Endothelial Fibrinolytic Capacity Predicts Future Adverse Cardiovascular Events in Patients With Coronary Heart Disease

Simon D. Robinson, Christopher A. Ludlam, Nicholas A. Boon, David E. Newby

Objective—The endothelium-derived fibrinolytic factor tissue plasminogen activator (t-PA) is a major determinant of vessel patency after coronary plaque rupture and thrombosis. We assessed whether endothelial fibrinolytic capacity predicts atherothrombotic events in patients with coronary heart disease.

Methods and Results—Plasma t-PA and plasminogen activator inhibitor (PAI)-1 concentrations were measured during intrabrachial substance P infusion in 98 patients with angiographically proven stable coronary heart disease. Forearm blood flow was measured during infusion of substance P and sodium nitroprusside. Cardiovascular events (cardiovascular death, myocardial infarction [MI], ischemic stroke [CVA], and emergency hospitalization for unstable angina) were determined during 42 months of follow-up. Patients experiencing a cardiovascular event (n=19) had similar baseline characteristics to those free of events. Substance P caused a dose-dependent increase in plasma t-PA concentrations (P<0.001). However, net t-PA release was 72% lower in the patients who experienced death, MI, or CVA, and 48% lower in those who suffered death, MI, CVA or hospitalization for unstable angina (P<0.05). Major adverse cardiovascular events were most frequent in those with the lowest fibrinolytic capacity (P=0.03 for trend); patients with the lowest quartile of t-PA release had the highest rate of adverse events (P=0.01).

Conclusion—Endothelial fibrinolytic capacity, as measured by stimulated t-PA release, predicts the future risk of adverse cardiovascular events in patients with coronary heart disease. We suggest that endothelial fibrinolytic capacity is a powerful novel determinant of cardiovascular risk. (Arterioscler Thromb Vasc Biol. 2007;27:1651-1656.)

Key Words: coronary heart disease ■ endothelium ■ fibrinolysis ■ survival ■ vasodilation

The endogenous fibrinolytic system protects the circulation from intravascular fibrin formation and thrombosis. In the presence of developing thrombus, the fibrinolytic factor tissue plasminogen activator (t-PA) is rapidly released from the vascular endothelium by the coagulation factors thrombin and factor Xa1 and causes a 1000-fold increase in the enzymatic conversion of plasminogen to plasmin.2,3 This ensures that rapid plasmin generation, fibrin degradation, and clot dissolution are tightly regulated and localized to sites of vascular injury and thrombus formation. Thus, the rapidity and extent of acute t-PA release from the endothelium is a critical factor in determining the efficacy of local endogenous fibrinolysis.4,5 Areas of endothelial denudation and thrombus deposition are a common finding on the surface of atheromatous plaques and are often subclinical.6 Through t-PA release, endogenous fibrinolysis is usually able to prevent thrombus propagation, vessel occlusion, and tissue infarction, although organization of the residual thrombus may lead to plaque growth and expansion.6 The resolution of thrombus after atheromatous plaque rupture, and the resulting clinical sequela, may therefore be critically dependent on the efficacy of endogenous fibrinolysis.7 Accordingly the capacity of the endothelium to release t-PA may predict the outcome of individual plaque events and long-term cardiovascular risk.

Intraarterial infusion of substance P8 and bradykinin9 cause a rapid and sustained release of t-PA from the endothelium, and acute stimulated t-PA release has been measured within the forearm8,9 and coronary11,12 circulations of man. Previous studies have demonstrated impaired forearm t-PA release in cigarette smokers13 and patients with hypertension14 who are at particular risk of coronary thrombosis and myocardial infarction (MI).15,16 Although this suggests that endogenous fibrinolysis plays an important role in the pathogenesis of coronary thrombosis, the relationship between the capacity to release t-PA and the future risk of adverse cardiovascular events is unknown. The aim of the present study was, therefore, to determine whether endothelial fibrinolytic capacity predicts the future risk of atherothrombotic events in patients with stable coronary heart disease (CHD).

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Methods

Patients
We recruited patients with angiographically proven CHD defined as ≥50% luminal stenosis of at least one major epicardial coronary vessel. All patients had stable anginal symptoms and had not undergone coronary revascularization within the preceding 3 months. Exclusion criteria were significant cardiac failure, renal impairment, systolic blood pressure <100 or >190 mm Hg, or diabetes mellitus. All studies were undertaken with the approval of the local Research Ethics Committee, the written informed consent of each subject, and in accordance with the Declaration of Helsinki.

