (10^{-2} poise), A is the area of the gel perpendicular to the flow (0.049 cm^2), and Δρ is the differential pressure (in dyne/cm²).16 All measurements were performed in duplicate by an investigator blinded to the origin of the plasma sample and expressed as an arithmetic mean unless otherwise stated. The intraindividual variability of results was 8.1%.

Turbidimetric Clot Lysis Assay
Plasmin-mediated fibrinolysis was evaluated with a slightly modified method by Williams et al.17 A total of 100 μL citrated plasma was diluted with 100 μL of a buffer (0.05 mol/L Tris HCl, 0.15 mol/L NaCl, pH 7.4) containing 20 mmol/L calcium chloride, 1 U/mL human thrombin (Sigma), and 1 μg/mL recombinant tissue plasminogen activator (tPA; Boehringer Ingelheim). Clot assembly kinetics were monitored spectrophotometrically at 405 nm in duplicate aliquots. Turbidimetric lysis time (t50) was defined as the time required for gel turbidity to decrease by 50% from the maximum optical density.14 The intraindividual variability of the measurements was 8.4%.

Methionine Loading Test
A standard oral methionine loading test (0.1 g/kg of body weight in 200 mL of orange juice) was performed in 23 individuals from the healthy control group and in 16 patients from the advanced CAD group to evaluate the effect of acute increase in tHcy levels. Fibrin clot properties and plasma tHcy levels were determined 15 minutes before and 4 hours after methionine ingestion. Subjects were not allowed to eat or drink any liquids other than water during the test.

Folic Acid Treatment
To evaluate the effect of plasma tHcy reduction on fibrin clot structure, we recruited the additional 20 asymptomatic men with hyperhomocysteinemia. Defined as plasma tHcy levels >14.5 μmol/L, which is the upper limit of the reference values for the general population in southern Poland.19 The subjects denied taking any medication. Hcy, folate, fibrinogen levels, lipoprotein profile, fibrin clot permeability, and susceptibility to lysis were determined both before and after a 4-week folic acid treatment (0.8 mg daily).

Statistical Analysis
Kolmogorov–Smirnov, Cramer Von Mises, and Anderson-Darling were used to determine normal distribution. Parametric t tests were used to test for differences for normally distributed data. Data are given as medians and ranges. We used a Wilcoxon matched pairs test to examine differences within the same individuals over time. Baseline values were compared by the Kruskal–Wallis ANOVA with multiple pairwise comparisons (Dwass-Steel-Critchlow-Fligner method) if the test was significant. We used Mann–Whitney U test to compare the subgroups with plasma tHcy of ≤ 14.5 or >14.5 μmol/L. Pearson correlation coefficients (r) were used to evaluate simple linear relationships between variables. Hierarchical forward stepwise multiple regression analysis was used to determine predictors of fibrin gel permeation (Ks) and plasmin-mediated fibrinolysis (t50). The level of statistical significance was set at P<0.05. To facilitate interpretation of the magnitude of the effect we also report 95% CIs around the estimate.

Results
The characteristics of the study groups are shown in Table 1. The groups did not differ with respect to age, smoking status, fibrinogen, and folate levels. There was no difference in lipid profile between controls and CAD patients. Compared with healthy controls, patients with DM had higher total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride levels along with lower high-density lipoprotein cholesterol levels. Subjects with HCH had significantly higher total cholesterol and LDL cholesterol levels than healthy...
controls. Compared with healthy controls, median plasma tHcy was higher in DM and hypercholesterolemic patients. Median clot permeability was lower in all 3 patient groups (ie, in advanced CAD, DM, or hypercholesterolemic subjects) compared with healthy individuals. Clot lysis time was longer in patients with DM and HCH only. In all study groups, folate levels correlated with tHcy levels ($r = -0.58$ to $-0.85$; $P \leq 0.01$).

**Healthy Controls**

As expected, fibrin clot permeability was negatively correlated with fibrinogen ($r = -0.77$; 95% CI, $-0.85$ to $-0.66$; $P < 0.0001$) and age ($r = -0.3$; 95% CI, $-0.49$ to $-0.08$; $P < 0.008$) but not with any of the lipid variables (Table 2).

