Relationships Between Homocysteine, Factor VIIa, and Thrombin Generation in Acute Coronary Syndromes

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Background—It has been suggested by clinical, epidemiological, and experimental in vitro studies that homocysteine potentiates thrombin generation. This prothrombotic effect however has not previously been demonstrated in patients presenting with acute coronary syndromes (ACS).

Methods and Results—Patients with ACS (n = 117) presenting with confirmed acute myocardial infarction (MI) (n = 57) or unstable angina pectoris (UAP) (n = 60) were consecutively recruited together with patients (n = 18) in whom the presenting chest pain was not of cardiac origin (NCP), included as controls. Plasma samples were collected on admission and before clinical intervention. Homocysteine was assayed by high performance liquid chromatography, and both Factor VIIa and prothrombin fragment F1+2 were analyzed by ELISA. There were significant elevations in F1+2 in MI (P < 0.001) and UAP (P = 0.003), and modest elevations in Factor VIIa in UAP (P < 0.05) compared with NCP but no differences in homocysteine levels among those groups. On dividing patients with ACS into quartiles of homocysteine, there was a stepwise increase in F1+2 (P < 0.0001) and of Factor VIIa (P < 0.05). There were significant correlations in ACS between homocysteine and F1+2 (r = 0.46, P < 0.0001), homocysteine and Factor VIIa (r = 0.24, P < 0.01), and F1+2 and Factor VIIa (r = 0.41, P < 0.0001). There was no correlation between homocysteine and either F1+2 (r = −0.15, P = 0.57) or Factor VIIa (r = 0.22, P = 0.37) in the NCP patients.

Conclusions—Elevated plasma homocysteine is associated with and may cause elevated Factor VIIa and thrombin generation in patients presenting with ACS. These findings suggest an explanation for the prothrombotic effect of homocysteine in ACS. (Circulation. 2000;101:372-377.)

Key Words: coagulation ■ risk factors ■ heart disease ■ endothelium

Recent epidemiological studies have shown that moderately elevated plasma homocysteine concentrations are associated with an increased risk for the development of atheromatous (cerebrovascular) and peripheral vascular disease and thrombotic vascular diseases (deep vein thrombosis). Coronary artery disease may encompass both an atheromatous lesion and thrombosis during acute coronary events. There is a known association between elevated homocysteine levels and the development of coronary artery disease.

Homocystinuria, the genetic form of hyperhomocysteinemia, is associated with a marked rise of plasma homocysteine levels and an early risk of mortality from thrombotic vascular disease. Although the exact mechanism of atherothrombosis associated with hyperhomocysteinemia is not clearly understood, several studies have pointed to an association with inhibition of thrombomodulin activity, reduction of protein C activation, increased platelet aggregation, and predisposition to endothelial cell injury. Most of these studies, however, were in vitro studies, where supraphysiological concentrations of homocysteine were used.

Disruption of coronary arterial atheromatous plaques is known to initiate a thrombotic process leading to complete or partial occlusion in the acute coronary syndromes (ACS) of myocardial infarction (MI) and unstable angina pectoris (UAP). Disrupted coronary plaques are thought to contain an abundance of tissue factor which, when exposed to circulating blood, forms a complex with Factor VII/VIIa initiating the coagulation cascade. Factor VIIa is generated by enzymatic cleavage of Factor VII by various circulating proteases, resulting in rapid generation of Factor IXa and Factor Xa. How these processes may be influenced by homocysteine in plasma in clinical syndromes characterized by arteriothrombotic disease is uncertain.

We report an investigation of the relationship between plasma homocysteine and activation of coagulation in patients presenting with acute coronary ischemia, studied on admission and before clinical intervention.

Patients and Methods

Study Population
Consecutive patients (n = 135) were recruited on admission to the Coronary Care Unit at Charing Cross Hospital in London
October 1995 and January 1997. Written informed consent was obtained from all the patients with the approval of the local ethical committee at Charing Cross Hospital. The patients were given a provisional diagnosis to guide appropriate treatment, but this diagnosis was subsequently reviewed during follow-up, and final diagnoses (MI, UAP and noncardiac chest pain [NCP]) were used in all analyses.

MI was diagnosed according to the World Health Organization criteria. UAP was diagnosed if the WHO criteria for MI were not met, all enzyme measurements (creatine kinase, aspartate transaminase, hydroxybutyrate dehydrogenase) were below twice their upper reference range throughout the routine sampling period (daily for 3 days). Evidence of ischemic heart disease was demonstrated by either follow-up cardiac event (death, coronary revascularization, or MI), a positive exercise treadmill test (>0.1 mV ST segment depression 80 ms after the J point) or demonstration of ischemia on thallium radio-isotopic scanning. NCP was diagnosed if angina was excluded by an alternative source of pain, a negative exercise treadmill test, or normal coronary anatomy on subsequent angiography. Patients were excluded from the study if they were receiving anticoagulant treatment before admission.

