Coagulopathy After Cardiac Surgery May Be Influenced by a Functional Plasminogen Activator Inhibitor Polymorphism

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BACKGROUND: Cytokine-mediated inflammation and coagulopathy may occur after cardiac surgery. In this study we investigated the temporal pattern of plasminogen activator inhibitor-1 (PAI-1) gene expression after cardiac surgery and its relation with PAI genotype, and obtained preliminary data regarding its relation to perioperative morbidity.

METHODS: The relative change in PAI-1 mRNA 1, 6, and 24 h after cardiopulmonary bypass (CPB) was measured from mononuclear cells in 82 patients undergoing elective cardiac surgery. DNA was analyzed for carriage of the 4G/5G PAI-1 polymorphism.

RESULTS: PAI-1 gene expression decreased after CPB in all patients. A larger reduction in PAI-1 gene expression was observed in homozygous carriers of the 5G allele. Homozygous carriers of the 5G allele were also more likely to receive transfusion of coagulation blood products. There was no relation between change in PAI-1 gene expression and duration of CPB.

CONCLUSIONS: PAI-1 gene expression decreased over time after CPB. We found a link between PAI-1 genotype, PAI gene expression, and transfusion of coagulation products after cardiac surgery.

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Originally isolated from human endothelial cells, plasminogen activator inhibitor-1 (PAI-1) functions primarily as an inhibitor of endogenous tissue plasminogen activator (tPA). A variety of cell types, including mononuclear and vascular smooth muscle cells, contribute to PAI-1 production. It is plausible that increased production of PAI-1 induces a prothrombotic state by inhibiting tPA-dependant plasmin production and, thus, fibrinolysis. Indeed, susceptibility to thrombosis has been confirmed in the setting of meningococcal septicemia, trauma, and deep vein thrombosis (1–3). Additionally, there are clinical data associating deficiencies in PAI-1 production with an increased likelihood of hemorrhagic events (4–6).

One explanation for interindividual variability in PAI-1 levels might be PAI-1 genotype. A common functional insertion/deletion (4G/5G) polymorphism has been described in the promoter region of the PAI-1 gene with homozygosity for the 4G variant associated with greater basal PAI-1 transcription and the 5G allele with lower levels of PAI-1 (7). The clinical relevance of PAI-1 allelic variability is further underscored by studies in meningococcal disease demonstrating increased mortality rates in patients homozygous for the PAI-1 4G variant (8). There is additional evidence linking this variant to increased PAI-1 protein levels, which themselves independently predict mortality (1). There is little data, however, on the implications of PAI-1 gene expression and PAI-1 genetic variants in clinical situations involving significant hemodynamic perturbations, blood loss, and vigorous activation of coagulation and inflammatory pathways. This scenario is typical of cardiac surgery using cardiopulmonary bypass (CPB).

The purpose of this study was to examine the temporal pattern of changes in PAI-1 messenger RNA (mRNA) in mononuclear cells in patients undergoing cardiac surgery with CPB, to determine whether PAI 4G/5G gene polymorphism influences PAI-1 mRNA expression, and to obtain preliminary information on the relation between PAI-1 mRNA and clinical outcomes.

METHODS

Patient Population

All procedures of the study were approved by our institutional ethics committee and written informed consent was obtained from each patient before inclusion into the study. Eighty-two patients scheduled for elective cardiac surgery using CPB were recruited for a study examining perioperative changes in cytokine

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mRNA (9). The current investigations were performed on stored blood samples from the latter study. Exclusion criteria included: renal impairment as defined by a creatinine >150 μmol/L, history of liver disease, coagulation disorders as defined by international normalized ratio >1.5, hematological malignancies, coronary artery bypass graft surgery without CPB, emergency surgery, and use of corticosteroids or anticoagulant drugs before surgery.

Clinical Care

Our perioperative clinical care has been described (9), which in brief, included anesthesia with propofol, fentanyl, pancuronium, and isoflurane. Nonpulsatile CPB was performed using a membrane oxygenator (Cobe, Denver, CO) with flow maintained between 2.0 and 2.4 $L \cdot min^{-1} \cdot m^{-2}$. Body temperature was kept between 32°C and 35°C during CPB. Heparin 3 mg/kg was given to obtain an activated clotting time of >480 s before CPB and reversed after CPB with protamine (1:1 ratio of protamine with heparin).

