Plasma resistance to activated protein C regulates the activation of coagulation induced by thrombolysis in patients with ischaemic heart disease

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Abstract
Objective—To determine whether there was a relation between plasma resistance to activated protein C and the coagulation activation induced during thrombolysis with 100 mg alteplase in 25 patients with acute ischaemic heart disease.

Methods—Blood samples were collected before (t = 0 h), during (t = 2.5 h), and after (t = 4 h, t = 12 h, and t = 24 h) thrombolysis to examine the relation between baseline activated protein C resistance ratio and markers of coagulation activation—that is, thrombin-antithrombin III-complexes and prothrombin fragment 1 + 2 generated during thrombolysis.

Results—There was a negative correlation between activated protein C resistance ratio and area under the curve of thrombin-antithrombin III-complexes (r = −0.60; P < 0.003) and there was a trend to a negative correlation between activated protein C resistance ratio and area under the curve of prothrombin fragment 1 + 2 (r = −0.37; P = 0.07). This accorded with the negative correlation between activated protein C resistance ratio and the peak value of thrombin-antithrombin III-complexes (r = −0.55; P < 0.005) and between activated protein C resistance ratio and the peak value of prothrombin fragment 1 + 2 (r = −0.42; P < 0.04).

Components of the protein C/S system or known inhibitors of activated protein C may influence the activated protein C resistance ratio. There were no associations between the activated protein C resistance ratio and protein C, protein C inhibitor, or plasminogen activator inhibitor type-1, whereas there was a trend to a negative correlation between activated protein C resistance ratio and protein S.

Conclusions—The results indicate that plasma resistance to activated protein C may be one of the main mechanisms regulating the activation of coagulation induced by thrombolysis. This study suggests that it may be possible to single out individuals with a high risk of reocclusion before the start of thrombolytic therapy.

Keywords: thrombolysis; coagulation; activated protein C resistance; ischaemic heart disease

Several large scale trials have shown that thrombolytic therapy reduces mortality after acute myocardial infarction, and today thrombolytic therapy is a well established treatment for acute myocardial infarction. Failure to reperfuse or reocclusion after successful reperfusion are still major challenges. Failure to reperfuse was reported in 15–40% of patients and in addition 5–20% of the patients in whom thrombolysis was successful had reocclusion.

Early reocclusion may be a result of activation of the coagulation system by thrombolysis. The activation of coagulation system is reflected by the generation of fibrinopeptide A, prothrombin fragment 1 + 2 (F 1 + 2), and thrombin-antithrombin III—complexes (TAT) during and after the infusion of thrombolytic agents. The mechanism responsible for this apparently paradoxical coagulation activation is incompletely understood, but observations indicate that plasmin generated during thrombolysis is the primary trigger. In addition to activation of coagulation it has recently been reported that thrombolysis generates an endogenous anticoagulant enzyme, activated protein C (APC), which may regulate activation of coagulation through the proteolytic degradation of central components of the coagulation cascade. Thus there is evidence that the activation of coagulation during thrombolysis is determined by a complex balance between plasm mediated coagulant and anticoagulant mechanisms.

To study whether factors in plasma regulate the activation of coagulation during thrombolysis we determined plasma resistance to activated protein C before thrombolytic therapy with alteplase in 25 patients with acute ischaemic heart disease and correlated the plasma activated protein C resistance values with the amount of coagulation reaction products formed during thrombolysis.

Patients and methods

PATIENTS
We studied 25 patients admitted to the coronary care unit with a tentative diagnosis of acute myocardial infarction and symptoms lasting less than five hours.
Originally, the cohort consisted of 34 patients, recruited from one arm of a randomised placebo controlled trial of thrombolysis. Nine patients were excluded from the present study: two patients died within a few hours after inclusion; in five patients no plasma samples were available for determination of plasma resistance to activated protein C, in one patient it was not possible to determine plasma resistance to activated protein C because there was marked prolongation of clotting time, and one patient who responded with abnormal high coagulation reaction products died of cardiac rupture 31 hours after inclusion. None of the patients received treatment with heparin or vitamin K antagonist before admission or at the time of admission to hospital. The activated partial-thromboplastin times (APTT) in the 25 patients determined at baseline (t = 0 h) were 29±3 s—44±8 s and the median value was 37±6 s. These values were similar to the values obtained in 25 healthy volunteers (range = 34±5 s—46±2 s; median value = 39±4 s).