Fibrin clot permeability, measured in duplicate, was negatively associated with plasma tHcy ($r = -0.78$, and $r = -0.75$, $P < 0.0001$ for 2 individual $K_s$ values; $r = -0.94$; 95% CI, $-0.96$ to $-0.90$, $P < 0.0001$ for the mean $K_s$ value; Figure 1A). There was also a strong positive correlation between clot permeability and folate levels ($r = 0.57$; 95% CI, 0.40 to 0.71; $P < 0.0001$). Clot lysis time positively correlated with age ($r = 0.40$; 95% CI, 0.19 to 0.57; $P = 0.0004$), fibrinogen ($r = 0.60$; 95% CI, 0.43 to 0.72; $P < 0.0001$), and Hcy ($r = 0.74$; 95% CI, 0.61 to 0.83; $P < 0.0001$; Figure 1B) and negatively with folate ($r = -0.46$; 95% CI, $-0.62$ to $-0.26$; $P < 0.0001$). Plasma tHcy was the only predictor of fibrin clot permeability ($R^2 = 0.88$; slope estimate $-0.26$; $P < 0.0001$) and clot susceptibility to lysis ($R^2 = 0.54$; slope estimate $0.13$; $P < 0.0001$) in a multiple regression model, including age and fibrinogen (Table 3).

**CAD Patients**

In patients with advanced CAD taking low-dose aspirin, clot permeability was also associated negatively with age ($r = -0.65$; 95% CI, $-0.81$ to $-0.40$; $P < 0.0001$), fibrinogen ($r = -0.81$; 0.96

**TABLE 1. Baseline Characteristics and Laboratory Test Results in the Four Groups of Participants**

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls (n=76)</th>
<th>CAD Patients (n=33)</th>
<th>DM Patients (n=16)</th>
<th>HCH Patients (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>51.5 (38–65)</td>
<td>55 (42–65)</td>
<td>52 (40–62)</td>
<td>48 (38–62)</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>4.94 (3.40–6.76)</td>
<td>5.03 (3.93–6.76)</td>
<td>5.97 (5.38–6.44)*</td>
<td>7.90 (7.10–8.50)*</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.37 (0.67–2.06)</td>
<td>1.39 (0.90–1.95)</td>
<td>0.93 (0.79–1.22)*</td>
<td>1.34 (1.18–1.56)</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>2.90 (0.77–4.41)</td>
<td>2.88 (1.38–4.30)</td>
<td>3.63 (2.89–4.01)*</td>
<td>5.85 (5.15–6.52)*</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>1.34 (0.30–2.39)</td>
<td>1.57 (0.84–3.60)</td>
<td>3.10 (2.49–3.85)*</td>
<td>1.65 (0.90–1.97)</td>
</tr>
<tr>
<td>Fibrinogen, g/L</td>
<td>2.51 (1.94–3.88)</td>
<td>2.66 (1.91–3.80)</td>
<td>2.55 (2.03–3.41)</td>
<td>2.74 (2.13–3.63)</td>
</tr>
<tr>
<td>Hcy, μmol/L</td>
<td>12.3 (5.8–24.3)</td>
<td>14.50 (8.80–25.00)</td>
<td>15.75 (12.20–21.80)*</td>
<td>16.80 (13.80–19.40)*</td>
</tr>
<tr>
<td>Folate, ng/mL</td>
<td>12.35 (4.10–8.30)</td>
<td>11.10 (4.20–4.90)</td>
<td>12.50 (8.40–3.90)</td>
<td>10.20 (8.70–14.10)</td>
</tr>
<tr>
<td>$K_s$, 10⁻⁹ cm²</td>
<td>9.35 (6.20–11.40)</td>
<td>8.70 (6.80–10.10)*</td>
<td>7.50 (7.00–8.30)*</td>
<td>7.80 (7.10–8.40)*</td>
</tr>
<tr>
<td>$t_{50%}$, min</td>
<td>7.9 (6.2–9.3)</td>
<td>7.6 (6.0–9.8)</td>
<td>8.7 (7.8–9.3)*</td>
<td>8.8 (8.0–9.4)*</td>
</tr>
<tr>
<td>HbA₁c, %</td>
<td>7.65 (6.9–8.5)</td>
<td>7.65 (6.9–8.5)</td>
<td>7.65 (6.9–8.5)</td>
<td>7.65 (6.9–8.5)</td>
</tr>
<tr>
<td>Current smokers, n</td>
<td>13</td>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Note:** Values are medians (ranges) or numbers of individuals. *$P<0.05$ vs healthy controls.