Messenger RNA Analysis

Blood samples were obtained for mRNA analysis at three time points: (a) before anesthesia induction, (b) 1 h, and (c) 6 h after discontinuing CPB. A 24-h sample was taken from a subgroup of patients who remained in the intensive care unit at 24 h. Mononuclear cells were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) using a standard protocol (10,11). The mRNA was extracted and quantified using real-time polymerase chain reaction (PCR) as previously described (9,12,13). In brief, mRNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany). Concentration and purity of RNA were determined by measuring the absorbance at 260 and 280 nm on a spectrophotometer (Eppendorf, Biophotometer). Total RNA was reversed transcribed to produce complementary DNA (cDNA) using a murine moloney leukemia virus reverse transcriptase. Reverse transcription was performed at 37°C for a duration of 60 min in a final volume of 30 μL containing: 500 ng sample RNA, 1.25 μL (250 U) murine moloney leukemia virus reverse transcription (Promega,) with 2 μL random hexamers ($pd(N)_6$), 0.2 mM dNTP's, 0.6 μL RNAsin 1 U/μL, 10 mM dithiothreitol, 4.5 μL 11% DMSO and 6 μL 5× RT buffer (250 mM Tris–HCL, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$). cDNA was used as a template for PCR. Quantification of mRNA using group-specific primer pairs and FAM probes (Assay by Demand, Applied Biosystems Perkin Elmer, Foster City, CA) for the detection of PAI-1 was carried out on an ABI PRISM GeneAmp 7000 Sequence Detection System. Real-time quantification was performed under the following conditions: 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Normalization of cDNA templates was achieved by glyceraldehyde-3-phosphate dehydrogenase quantification. The comparative threshold cycle (C_T) method of quantification was used comparing time and baseline (ABI Prism 7700 Sequence Detection System User Bulletin 2, PE Applied Biosystems).

In short, gene expression is measured as increased fluorescence corresponding to amplification of the target mRNA. The cycle in which fluorescence exceeds the set detection threshold is termed the "threshold cycle" (C_t). The higher the starting quantity of the target mRNA, the earlier a significant increase in fluorescence, and the smaller the C_t value obtained. The C_t values of the timed samples (i.e., 1, 6, and 24 h) were compared to the preoperative baseline sample to determine the proportional change in mRNA after CPB. This methodology does not provide absolute quantification of mRNA at any time point. This method has been previously described (13–15).

DNA Analysis

A second blood sample before anesthesia induction was obtained for DNA analysis of the 4G/5G PAI polymorphism. Taqman technology was used to genotype the PAI-1 4G/5G polymorphism. Fluorescent dye-labeled Taqman MGB probes were designed using Assay by Design (Applied Biosystems/Perkin Elmer, CA). The primer sequences were as follows: Forward Primer 5′-AGCCAGACAAGGTTGT TGACA-3′, Reverse Primer 5′ GCCGCCTCCGATGATACAC-3′. The fluorescent probes were as follows: Allele 1 Probe 5′-CTGACT***CCCC***ACGTGT-3′, *5′Fluor Label,6-FAM*, Allele 2 Probe 5′-CTGACT***CCCCC***ACGTGT-3′ *5-Fluro Label, VIC.* PCR amplification was performed using 12.5 μL of Taqman universal PCR master mix (Applied Biosystems/Perkin Elmer), 0.2 μM of FAM probe, 0.2 μM of VIC Probe, 0.9 μM of forward primer, 0.9 μM of reverse primer, and 9.875 μL of H_2O. The cycling conditions consisted of a denaturation step at 95°C for 10 min, 40 cycles at 92°C for 15 s, a 60-s annealing step for 40 cycles at 62°C, and finally a holding temperature of 15°C. Allelic discrimination was determined using an ABI prism 7000 sequence detection system (Applied Biosystems/Perkin Elmer). The results of this test indicated whether patients were homozygous (i.e., 4G/4G, 5G/5G) or heterozygous (i.e., 4G/5G) for these commonly occurring polymorphic alleles based on the binding of the fluorescent dye labeled Taqman probes.