The patients were treated with a 5000 IU bolus injection of intravenous heparin (porcine mucosa, Leo Pharmaceuticals, Copenhagen, Denmark) followed by a 3 h treatment with an alteplase, administered as an initial bolus injection of 10 mg, followed by a continuous infusion of 50 mg and 20 mg in each of the subsequent hours (total amount 100 mg). After the alteplase was given all the patients were treated with 1000 IU of heparin per hour for the next 21 hours.

The study was approved by the local ethics committee and performed according to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants before they entered the study.

**BLOOD SAMPLING**

Blood samples were collected from a venous cannula inserted into the forearm contralateral to the infusion arm before (t = 0 h), during (t = 2±25 h), and after (t = 4 h, 12 h, and 24 h) treatment with alteplase. Blood samples at 0 h were collected before heparin treatment. The first 10 ml of blood was discarded and blood samples for determination of TAT and F 1 + 2 were collected into 5 ml siliconised evacuated glass tubes containing sodium citrate (0.5 ml of 0.129 mol/l sodium citrate; Vacutainer A 3206 SBW-E, Becton Dickinson, Heidelberg, Germany) by a standard technique. Immediately after blood collection 10 μl of a 5·0 mmol/l solution of D-Phe-Pro-Arg-CH₂Cl (P-PACK, Calbiochem, San Diego, CA, USA) was added to prevent in vitro activation reaction. Plasma resistance to activated protein C was determined at baseline (t = 0 h) in citrated plasma without P-PACK and baseline plasma concentrations of protein C, protein S, protein C inhibitor, and plasminogen activator inhibitor type-1 were determined in EDTA-plasma without P-PACK. The blood samples were immediately placed on crushed ice until centrifugation (2000 g for 20 min at 4°C). Plasma specimens of 500 μl in plastic tubes were frozen and kept at −80°C until assay. Determination of plasma resistance to activated protein C is liable to errors if the plasma samples are contaminated with platelets. Thrombin generated from a coagulation load of at least 1000 g and a centrifugation time of 20 min or above this should be used, and plasma should be carefully sampled to avoid platelet contamination.

**ASSAYS**

Plasma concentration of TAT and F 1 + 2 were determined by enzyme-linked immunosorbent assays (ELISA; Behringwerke, Marburg, Germany) and expressed in μg/l and nmol/l, respectively. Plasma resistance to activated protein C was determined by a modified APTT test (Coastest, activated protein C resistance, Chromogenix, Mölndal, Sweden). Briefly, 50 μl plasma and 50 μl APTT-reagent (phospholipids and activator) were incubated at 37°C for 5 min. Fifty μl of CaCl₂ (APTT) or activated protein C/CaCl₂ (APTT+activated protein C) was added and the coagulation time was determined by the use of a Lode coagulometer (Groningen, The Netherlands). Activated protein C resistance ratio (APC-R) was calculated as the ratio of (APTT+activated protein C)/APTT. The analytic imprecision of the assay was examined at two levels by control plasma delivered by the manufacturer. The interassay coefficient of variance was 3±1% (mean APC-R = 3±32; SD = 0±10; N = 7) and 4±0% (mean APC-R = 1±90; SD = 0±08; N = 7).