**TABLE 2. Linear Regression Analysis of the Determinants of Fibrin Clot Permeability ($K_s$) and Clot Lysis Time ($t_{50\%}$): Pearson’s Correlation Coefficients ($r$) and 95% CIs**

<table>
<thead>
<tr>
<th>Determinant</th>
<th>Healthy Subjects (n=76) r (95% CI)</th>
<th>CAD Patients (n=33) r (95% CI)</th>
<th>DM Patients (n=16) r (95% CI)</th>
<th>HCH Patients (n=15) r (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_s$</td>
<td>-0.94 (-0.96 to -0.90)</td>
<td>-0.74 (-0.61 to 0.83)</td>
<td>-0.74 (-0.68 to 0.91)</td>
<td>-0.74 (-0.62 to 0.91)</td>
</tr>
<tr>
<td>tHcy</td>
<td>-0.74 (-0.61 to 0.83)</td>
<td>-0.74 (-0.68 to 0.91)</td>
<td>-0.74 (-0.62 to 0.91)</td>
<td>-0.74 (-0.60 to 0.91)</td>
</tr>
<tr>
<td>Folate</td>
<td>0.57 (0.40 to 0.71)</td>
<td>-0.46 (-0.62 to 0.26)</td>
<td>0.75 (-0.55 to 0.87)</td>
<td>-0.71 (-0.85 to 0.48)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>-0.77 (-0.85 to -0.66)</td>
<td>0.60 (-0.43 to 0.72)</td>
<td>-0.81 (-0.90 to -0.65)</td>
<td>0.48 (-0.16 to 0.71)</td>
</tr>
<tr>
<td>Age</td>
<td>-0.3 (-0.85 to -0.66)</td>
<td>0.40 (-0.85 to -0.49)</td>
<td>-0.81 (-0.90 to -0.65)</td>
<td>0.49 (-0.16 to 0.71)</td>
</tr>
<tr>
<td>HbA₁c</td>
<td>-0.49 (-0.49 to -0.08)</td>
<td>0.19 (-0.19 to 0.57)</td>
<td>-0.81 (-0.81 to -0.40)</td>
<td>0.17 (-0.17 to 0.71)</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>-0.91 (-0.97 to -0.76)</td>
</tr>
</tbody>
</table>

All indicated correlations were significant ($P<0.05$). NC indicates no correlation.
95% CI, −0.90 to −0.65; P<0.0001), tHcy (r = −0.7; 95% CI, −0.84 to −0.47; P<0.0001; Figure 1C), and positively with folate (r=0.75; 95% CI, 0.55 to 0.87; P<0.0001; Table 2). In CAD patients, fibrinogen and tHcy were stronger predictors of the permeation coefficient (R^2=0.84; P<0.0001) than was fibrinogen alone (R^2=0.66; P<0.0001). As in healthy controls, in CAD patients, lysis time correlated positively with age (r=0.49; 95% CI, 0.17 to 0.71; P=0.004), fibrinogen (r=0.48; 95% CI, 0.16 to 0.71; P=0.005), and tHcy (r=0.83; 95% CI, 0.68 to 0.91; P<0.0001; Figure 1D) and negatively with folate.

**TABLE 3. Independent Predictors of Fibrin Gel Permeation (K_s) and Clot Susceptibility to Lysis (t_50%) in the Four Studied Groups in Multiple Stepwise Regression Analysis Models**