**Clinical Data**

Full clinical, biochemical, management and result of investigations were recorded on all patients. Clinical details included risk factor assessment for coronary artery disease. Smoking status was classified as current smokers and nonsmokers; current smokers included individuals who stopped <4 weeks before enrollment in the study. A known history of hypertension and/or diabetes mellitus and treatment received before admission were also recorded. Routine lipid profile estimation was performed on admission for all the patients documenting their total cholesterol, triglycerides, and HDL levels.

**Protocol**

Venous blood samples were obtained on admission by trained medical or senior nursing staff from a cuffed antecubital vein before initiation of thrombolysis or anticoagulant treatment. Care was taken to obtain this sample with a fresh needle and syringe. Blood was transferred into bottles containing 0.105 mol/L sodium citrate for Factor VIIa and F1+2 assay and bottles containing EDTA (7.5%) for homocysteine assay (Becton Dickinson). Within 15 minutes of collection, platelet-poor plasma was obtained by centrifugation at room temperature for 15 minutes at 3000g, flash frozen on dry ice, and then transferred to a −80°C freezer. Blind analysis of all samples was performed in batches at completion of sample collection. The protocol for sample collection and storage was formulated during a prior study published elsewhere. Emphasis was put on clean venipuncture to minimize artifacts. This was a particular concern because sampling was performed in the environment of a busy coronary care unit. To provide reassurance that artifactual elevations in samples was minimal, blood samples were also taken from a group (n = 11) of age-matched healthy controls; these samples were similarly processed and assayed. The results of the assay of these samples were not significantly different from those of the NCP group (which were collected in the coronary care unit and subsequently assayed blind), suggesting that the sampling strategy was adequate.

**Homocysteine Measurements**

Plasma total homocysteine, which includes the sum of protein-bound and free homocysteine, was measured by high-performance liquid chromatography with fluorescence detection. The intra- and interassay coefficients of variation were ≤5%. Plasma homocysteine was recorded in micromoles per liter.

**Factor VIIa and Prothrombin Fragment F1+2 Measurements**

These were performed with specific ELISAs (developed in-house and fully reported elsewhere). The assay for F1+2 was performed as originally described and has intra- and interassay coefficients of variation of ~10% and ~15%, respectively. The normal laboratory mean (±SEM) is 31.0±3.7 ng/mL, which corresponds to 0.88±0.11 mmol/L. The Factor VIIa assay was modified, as described by replacement of the standard, which altered the normal mean to 0.25±0.10 ng/mL (0.005±0.003 mmol/L). The intra- and interassay coefficients of variation are ~4% and ~10%, respectively. The ELISA to Factor VIIa measures free Factor VIIa in plasma. Free Factor VIIa will have been generated and released from a cell surface on which tissue factor has been expressed. By its nature, the Factor VIIa ELISA cannot measure any Factor VIIa still bound to the cell surface.

**Statistical Methods**

Because the distribution of values for plasma homocysteine, F1+2, and Factor VIIa were positively skewed, the variables were logarithmically transformed; geometric means (95% CI), unless otherwise stated, are presented throughout the text, tables, and figures. Median and interquartile (25th to 75th) ranges of plasma homocysteine were used to categorize the patients into quartiles in MI (12.7, 11.6 to 16.8 μmol/L), UAP (13.1, 10.2 to 16.5 μmol/L), and ACS (12.9, 10.9 to 16.5 μmol/L). Patients with a final diagnosis of NCP were used as controls to establish that in those patients with definite ACS, coagulation activation had occurred and sampling artifacts had been kept to a minimum. The median homocysteine level of 12.0 μmol/L was used to categorize the NCP patients above and below this level.

**Results**

Clinical details of the 117 ACS and 18 NCP patients are given in Table 1. Mean age, sex, and risk factor distribution of the patients with ACS was not statistically different from those with NCP.

The results for plasma homocysteine, Factor VIIa, and F1+2 concentration in ACS are presented in Table 2, stratified by age, sex, smoking status, cholesterol, HDL, hypertension, and diabetes mellitus. Homocysteine showed significant difference with increasing age. Factor VIIa did not vary significantly with any of these factors. There was a highly significant difference in F1+2 levels with age, a finding that has been often reported. F1+2 was also noted to be slightly higher in women and in patients with hypertension.