Plasma concentration of protein C was determined by an enzyme-linked immunosorbent assay (ELISA) according to a previously described procedure and expressed relative (%) to pooled normal plasma. Plasma concentration of protein S was determined by electroimmunodiffusion using 1·2% polyclonal antibody against human protein S (Dako, Glostrup, Denmark) in a 1% agarose gel and expressed relative (%) to pooled normal plasma. Plasma concentration of protein C inhibitor was determined by electroimmunodiffusion using 1·5% antiserum against human protein C inhibitor (Nordic, TIlburg, The Netherlands) in a 1% agarose gel and expressed relative (%) to pooled normal plasma. Plasma concentration of plasminogen activator inhibitor type-1 antigen was determined by using an ELISA (Tintelleize, Biopool, Umeå, Sweden) and expressed in μg/ml. All assays were done in duplicate.

**STATISTICAL ANALYSIS**

We used non-parametric statistics to evaluate the results, because most of our data showed non-Gaussian distribution and our group of patients was small. The five serial measurements of TAT and F 1 + 2 were evaluated by Friedman’s two-way analysis of variance; when the results were significant, differences within the groups were evaluated by Wilcoxon’s signed rank sum test to compare appropriate time points. Box and whisker plots were used to present the serial measurements of TAT and F 1 + 2. The box is defined by the lower and upper quartiles and the solid square
in the box indicates the median value. The whiskers attached to the box indicate the minimum and maximum values. Otherwise, data are presented as median values with ranges in brackets. Spearman rank order correlation was used to test associations between two variables. To examine the relation between APC-R and the coagulation reaction products, we used two summary measures of the coagulation reaction products—that is, the average area under the curve in the 24-hour treatment period and the peak value after initiation of treatment.\textsuperscript{22} A P value < 0.05 was regarded as statistically significant.

**Results**

The baseline characteristics of the patients are presented in the table.

**COAGULATION ACTIVATION INDUCED BY THROMBOLYSIS**

The coagulation activation in the patients was reflected by significant changes in the plasma concentrations of TAT and F 1 + 2 in the first 24 hours after starting thrombolysis (figs 1 and 2). We observed a significant increase in the median plasma concentrations of TAT and F 1 + 2 in the first four hours after the start of thrombolysis followed by a decrease and return to the pretreatment concentrations.

![Figure 1: Box and whisker plot showing the response of the coagulation reaction product, thrombin-antithrombin III-complexes during the first 24 hours after the start of thrombolysis with 100 mg alteplase in 25 patients with ischaemic heart disease. *\textsuperscript{TAT}<br>\textsubscript{Ant} v TAT\textsubscript{Ant} \textsubscript{1}, P < 0.0002; **TAT\textsubscript{Ant} \textsubscript{1} v TAT\textsubscript{Ant} \textsubscript{2}, P < 0.0003. TAT, thrombin-antithrombin III-complexes.](http://heart.bmj.com/)

![Figure 2: Box and whisker plot showing the response of the coagulation reaction product, prothrombin fragment 1 + 2, during the first 24 hours after the start of thrombolysis with 100 mg alteplase in 25 patients with ischaemic heart disease. *\textsuperscript{F1 + F2, P < 0.0001; **F1 + F2, v F1 + 2, P < 0.0001. F 1 + 2, prothrombin fragment 1 + 2.](http://heart.bmj.com/)**

**ASSOCIATIONS BETWEEN APC-R AND THE COAGULATION REACTION PRODUCTS**

There was a negative correlation between APC-R and area under the curve of TAT (r\textsubscript{t} = − 0.60; P < 0.003), between APC-R and the peak value of TAT (r\textsubscript{t} = − 0.55; P < 0.005), and a negative correlation between APC-R and area under the curve of F 1 + 2 (r\textsubscript{t} = − 0.37; P = 0.07), between APC-R and the peak value of F 1 + 2 (r\textsubscript{t} = − 0.42; P < 0.04). The associations between APC-R and the coagulation reaction products are shown in figs 3 and 4.