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Independent Predictors of K_s</th>
<th>Independent Predictors of t_50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>Hcy</td>
<td>Hcy</td>
</tr>
<tr>
<td>R^2=0.88</td>
<td>R^2=0.54</td>
<td></td>
</tr>
<tr>
<td>Slope estimate = 0.26</td>
<td>Slope estimate = 0.13</td>
<td></td>
</tr>
<tr>
<td>SE 0.011</td>
<td>SE 0.014</td>
<td></td>
</tr>
<tr>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>CAD patients</td>
<td>Hcy</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>R^2=0.84</td>
<td>R^2=0.69</td>
<td></td>
</tr>
<tr>
<td>Slope estimate = −0.11</td>
<td>Slope estimate = −1.1</td>
<td>Slope estimate = 0.19</td>
</tr>
<tr>
<td>SE 0.019</td>
<td>SE 0.135</td>
<td>SE 0.023</td>
</tr>
<tr>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>HbA_1c</td>
<td>HbA_1c</td>
</tr>
<tr>
<td>R^2=0.94</td>
<td>R^2=0.95</td>
<td></td>
</tr>
<tr>
<td>Slope estimate = 0.99</td>
<td>Slope estimate = −0.97</td>
<td></td>
</tr>
<tr>
<td>SE 0.066</td>
<td>SE 0.071</td>
<td></td>
</tr>
<tr>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>HCH</td>
<td>Fibrinogen</td>
<td>LDL-C</td>
</tr>
<tr>
<td>R^2=0.66</td>
<td>R^2=0.65</td>
<td></td>
</tr>
<tr>
<td>Slope estimate = −0.64</td>
<td>Slope estimate = 0.96</td>
<td></td>
</tr>
<tr>
<td>SE 0.127</td>
<td>SE 0.195</td>
<td></td>
</tr>
<tr>
<td>P=0.0002</td>
<td>P=0.0003</td>
<td></td>
</tr>
</tbody>
</table>
(r = -0.71; 95% CI, -0.85 to -0.49; P < 0.0001). In CAD patients, tHcy was the only predictor of lysis time in a regression model including age and fibrinogen (R² = 0.69; slope estimate 0.19; P < 0.0001; Table 3). There was no association between lipid profiles and the permeation or lysis time in this patient group.

**Diabetes Mellitus**

There was a significant negative correlation between permeation and age (r = -0.70; 95% CI, -0.89 to -0.32; P = 0.002), fibrinogen (r = -0.79; 95% CI, -0.92 to -0.48; P < 0.0003), and HbA1c (r = -0.91; 95% CI, -0.97 to -0.76; P < 0.0001; Table 2). Significant positive correlations were observed for the lysis time and age (r = 0.59; 95% CI, 0.14 to 0.84; P = 0.02), fibrinogen (r = 0.71; 95% CI, 0.34 to 0.89; P = 0.002), and HbA1c (r = 0.97; 95% CI, 0.91 to 0.99; P < 0.0001). There were no associations between permeability and plasma tHcy or folate levels in diabetics (P = 0.09). Lysis time showed associations with tHcy (r = 0.51; 95% CI, 0.02 to 0.80; P = 0.044; Table 2) and triglycerides (r = -0.52; 95% CI, -0.81 to -0.04; P = 0.037).

In this group, HbA1c was the only predictor of permeability coefficient and lysis time in a stepwise regression model including age, fibrinogen, and tHcy (Table 3).

**Hypercholesterolemia**

In patients with total cholesterol levels >7.0 mmol/L, clot permeability correlated significantly not only with age (r = 0.62; 95% CI, 0.15 to 0.86; P = 0.02) and fibrinogen (r = -0.81; 95% CI, 0.94 to 0.52; P = 0.0002) but also with total cholesterol (r = -0.78; 95% CI, -0.92 to -0.45; P = 0.001) and LDL cholesterol (r = -0.79; 95% CI, -0.93 to -0.47; P = 0.0004; Table 2). There was no association between permeability and tHcy levels in this group. Permeation coefficient was predicted independently by fibrinogen (R² = 0.66; slope estimate -0.64; P = 0.0002), in a multiple regression model also including tHcy and age (Table 3). Lysis time correlated positively with fibrinogen (r = 0.78; 95% CI, 0.44 to 0.92; P = 0.001), total cholesterol (r = 0.88; 95% CI, 0.67 to 0.96; P < 0.0001), LDL cholesterol (r = 0.81; 95% CI, 0.50 to 0.93; P = 0.0002), and with tHcy levels (r = 0.58; 95% CI, 0.09 to 0.84; P = 0.024) but not with age. In a stepwise regression model, only LDL cholesterol was an independent predictor of fibrinolytic time (R² = 0.65; slope estimate 0.96; P = 0.0003).