Venous Sampling and Assays
Venous blood was collected into tubes containing gel, ethylene diamine tetra-acetic acid, acidified buffered citrate, and trisodium citrate. Platelet-free plasma and serum were stored at -80°C before assay. Plasma t-PA and PAI type 1 (PAI-1) antigen concentrations (Coatiza, Chromogenix) were determined using enzyme-linked immunosorbent assays.8,13 Highly sensitive assays of C-reactive protein (hs-CRP) were undertaken on serum using the method of particle-enhanced immunonephelometry (Behring BN II nephelometer). Serum biochemical analysis and hematocrit estimations were undertaken by the hospital Clinical Biochemistry and Hematology Laboratories, respectively.

Forearm Study Protocol
Subjects abstained from alcohol for 24 hours and from food, tobacco, and caffeine-containing drinks for at least 4 hours before each study visit. Cardioactive medications were withheld on the morning of each study. All studies were carried out in a quiet temperature controlled room maintained at 22 to 25°C. Patients rested recumbent, each study. All studies were carried out in a quiet temperature controlled room maintained at 22 to 25°C. Patients rested recumbent, strain gauges and cuffs were applied. A 17-G venous cannula was inserted into the antecubital vein of each arm and the brachial artery of the nondominant arm was cannulated with a 27-G needle (Cooper’s Needle Works Ltd). Forearm blood flow (FFB) was measured in both forearms by venous occlusion plethysmography. Forearm blood flow was measured at baseline and with each dose of the vasodilators were analyzed as the ratio between the infused and control arms expressed as a percentage of the ratio measured during the baseline control period.20 Estimated net release of t-PA was defined previously as the product of the infused forearm plasma flow, and the concentration difference between the infused and noninfused forearms.5,8,13 Cumulative event rates were estimated by Kaplan–Meier survival curves, and probability values were determined with the log-rank test. In those patients who experienced multiple events, survival analysis was restricted to the first event. For Kaplan–Meier analyses, subjects were divided into quartiles according to estimated net release of t-PA antigen. All statistical analysis was completed using GraphPad Prism (GraphPad Software) with statistical significance being assigned at the 5% level.

Results
Endothelial fibrinolytic capacity was measured in 98 subjects with angiographically proven CHD who were followed up for a median of 42 months (range 5 to 51 months). Over the follow-up period, 2 patients died from cardiovascular disease, 2 suffered a MI, 2 had an ischemic CVA, and 13 had an emergency admission for unstable angina. In general, patients experiencing cardiovascular events had similar baseline characteristics and use of secondary preventative medications to those free from events (Table), although plasma glucose levels were slightly higher in those subsequently experiencing clinical events (6.1 ±0.9 versus 5.6 ±0.8 mmol/L, respectively, P=0.03).

Substance P caused a dose-dependent increase in plasma t-PA (P<0.001, ANOVA) but not PAI-1 (P=ns) concentrations. The concentration differences of t-PA antigen between the forearms and estimated net release of t-PA both increased dose dependently (P<0.001 for both, ANOVA) although this increase was reduced in subjects who subsequently experienced cardiovascular events (P<0.05, ANOVA; Figure 1). Specifically, net release of t-PA during substance P infusion was 72% lower in those subjects suffering cardiovascular death, MI, or CVA (P=0.02, ANOVA), and 48% lower in those with cardiovascular death, MI or CVA, or hospitalization for unstable angina (P=0.008, ANOVA). Major adverse cardiovascular events were most frequent in those with the lowest fibrinolytic capacity (P=0.03 for trend) such that patients with the lowest quartile of t-PA release had the highest rate of adverse events (P=0.01; Figure 2).

Although baseline parameters were similarly distributed between subjects with and without events, baseline plasma PAI-1 antigen and female gender were positively correlated with t-PA release (P<0.05), whereas cigarette smoking was associated with a reduction in substance P–induced t-PA release (P=0.05). There was no association between acute t-PA release and plasma glucose concentrations (159±24 versus 168±26, P=0.80; area under the curve for t-PA release in subjects < versus > median glucose concentration).
There was no difference in resting forearm blood flow between those subjects with and without atherothrombotic events (2.9 \pm 0.3 \text{mL/100 mL tissue/min} versus 2.8 \pm 0.3 \text{mL/100 mL tissue/min}, respectively; \textit{P} = 0.37). Substance P and sodium nitroprusside caused dose-dependent increases in infused forearm blood flow in all subjects (\textit{P} < 0.001, ANOVA). There were no differences in either endothelium-dependent or -independent blood flow responses between subjects in either group (Figure 3).