Outcome Measures

Lactate levels were measured routinely from arterial blood specimens every 4–6 h after surgery. Hyperlactatemia was considered significant when lactate levels exceeded 4 mmol/L on any sample within 24 h after CPB. Need for vasoconstrictor drugs (norepinephrine/epinephrine) to maintain the systemic mean blood pressure >70 mm Hg within 24 h was also recorded. There was no algorithm for administration of vasoactive drugs. Hemoglobin levels and platelet levels were measured immediately postoperatively and at 12 h after CPB. Additional levels were obtained within 24 h, when considered necessary by the physician caring for the

Table 1. Patient Demographics and Operative Details

No. of patients	$N = 82$
Age (yr)	63 ± 1.1
Sex (M:F)	62:18
Body surface area (m^2)	1.91 ± 0.02
Weight (kg)	80.1 ± 1.5
Hypertension	37 (45)
Prior myocardial infarction	27 (33)
Diabetes mellitus	13 (15)
Prior aspirin	53 (65)
Prior clopidogel	13 (15)
Tranexamic acid	43 (52)
Aprotinin	3 (4)
CABG	57 (70)
Valve	20 (24)
CABG&valve	5 (6)
CPB duration (min)	102 ± 3.7
Aortic cross clamp duration (min)	62 ± 2.8
Heparin (mg)	279 ± 7.7
Protamine (mg)	283 ± 7.0
Chest tube blood loss mL (0–24 h)	1334 ± 70
Hemoglobin g/dL	8.48 ± 0.16
Platelet count $1000/mm^3$	142 ± 5.1
Deaths	1

Values quoted as mean ± SE for continuous variables, percentages for categorical data in parentheses.

M:F = male:female; CABG = cardiopulmonary bypass graft; CPB = cardiopulmonary bypass.

patient. Administration of packed red blood cells, hemostatic blood products, and antifibrinolytic drugs were determined by the clinician caring for the patient independent of the investigators and blinded to PAI-1 genotype or other data.

Data Analysis

The relative change in PAI mRNA at each time point compared with baseline is expressed as median with interquartile range. The difference in comparative changes in PAI-1 mRNA was analyzed by Wilcoxon's signed rank test for comparison of matched pairs. Patients were categorized according to requirement for vasoconstrictor infusion, the occurrence of hyperlactatemia, and the association between PAI gene expression. These patient groups were compared using Wilcoxon's ranked sum test. The distribution of PAI-1 alleles was analyzed by χ^2 or Fisher's exact test as appropriate. The relation between postoperative blood loss and requirement for transfusion of coagulation products, the carriage of specific PAI-1 alleles, and changes in PAI-1 mRNA were analyzed by Wilcoxon's ranked sum test and Spearman rank correlation coefficient.

A multivariate analysis (logistic regression) to assess the predictors for transfusion of coagulation products was performed. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic information is presented in Table 1. Thirty-two percent of the 82 patients required vasoconstrictor drugs (either norepinephrine or epinephrine) to maintain a mean arterial blood pressure of 70 mm Hg in the first 24 h after CPB. Twenty-four

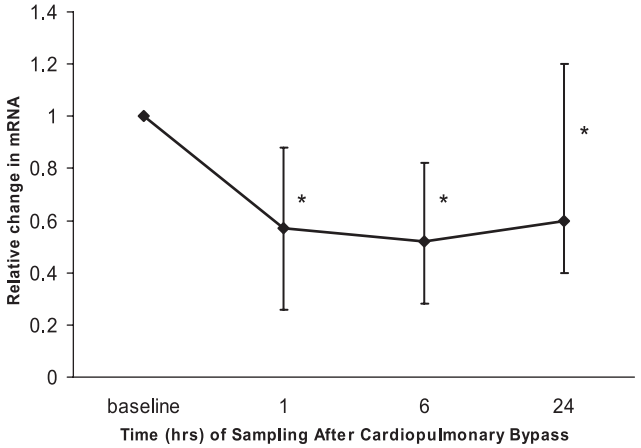

Figure 1. Proportional change from baseline in plasminogen activator inhibitor-1 (PAI-1) mRNA 1, 6, and 24 h after cardiopulmonary bypass (CPB). The values are expressed as median and interquartile range. Comparisons were made with Wilcoxon's signed rank test.

percent of patients developed hyperlactatemia within 24 h of CPB. Three patients received aprotinin and 43 patients received tranexamic acid in the perioperative period. No patient received both antifibrinolytics.

Samples were available for mRNA analysis from all 82 patients at baseline, in 81 patients 1 h after CPB, in 79 patients at 6 h after CPB, and in 26 patients 24 h after CPB. Overall PAI-1 gene expression decreased over time (Fig. 1). Compared with baseline (where mRNA was assigned a value of 1), PAI-1 mRNA 1 h after CPB was 0.57 (0.26–0.88) (median, interquartile range, $P = 0.002$). At 6 h post-CPB PAI-1 mRNA was 0.52 (0.28–0.82) ($P = 0.002$ compared with baseline). At 24 h post-CPB, PAI-1 mRNA was 0.57 (0.39–1.15), ($P = 0.002$, compared with baseline). There was no association between change in PAI-1 gene expression post-CPB and requirement for vasoconstrictors and hyperlactatemia ($P = 0.9$).