**Hyperhomocysteinemia**

Compared with healthy controls with normal tHcy levels (≤14.5 μmol/L), clots formed from plasma from healthy controls with tHcy levels >14.5 μmol/L were 20% less permeable (7.95 versus 9.9 × 10⁻⁵ cm²; P < 0.0001; Figure 2A) and more susceptible to fibrinolysis (8.45 versus 7.5 minutes; P < 0.0001; Figure 2B). Similar effects of hyperhomocysteinemia on clot permeability and lysis were observed in a group of CAD patients (Figure 2).

**Acute Hyperhomocysteinemia**

After methionine load, plasma tHcy was significantly higher compared with the baseline values both in healthy controls (25.2 versus 12.3 μmol/L; P = 0.00003) and in CAD patients (14.5 versus 16.5 (14.9 to 25.0) μmol/L; P = 0.0004). Permeability and lysis time remained unchanged after methionine loading (P > 0.05) and showed no association with tHcy after methionine ingestion (data not shown).

**Folate-Induced Hcy Reduction**

Folic acid administration for 4 weeks resulted in a marked increase in folate concentrations by >300% and a significant 30% reduction in plasma tHcy levels, whereas lipid profiles and fibrinogen concentration remained unaltered (Table 4). After folic acid therapy, clot permeability increased and susceptibility to lysis improved significantly (Table 4). Base-
Fibrin clot properties have been evaluated using several approaches, ranging from a variety of purified systems to assays using citrated or dialyzed plasma.8–18,24 Determination of the permeation coefficient is the most commonly used method to assess clot porosity, which has been shown to be the major determinant of clot structure in various populations, including patients at increased risk of cardiovascular events and their relatives.9,10,16 To evaluate clot permeability, we adopted an ex vivo assay using citrated plasma that has successfully been used in previous studies.11,15,25

We assessed clot susceptibility to fibrinolysis in an ex vivo assay in which fibrin assembly and lysis occurred simultaneously. Lysis was monitored in the presence of the recombinant tPA at a final concentration of 0.5 μg/mL that exceeds levels found under physiological conditions but is similar to that during thrombolytic treatment. In a similar model of spectrophotometrically assessed lysis, it has been shown that varying concentrations of plasmin, a product of tPA-mediated plasminogen activation, affects the time course of fibrinolysis.26 We have chosen the tPA concentration of 0.5 μg/mL that ensured a steep increase in absorbance followed by its relatively rapid decrease, reflecting lysis efficacy in the assay, because we expected rather subtle differences in lysis time between consecutive plasma samples in which tHcy levels ranged from 6 to <30 μmol/L. We have seen very long lysis times with low reproducibility in the experiments performed in the presence of a 10-fold lower tPA concentration.

Most studies suggest that dense fibrin networks are characterized by reduced lysis rate most likely because of a less efficient transport of fibrinolytic agents through a fibrin clot.27–29 The fibrin network configuration rather than fibrin diameter has been reported to have a stronger effect on the fibrinolysis rate.30 Impaired fibrinolysis in subjects with hyperhomocysteinemia most likely reflects a smaller pore size in fibrin network, as it was shown in patients with diabetes.11

### TABLE 4. Characteristics of Asymptomatic Subjects With Hyperhomocysteinemia (n=20) Before and After Four Weeks of Folic Acid Treatment (0.8 mg/day)

<table>
<thead>
<tr>
<th>Value</th>
<th>Baseline</th>
<th>After Treatment</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>50.5 (38.0–63.0)</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>4.67 (4.13–5.98)</td>
<td>4.67 (4.13–6.03)</td>
<td>0.95</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.36 (0.94–1.60)</td>
<td>1.41 (0.90–1.61)</td>
<td>0.02</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>2.76 (2.00–3.94)</td>
<td>2.68 (2.01–4.00)</td>
<td>0.79</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>1.38 (0.80–1.84)</td>
<td>1.30 (0.66–1.84)</td>
<td>0.009</td>
</tr>
<tr>
<td>Fbg, g/L</td>
<td>3.36 (2.25–4.30)</td>
<td>3.27 (2.41–4.22)</td>
<td>0.33</td>
</tr>
<tr>
<td>Hcy, μmol/L</td>
<td>17.95 (15.1–22.8)</td>
<td>11.9 (8.4–14.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Folate, ng/mL</td>
<td>9.3 (6.1–15.2)</td>
<td>28.5 (22.7–43.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$K_v$, $10^{-3}$ cm$^2$</td>
<td>7.6 (6.5–8.5)</td>
<td>8.45 (7.7–10.1)</td>
<td>0.0002</td>
</tr>
<tr>
<td>$t_{50%}$, min</td>
<td>8.7 (8.0–9.8)</td>
<td>7.85 (7.1–8.6)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are medians (ranges).
TC indicates total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, LDL cholesterol; TG, triglycerides; Fbg, fibrinogen; $K_v$, permeability coefficient; $t_{50\%}$, lysis time.