### Discussion

This is the first study to assess the relationship between endothelial fibrinolytic capacity and the future risk of atherothrombotic cardiovascular events. The population that we chose to study was a homogenous cohort of patients with stable CHD and a typical distribution of conventional cardiovascular risk factors. Even though this was a relatively low risk population receiving optimal secondary preventative therapy, endothelial fibrinolytic capacity has emerged as a powerful novel determinant of future cardiovascular risk.

In a metaanalysis of prospective observational studies,\textsuperscript{21} the risk of CHD is increased in subjects with plasma t-PA antigen concentrations in the highest tertile compared with those in the lowest tertile. This may seem counterintuitive but in part reflects the concomitant rise of plasma PAI-1 concentrations and the associated reduction in t-PA activity. Moreover, in the basal state, the endogenous fibrinolysis system is effectively inactive and does not influence in situ thrombus formation. Neither basal plasma t-PA nor PAI-1 concentrations control the local vascular fibrinolytic capacity, which is

### Baseline Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>All Patients (\textit{n}=98)</th>
<th>Subjects With Events (\textit{n}=19)</th>
<th>Subjects Without Events (\textit{n}=79)</th>
<th>\textit{P} Value*</th>
</tr>
</thead>
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<tr>
<td>Age, years</td>
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<td>62 \pm 8</td>
<td>59 \pm 7</td>
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<td>17/2</td>
<td>63/16</td>
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<td>BMI, kg/m\textsuperscript{2}</td>
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<td>28 \pm 4</td>
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</tr>
<tr>
<td>Systolic BP, mm Hg</td>
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<td>138 \pm 20</td>
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<tr>
<td>Diastolic BP, mm Hg</td>
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<td>77 \pm 10</td>
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<td>Pulse, bpm</td>
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<td>57 \pm 9</td>
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<tr>
<td>Cigarette smoker, %</td>
<td>31</td>
<td>32</td>
<td>30</td>
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</table>

**Values are mean \pm SD.**

*Unpaired \textit{t} test or Fishers exact test for subjects with, vs without, events (death from a cardiovascular cause, MI, ischemic CVA, hospitalisation for unstable angina). As C-reactive protein values were not normally distributed they were log transformed before statistical analysis.

BMI indicates body mass index; bpm, beats per minute; MI, myocardial infarction; CHD, coronary heart disease; FHx, family history; CVD, cerebrovascular disease; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; chol, cholesterol; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor\textsuperscript{-1}.\textsuperscript{1}
determined by the acute release of t-PA from the endothelium. This underscores the importance of assessing the pathophysiologically relevant measure of acute stimulated endothelial t-PA release.

The positive association between forearm t-PA release and female gender is in keeping with previous reports. Furthermore, we and others have also previously demonstrated that changes in its fibrinolytic capacity are indicative of a systemic effect and not simply local plaque burden. These considerations are, therefore, in keeping with the hypothesis that atherosclerosis is a systemic disorder, and that acute t-PA release should be considered a distinct marker of endothelial function.

Intravascular thrombus formation is a key feature of clinical atherosclerotic events associated with eroded or unstable coronary plaques. The importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct related artery after acute myocardial infarction, occurring in up to 30% of patients within the first 12 hours. Any reduction in the acute dynamic fibrinolytic response decreases the capacity to lyse intraluminal thrombus and the likelihood of restoring vessel patency. In this prospective observational cohort study, we have further demonstrated that the capacity to release t-PA appears to be a major determinant of the risk of cardiovascular events and suggests that endothelial fibrinolytic capacity has a crucial role in the pathogenesis of atherothrombosis.