### Table 1. Clinical Characteristics of Patients Presenting With Myocardial Infarction, Unstable Angina, and Noncardiac Chest Pain

<table>
<thead>
<tr>
<th></th>
<th>ACS (n=117)</th>
<th>MI (n=57)</th>
<th>UAP (n=60)</th>
<th>NCP (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>66.5±1.3</td>
<td>67.3±1.9</td>
<td>65.7±1.8</td>
<td>65.9±1.2</td>
</tr>
<tr>
<td>Sex (male), %</td>
<td>62</td>
<td>62</td>
<td>62</td>
<td>60</td>
</tr>
<tr>
<td>Smoking (current), %</td>
<td>28</td>
<td>34</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>33</td>
<td>33</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>Cholesterol=6.0 mmol/L, %</td>
<td>58</td>
<td>59</td>
<td>58</td>
<td>22</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>12</td>
<td>7</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>IHD, %</td>
<td>28</td>
<td>20</td>
<td>37</td>
<td>38</td>
</tr>
<tr>
<td>Creatinine, mmol/L*</td>
<td>105±2</td>
<td>105±3</td>
<td>105±3</td>
<td>97±17</td>
</tr>
</tbody>
</table>

*Mean±SEM.

IHD indicates ischemic heart disease.
The respective relationships of Factor VIIa and F1+2 to final diagnosis were examined. F1+2 levels were significantly \( (P<0.001) \) higher in patients with MI (44.8, 37.0 to 53.4 ng/mL) than in patients with NCP (25.1, 20.0 to 31.6 ng/mL), but not significantly different in patients with UAP (39.3, 33.2 to 46.6 ng/mL), Figure 1a. Factor VIIa was significantly higher in patients with UAP (0.38, 0.29 to 0.51 ng/mL) than in patients NCP (0.22, 0.16 to 0.31 ng/mL) but not different in patients with MI (0.31, 0.23 to 0.41 ng/mL), Figure 1b. Evidence of a relationship between changes in levels of F1+2 and Factor VIIa was then sought through correlation analysis. There were significant correlations between F1+2 and Factor VIIa levels when considering results in MI \( (r=0.38, P=0.01, n=58) \), in UAP \( (r=0.45, P=0.003, n=60) \), and when the ACS were considered as one group \( (r=0.41, P<0.0001, n=117) \). There was no correlation between F1+2 and Factor VIIa levels in the NCP group \( (r=-0.06, P=0.59) \). These observations confirm a hypercoagulable state in these patients presenting with ACS but not in patients with NCP.

Plasma homocysteine concentrations were similar in both MI (13.8, 12.5 to 15.3 \( \mu \)mol/L) and UAP (13.3, 12.2 to 14.5 \( \mu \)mol/L), but they were not significantly higher than NCP (12.0, 9.7 to 14.8 \( \mu \)mol/L), Figure 1c.

The relationship between the coagulation activation markers (F1+2 and Factor VIIa) and homocysteine was studied by dividing the latter into quartiles. It can be seen from Figure 2a that in MI there is a stepwise increase in F1+2 with increasing quartiles (ANOVA, \( P=0.05 \)): there is a significant difference \( (P=0.007) \) between F1+2 levels in the lowest (33.5, 27.2 to 41.2 ng/mL) and highest (35.3, 24.0 to 52.0 ng/mL) homocysteine quartiles, Figure 2b. When the results for all...
patients with ACS were combined (MI plus UAP), there was a significant graded increase in F1+2 between the homocysteine quartiles (ANOVA, $P<0.0001$). The difference between the upper (63.4, 49.0 to 81.9 ng/mL) and lower (31.1, 27.1 to 35.7 ng/mL) homocysteine quartiles was highly significant ($P<0.0001$; Figure 2c). When all results for homocysteine and F1+2 in the ACS were compared by correlation analysis, there was a significant correlation ($r=0.46$, $P<0.0001$, $n=117$).

A similar analysis of relationship between quartiles of homocysteine and Factor VIIa levels showed no change in MI (Figure 3a). There was, however, a significant increase overall in Factor VIIa levels in UAP (ANOVA, $P<0.05$) and between lowest levels (0.25, 0.16 to 0.37 ng/mL) and highest levels (0.71, 0.44 to 1.13 ng/mL) homocysteine quartiles was highly significant ($P<0.0001$; Figure 3c). When all results for homocysteine and Factor VIIa in the ACS were compared by correlation analysis, there was a significant correlation ($r=0.46$, $P<0.0001$, $n=117$).

Discussion
The blood coagulation cascade is known to be activated in the acute phase of MI and UAP. Plasma levels of Fibrinopeptide A, F1+2, have been shown to be elevated, signaling activation of the end stages of the coagulation process. Plaque rupture and fissuring is thought to be a critical event in the
pathogenesis of the ACS. This is associated with exposure of tissue factor, which can trigger the coagulation cascade via Factor VIIa generation. However, previous studies, which have investigated Factor VIIa levels, have generally failed to demonstrate changes that could be responsible for the readily detectable increase in thrombin generation (F1+2 elevation). An exception was a small previous study from this center, which showed elevated Factor VIIa levels, particularly in UAP.