To convert plasma cholesterol from mmol/L to mg/dL, multiply by 38.5.

line tHcy (slope estimate $-0.21$; SE 0.03; P<0.0001) and age (slope estimate $-0.03$; SE 0.009; P=0.006) were predictors of clot permeability ($R^2=0.86$; P<0.0001), whereas only tHcy (slope estimate 0.21; SE 0.03; P<0.0001) predicted fibrinolysis time ($R^2=0.77$; P<0.0001) in a multiple regression model also including age and fibrinogen. In contrast to the other variables, post-treatment tHcy levels correlated with permeability ($r=-0.91$; 95% CI, −0.96 to −0.78; P<0.0001) and fibrinolysis time ($r=0.53$; 95% CI, 0.11 to 0.79; P=0.02).

### Discussion

The results of our study show that plasma tHcy levels correlate significantly with plasma fibrin clot permeation and clot susceptibility to fibrinolysis both in apparently healthy subjects and in patients with advanced CAD. These associations were observed regardless of whether the subjects studied took low-dose aspirin or not. DM and HCH obscured the association between tHcy levels and fibrin clot properties. Correlations between serum folate levels and fibrin clot properties are most likely attributable the strong negative association between plasma tHcy and folate. Our data corroborate and expand the previous findings from animal models and in vitro experiments, suggesting that hyperhomocysteinemia may alter clot properties because of an acquired dysfibrinogenemia.12

A recent meta-analysis convincingly demonstrated that plasma fibrinogen is associated with the risks of CAD, stroke, or vascular and nonvascular mortality; however, the power of this association differed among studies.20 Moreover, fibrinogen was found to be associated with long-term cardiovascular risk in stable angina patients.21 In most studies, fibrinogen levels ranged within the reference values, and the difference between levels in cases and controls was usually ≈0.2 g/L.21 We did not observe the significant differences in plasma fibrinogen levels between patients with CAD or diabetes and age- and sex-matched healthy controls, although they had been found in several previous studies in CAD patients but not in all.22 In the present study, the groups were quite homogenous with respect to male sex, body mass index, low percentage of smokers, and no comorbidities that might increase fibrinogen levels. This may explain not only similar but also relatively low fibrinogen levels in patients and in controls. Fibrinogen levels can be reduced by moderate alcohol consumption or regular exercise. In view of difficulties to reliably quantify these factors, their potential impact on fibrinogen measured in the groups studied cannot be excluded. One might speculate that aspirin taken by CAD patients can reduce levels of fibrinogen, an inflammatory marker. However, low-dose aspirin appears unlikely to exert such effect in stable angina patients.21 Together, our results provide additional evidence for a pivotal role of fibrinogen levels in fibrin clot properties and suggest that altered fibrin clot properties, especially enhanced clot resistance to lysis, represent a common denominator of several factors known to increase the risk of atherothrombotic vascular disorders. We believe that hyperhomocysteinemia belongs to a group of important risk factors, including elevated fibrinogen and prothrombin levels,23 that increase clot rigidity, low permeability, and reduced susceptibility to lysis.

Fibrin clot properties have been evaluated using several approaches, ranging from a variety of purified systems to assays using citrated or dialyzed plasma.8–18,24 Determination of the permeation coefficient is the most commonly used method to assess clot porosity, which has been shown to be the major determinant of clot structure in various populations, including patients at increased risk of cardiovascular events and their relatives.9,10,16 To evaluate clot permeability, we adopted an ex vivo assay using citrated plasma that has successfully been used in previous studies.11,15,25