Endothelial dysfunction is characterized by the disruption of multiple homeostatic pathways predisposing to vasoconstriction, platelet activation, and thrombosis. To date, most clinical studies on endothelial function have focused on endothelium-dependent vasodilatation and future atherothrombotic events remains unclear. Moreover, the regulation of vessel tone may not be the facet of vascular function most closely allied to the future risk of atherothrombotic events. In this current study, we did not observe a difference in endothelium-dependent or -independent blood flow responses between subjects with and without subsequent cardiovascular events. This disparity may reflect a lack of power or the use of differing endothelium-dependent vasodilators. Thus far, studies assessing the prognostic value of endothelium-dependent vasodilatation have used acetylcholine. We chose to use substance P because this is a potent

Figure 1. Concentration differences between the forearms of plasma tissue plasminogen activator (t-PA) antigen (lower panels) and estimated net release of t-PA antigen (upper panels) in subjects with (●), and without (○), death, MI, or CVA (left hand panels) or death, MI, CVA, or hospitalization for unstable angina (right hand panels). *P=0.02, ANOVA (●) vs (○); †P=0.004, ANOVA (●) vs (○); ‡P=0.008, ANOVA (●) vs (○).

Figure 2. Cumulative proportion of patients without cardiovascular events during follow-up (Kaplan-Meier). Subjects are divided into quartiles for estimated net release of t-PA antigen. P=0.03, log rank for trend; P=0.01, lowest vs upper quartiles.

Figure 3. Forearm blood flow (FBF) during incremental doses of substance P (left), sodium nitroprusside (right) in subjects with (●) and without (○) death, MI, CVA, or hospitalization for unstable angina. P<0.001, ANOVA dose response; P=ns, (●) vs (○), for both vasodilators.
stimulant of endothelial t-PA release, and acetylcholine does not cause demonstrable t-PA release. Reports of preserved endothelium-dependent vasodilatation in smokers and in patients with hypertension despite reduced acute t-PA release suggest that, in some circumstances, reduced t-PA release may be a more sensitive marker of endothelial dysfunction. These data also highlight the complexity of vascular biology and demonstrate that endothelial dysfunction is not a single clinical entity encompassing a uniform pathophysiological response to vascular injury.

**Study Limitations**

Although we cannot determine basal t-PA release using the venous technique described, calculation of net t-PA release provides an accurate assessment of stimulated t-PA release with good reproducibility and basal release contributes only a small proportion of the overall venous plasma t-PA concentration. Furthermore, previous work has demonstrated that basal plasma t-PA concentrations do not control the local vascular fibrinolytic capacity which is in fact determined by the acute release of t-PA from the endothelium.

Plaque growth is induced by episodic subclinical plaque disruption and if local t-PA release is impaired, the continued presence of thrombus may favor smooth muscle migration, the production of new connective tissue, and plaque expansion. In keeping with this hypothesis, genetic murine models of plasminogen deficiency as well as PAI-1 overexpression have shown that reduced fibrinolytic potential is associated with enhanced macrovascular fibrin deposition and accelerated atherogenesis. As we do not have follow-up angiographic data, we do not know whether those subjects with the lowest local t-PA release or recurrent cardiovascular events exhibited a greater progression of angiographic disease over the study period. We have previously demonstrated an inverse correlation between acute coronary t-PA release and local atheromatous plaque burden, and prospective studies of coronary t-PA release and quantification of disease progression and would be of interest.

Comparable numbers of patients in each group were receiving secondary preventative medications including ACE inhibitors. Although treatment with ACE inhibitors enhances bradykinin-mediated t-PA release, these do not influence t-PA release in response to infusion of substance P. In keeping with the risk factor profile and use of secondary preventative therapies in our study cohort, relatively few events occurred over the study period. Despite this, similar results were noted for outcomes regardless of whether hospitalization for unstable angina was included. Although the risk factor profile and severity of coronary artery disease were similar in those patients with or without events during follow-up, the relatively modest study numbers and follow-up period mean we were unable to explore in detail potential interactions and independence of endothelial fibrinolytic capacity with conventional cardiovascular risk factors or the extent of coronary artery disease. Indeed, given the modest number of subjects and clinical end points, our results should be interpreted with caution. It would be desirable to increase subject numbers and clinical end points further, but the invasive nature and complexity of undertaking such detailed physiological studies presents many challenges when attempting to apply this technique to larger population-based clinical studies.

**Conclusion**

t-PA release from the endothelium is a distinct marker of endothelial function. In patients with stable CHD, we have shown that a reduction in acute t-PA release predicts an increased risk of adverse cardiovascular events. Further studies of the factors modifying the endogenous fibrinolytic capacity have the potential to provide major new insights into the pathophysiology of CHD and to shape future therapeutic interventions.

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**Disclosures**

None.

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