The design of our study differs from most investigations in that we have recruited all patients admitted to our coronary care unit and used patients diagnosed with NCP as a control group. This group was small because it comprised those patients who presented with suspected ACS but who were shown not to have the syndromes. It was mainly used as a control to establish that in those patients with definite ACS, coagulation activation had occurred and that sampling artifacts had been kept to a minimum. The main focus of this study was therefore not to study the relation between the ACS and NCP groups but the relationship between the degree of coagulation activation and homocysteine concentrations in patients in whom a thrombotic process had taken place. Although some formal analysis of results from the NCP group is presented, it must be stressed that the group size makes the interpretation of these results tentative.

We have been able to show a convincing association between Factor VIIa and F1+2 in the acute phase of the ACS. This association, manifest by correlation analysis, persists on subgroup analysis, despite the generally less clear elevation of Factor VIIa than F1+2 in MI. This latter finding could help explain the failure to demonstrate elevation of Factor VIIa in other studies of ACS. There is an important methodological aspect involved in the determination of Factor VIIa as an activation marker, which distinguishes it from other activation markers (eg, F1+2). Cellular expression of tissue factor is required for activation of Factor VII. An ability to detect Factor VIIa in plasma implies that sufficient Factor VIIa is released from the cell surface during the activation process to enable detection. Support for this possibility of detection in plasma is provided by results of in vitro activation experiments conducted in plasma. Addition of tissue factor and phospholipid vesicles to recalcified plasma results in generation in more Factor VIIa on a molar basis than the added tissue factor (H. Philippou, PhD, and D.A. Lane, PhD, unpublished observations, 1999). This suggests that each molecule of tissue factor can undergo multiple rounds of Factor VII activation, progressively releasing Factor VIIa. It might be that the low level activation of Factor VII observed in this clinical context might arise from the difficulty in its detection because of its slow release from the cell surface; this might also explain the lower levels of Factor VIIa observed in plasma in MI compared with UAP. There are additional methodological issues in the measurement of Factor VIIa levels. It is not clear whether our use of an ELISA for quantification of Factor VIIa levels was important for the demonstration of a relationship between Factor VIIa and F1+2 levels, because at the inception of this study we did not have available the functional assay for Factor VIIa. Regardless of these methodological points, our results provide an explanation for increased F1+2 levels in the ACS, by linking the initial cellular response (believed to be exposure of tissue factor) with the outcome (increased thrombin generation) through an essential proteinase (Factor VIIa) involved in activation of blood coagulation.

The main novel feature of our study has been to relate homocysteine levels to coagulation activation. A significant association between plasma homocysteine levels and coagulation activation has been demonstrated both in relation to changes in F1+2 and Factor VIIa levels. F1+2 plasma levels correlated positively and in a concentration-related manner to plasma homocysteine levels. This pattern was seen in patients with MI and UAP but not in patients with NCP (however, note the caution about the sample size). The association between F1+2 levels and homocysteine has also previously been shown by Kyrle et al in patients with prior history of venous thromboembolism. In a similar patient group, Cattaneo et al did not find any correlation.
study, however, blood samples were taken at least 4 months after the last thrombotic event. Furthermore, there was no difference in FV + FII levels between patients with prior history of thromboembolism and healthy controls, suggesting no obvious activation of coagulation. This contrasts to the present study, in which we have sampled on presentation and before any intervention in an attempt to study patients in the acute hypercoagulable state. The association between homocysteine and Factor VIIa levels is less prominent in the current study, possibly because of the relatively low elevation of Factor VIIa levels arising for the reasons discussed above.

How could homocysteine interact with coagulation to enhance activation? Fryer et al have suggested that high levels of plasma homocysteine may initiate coagulation by enhancing endothelial cell expression of tissue factor activity, and this certainly seems plausible in the light of the present observation of a correlation between homocysteine and Factor VIIa. Inhibition of protein C activation and downregulation of thrombomodulin is also well-documented.

This study has limitations: its power is limited by its size, this has prevented a detailed statistical analysis of possible confounders. The size of the NCP group has prevented firm conclusions from being drawn on differences between ACS and NCP patients regarding associations between coagulation activation markers and homocysteine.

Despite these limitations, this study has shown for the first time that elevated plasma homocysteine in patients presenting with ACS appears to be associated with increased coagulation activation as judged by increases in FV + FII and Factor VIIa levels. Although there is plausible experimental evidence to suggest that these are causally related, further experimental and larger scale clinical studies are required to confirm our findings. If such confirmation is obtained, it would suggest therapeutic modulation of plasma homocysteine as a means of reducing increased thrombin generation in ACS.

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References

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