**RELATIONS BETWEEN COMPONENTS OF THE PROTEIN C/S SYSTEM, PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1, AND APC-R**

In order to elucidate the mechanism regulating activated protein C, we also investigated whether the main components of the protein C/S-system or known inhibitors of activated protein C determined at baseline (t = 0 h) had any influence on APC-R. The median plasma concentration of protein C determined before the start of thrombolysis was 119% (96%–170%). There was no correlation between protein C and APC-R (r\textsubscript{t} = − 0.11; P = 0.62). The median plasma concentration of protein S determined before initiation of thrombolysis was 119% (96%–161%). There was no correlation between protein S and APC-R (r\textsubscript{t} = 0.01; P = 0.93), and there was a trend to a negative correlation.
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Figure 3 Scatter plot of the associations between activated protein C resistance ratio determined before initiation of thrombolysis with alteplase in 25 patients with acute ischaemic heart disease and the area under the curve of thrombin-antithrombin-III complexes in the first 24 hours of treatment ($r = -0.60$; $P < 0.003$) (A) and between activated protein C resistance ratio and the peak value of thrombin-antithrombin-III-complexes ($r = -0.55$; $P < 0.005$) (B). APC-R, activated protein C resistance ratio; AUC, area under the curve; TAT, thrombin-antithrombin-III-complexes.

Figure 4 Scatter plot of the associations between activated protein C resistance ratio determined before initiation of thrombolysis with alteplase in 25 patients with acute ischaemic heart disease and the area under the curve of prothrombin fragment $1 + 2$ in the first 24 hours of treatment ($r = -0.37$; $P = 0.07$) (A) and between activated protein C resistance ratio and the peak value of prothrombin fragment $1 + 2$ ($r = -0.42$; $P < 0.04$) (B). APC-R, activated protein C resistance ratio; AUC, area under the curve; F1 + 2, prothrombin fragment $1 + 2$.

between protein S and APC-R ($r = -0.39$; $P = 0.05$). The median plasma concentration of protein C inhibitor determined before initiation of thrombolysis was 99% (36%-163%). There was no correlation between protein C inhibitor and APC-R ($r = 0.06$; $P = 0.75$). The median plasma concentration of plasminogen activator inhibitor type-1 antigen determined before initiation of thrombolysis was 268 ng/ml (7.3 ng/ml–2260 ng/ml). There was no correlation between plasminogen activator inhibitor type-1 antigen and APC-R ($r = -0.15$; $P = 0.51$).

The median value of APC-R determined before initiation of thrombolysis and heparin treatment in our 25 patients with ischaemic heart disease was 3.19 (2.16–3.94). Our reference interval determined in 25 healthy volunteers was 2.99–4.78, with a median value of 4.03.

Discussion

Early reocclusion after successful reperfusion of the infarct related coronary artery may be a result of the coagulation activation caused by thrombolysis and it has been reported that plasmin is a key enzyme in this activation process. Thrombolysis also induces generation of anticoagulant endogenous activated protein C, which may put a brake on the degree of activation of the coagulation system. We have now shown that plasma resistance to activated protein C may be an important mechanism in the regulation of coagulation activation during thrombolysis with alteplase in patients with acute ischaemic heart disease.

Plasma resistance to activated protein C is determined as the anticoagulant response, measured by a modified APTT test, after a standard amount of activated protein C has been added to plasma. Addition of activated protein C to plasma enhances degradation of activated coagulation factor V and activated coagulation factor VIII and thereby prolongs the coagulation time. Plasma resistance to activated protein C is expressed as the APC-R, in which high values indicate low plasma resistance to activated protein C and low values indicate high plasma resistance to activated protein C.