We assessed clot susceptibility to fibrinolysis in an ex vivo assay in which fibrin assembly and lysis occurred simultaneously. Lysis was monitored in the presence of the recombinant tPA at a final concentration of 0.5 μg/mL that exceeds levels found under physiological conditions but is similar to that during thrombolytic treatment. In a similar model of spectrophotometrically assessed lysis, it has been shown that varying concentrations of plasmin, a product of tPA-mediated plasminogen activation, affects the time course of fibrinolysis.26 We have chosen the tPA concentration of 0.5 μg/mL that ensured a steep increase in absorbance followed by its relatively rapid decrease, reflecting lysis efficacy in the assay, because we expected rather subtle differences in lysis time between consecutive plasma samples in which tHcy levels ranged from 6 to <30 μmol/L. We have seen very long lysis times with low reproducibility in the experiments performed in the presence of a 10-fold lower tPA concentration.
Low-dose aspirin has been reported to increase fibrin clot permeability in healthy individuals.31 In our study, clots formed in plasma from patients with CAD taking aspirin were less permeable, whereas lysis time was essentially unaffected compared with age- and sex-matched healthy controls with similar fibrinogen concentrations and lipid profiles. This finding suggests that other determinants of CAD-related changes in blood coagulation, such as tHcy, have a major impact on fibrin clot structure that cannot be easily overcome by aspirin administration.

Several limitations of our study warrant consideration. First, our analysis was based on a determination of each variable at a single time point. Second, scanning electron microscopy could provide direct data on fiber thickness and pore size to confirm the results of clot permeation. However, this imaging technique requires that clots are fixed and processed, therefore measurements are difficult to extrapolate to in vivo conditions. Sauls et al12 showed that clots formed in plasma from hyperhomocysteinemic rabbits have tighter and rather disorganized structure compared with the control clots. Third, our study was limited to the population of male subjects, and it is uncertain whether the results can be generalized to women.

Molecular mechanisms underlying Hcy-related changes in clot properties are unclear. Under normal conditions, most of circulating tHcy is S-linked or N-linked to blood proteins.32 It has been demonstrated that the binding of Hcy by disulfide or amide linkages alters protein function. The ε-amino group of lysines in the fibrinogen molecule can be covalently modified by Hcy–thiolactone present in plasma,37 and this modification considerably changes fibrinogen properties.34,35 Small amounts of N-linked Hcy, but not S-linked Hcy, are present in native fibrinogen.32 Because lysine residues in the fibrinogen molecule mediate the binding of plasminogen and tPA to fibrin,38 lysine modification may impair plasminogen activation and fibrin cleavage by plasmin. It cannot be excluded that other components of the fibrinolytic system may also be N-homocysteinylated or S-homocysteinylated. For example, fibronectin has the ability to bind Hcy via a disulfide linkage, which affects the fibrin clot architecture.49 However, recent data suggest that the N-homocysteinylation of lysine residues in fibrinogen by Hcy–thiolactone affects its conversion to fibrin and the structure of fibrin clots.40 Protein N-homocysteinylation is a relatively slow process that increases significantly after chronic but not acute exposures to elevated tHcy levels.32 This is a most likely explanation why elevated tHcy levels observed after methionine loading test did not affect fibrin clot characteristics in our experiments. Together, these observations suggest that the association between tHcy and clot structure observed here are most likely attributable to Hcy–thiolactone-mediated N-homocysteinylation of fibrin. Although interactions of fibrinogen with Hcy via oxidative or thiol/disulfide exchange reactions might also play a role, this is unlikely because S-linked Hcy does not occur in native fibrinogen.

The lack of association between tHcy levels and clot properties in patients with diabetes or marked HCH reported here suggests that other nonenzymatic protein modifications, such as by glucose or by products of lipid oxidation, respectively, may affect fibrin clot structure. Because folate supplementation can efficiently reduce plasma tHcy levels, one could expect increased clot permeability and susceptibility to lysis in hyperhomocysteinemic subjects treated with folic acid. Indeed, we found that a 4-week folic acid administration leads to such beneficial changes in fibrin clot properties.

In conclusion, our observations indicate that tHcy has an effect on fibrin clot structure in humans and suggest that alterations in clot structure represent a novel prothrombotic effect of Hcy. Our data in conjunction with studies published by other investigators are consistent with a mechanism in which N-homocysteinylation of fibrinogen by Hcy–thiolactone impairs its biological function. Further studies are needed to determine a role of Hcy-related changes in fibrin network properties in atherothrombosis.

References


Plasma Homocysteine Affects Fibrin Clot Permeability and Resistance to Lysis in Human Subjects

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