ASSOCIATION BETWEEN APC-R AND COAGULATION ACTIVATION

In accordance with a number of previous studies, including our own pilot study, we observed that thrombolysis with alteplase caused an activation of coagulation as demonstrated by a significant increase in TAT and F1 + 2 (figs 1 and 2). The results of our present study indicate that it is a genuine plasma factor that determines the degree of coagulation activation during thrombolysis with alteplase. We observed a negative correlation between APC-R determined before thrombolysis with alteplase and area under the curve of TAT, the peak value of TAT and area under the curve of F1 + 2, and the peak value of F1 + 2 generated during the 24 hours treatment (figs 3 and
4). Probably this regulatory mechanism is of general importance, and not specifically related to treatment with alteplase, because we have observed a similar association between APC-R and activation of coagulation in six patients treated with streptokinase (results not shown). These negative correlations demonstrate that increased plasma activated protein C resistance (decreased APC-R) is associated with an increased coagulation activation during thrombolytic therapy. It is surprising that plasma resistance to activated protein C determines to such a degree the coagulation activation, but this observation supports the view of the protein C/S-system as a key anticoagulant system.23 Our observation of increased coagulation reaction products in patients with increased plasma resistance to activated protein C accords with recent reports suggesting an enhanced activation of coagulation in homozygous carriers of factor V Leiden point mutation.24

POSSIBLE CLINICAL IMPLICATIONS

The possibility of predicting the degree of coagulation activation caused by thrombolysis from APC-R determined before the start of therapy is interesting. Our results obtained by the serial measurements of TAT and F 1 + 2 demonstrate that patients with the lowest APC-R respond with the most excessive increase in TAT and F 1 + 2 during thrombolytic therapy (figs 3 and 4). This observation suggests that APC-R determined before the start of thrombolysis may be used to tailor antithrombotic treatment to the individual and that such a strategy may reduce the incidence of early reocclusion. It should be noted that plasma resistance to activated protein C is easily determined (within 30 min.) by a modified APTT test. There was no association between baseline APTT and the coagulation activation (results not shown) and to our knowledge our study is the first study to report that it may be possible to predict the degree of coagulation activation by determination of APC-R at baseline.

CAUSES OF INCREASED PLASMA RESISTANCE TO ACTIVATED PROTEIN C

Mutation in coagulation factor V is the most definitive cause of increased plasma resistance to activated protein C,25 but otherwise the physiological regulation of activated protein C in plasma is poorly understood. The mutation is particularly common among patients with venous thrombosis,26 whereas the prevalence of heterozygosity for the mutation of factor V Arg506 to Gln in patients with ischaemic heart disease is 5–6%.27–29 Because there were no cellular elements in our samples, we were not able to determine the prevalence of this mutation in our patients. However, it is unlikely that the prevalence is higher than previously reported because our patients had APC-R \( \geq 2.16 \), and most of patients with mutation in coagulation factor V have APC-R below 2.0.26

The mechanism of an enhanced APC-R as observed in the present study remains unknown but we have examined whether specific plasma proteins involved in the regulation of the protein C/S system had any influence on APC-R. There was no correlation between protein C and APC-R, whereas we found a trend to a negative correlation between protein S and APC-R. This association may represent a compensatory increase of protein S as a consequence of increased plasma resistance to activated protein C. Protein C inhibitor and plasminogen activator inhibitor type-1 are serpins which have the capacity to inhibit activated protein C,30,31 but it is not known whether these inhibitors are of clinical importance. We did not observe any association between protein C inhibitor and APC-R or between plasminogen activator inhibitor type-1 antigen and APC-R.

LIMITATIONS OF THE STUDY

Unfortunately, few of our patients had low values of APC-R. These patients in particular respond with marked activation of the coagulation system. Thus inclusion of more patients could strengthen the analysis of our results. However, our result is strengthened by the fact that the association was found with two variables (TAT and F 1 + 2) known to reflect the degree of coagulation activation and evaluated by two summary measures (area under the curve and peak value) and not single measurements.

In addition, it is important to stress that our study was designed to investigate the relation between APC-R and the coagulation activation after thrombolysis and not to evaluate any association with clinical endpoints. Therefore, there is a need for larger studies to investigate the relation between APC-R, coagulation activation, and clinical endpoints after thrombolysis.

CONCLUSION

In conclusion, we report a hitherto undescribed mechanism (plasma resistance to activated protein C) that is involved in the regulation the coagulation activation triggered by thrombolysis. This mechanism is inherent in human plasma and it is associated with resistance to activated protein C.

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**Notes**