The frequencies of the 4G and 5G alleles were 0.54 and 0.46, respectively. Sixteen percent of the patients were homozygotes for the 5G variant, 23% were homozygous for the 4G variant, and 61% were heterozygotes. The allelic distribution was in Hardy Weinberg equilibrium and conformed to previously reported frequencies in a Caucasian population (16). Patients were further dichotomized into quartiles to identify those with the highest PAI-1 gene expression after the CPB period relative to baseline (Table 2). The upper quartile was compared to the lower three quartiles. An association was found between PAI-1 genotype and PAI-1 mRNA production: no patient was homozygous for the 5G genotype but 21% of those homozygous for the 4G allele had PAI-1 mRNA in the upper quartile 1 h after CPB ($P = 0.01$). However, this association was not observed at the 6 h or 24 h time point after CPB.

Nine patients received transfusion of coagulation blood products after CPB (Table 3). Five received both fresh frozen plasma and platelets and four received

Table 2. 4G/5G Genotype Frequencies in Relation to PAI-1 mRNA 1 h After Cardiopulmonary Bypass

Population based on PAI mRNA level at 1 h	*N*	5G/5G	4G/5G	4G/4G	*P*
N		13	49	19	
High PAI mRNA	20	0 (0)	16 (33)	4 (21)	
Low PAI mRNA	61	13 (100)	33 (67)	15 (79)	0.01*

Comparisons by χ^2 test. *N* = number of patients, PAI-1 = plasminogen activator inhibitor-1, mRNA = messenger RNA.

High PAI mRNA refers to patients in the group with the highest quartile of PAI-1 mRNA values. Low PAI mRNA refers to the lowest three quartiles.

The values in parentheses are percentages of each genotype.

Table 3. Comparison of 5G/5G Genotype to Coagulation Blood Products Transfused

Homozygous for 5G	Yes	No	p value
N	13	69	
Chest tube blood loss in 24 h after CPB (mL)	1594 ± 174	1285 ± 75	0.18
Received coagulation products	5 (38)	4 (6)	0.003
Hemoglobin level (g/L)	83 ± 4	85 ± 2	0.6
Platelet level × 10^9/l	144 ± 12.9	141 ± 5.6	0.8

N = number of patients. Hemoglobin levels and platelet levels = minimum levels obtained within 24 h postcardiopulmonary bypass (CPB). Values quoted as number of patients with percentages in parentheses for categorical data. Values quoted as mean ± SE for continuous variables.

platelets only. Patients homozygous for 5G were more likely to receive blood coagulation products than those who were not ($P = 0.003$). In a multivariate logistic regression analysis of the requirement for transfusion of coagulation products which included PAI-1 genotype, PAI-1 mRNA values, use of antifibrinolytics, and duration of CPB only PAI-1 5G homozygous status was associated with transfusion of coagulation products ($r^2 = 0.16$, $P = 0.003$; odds ratio = 10; 95% confidence interval 2.3–50; and area under a receiver operator characteristic curve 0.722).

DISCUSSION

In this observational study of elective cardiac surgery patients, we demonstrate a consistent decrease in PAI-1 mRNA production after CPB with the extent of the decrease related to PAI-1 genotype. In addition, we describe an association between PAI-1 4G/5G genotype and a propensity to receive coagulation blood products early after cardiac surgery.

Previous studies measuring PAI-1 concentrations after cardiac surgery have produced inconsistent results (17–19). Valen et al. (19) found that CPB was associated with an increase in tPA and a decrease in PAI-1 levels. In contrast, Chandler et al. (20) noted a heterogeneous fibrinolytic response after CPB with either an increase or no change in PAI-1. The inconsistency may be partly explained by the assays used. PAI-1 protein levels in plasma can be assayed as either PAI-1 antigen or PAI-1 activity. However, both methods lack sensitivity (21,22), and consequently there is no "gold standard" methodology of PAI-1 quantification. Quantification of changes in mRNA production, using the technique of real time-PCR, is an exquisitely sensitive measure of gene expression in comparison to the protein assays (23). Additionally, real-time-PCR may be particularly applicable when assessing the influence of genetic variation on gene expression, as the mRNA product is not affected by post-transcriptional modifications as is the case with the protein product. Furthermore, reduced gene expression might be difficult to quantify with a protein-based assay, as inhibition of gene expression may result in undetectable protein levels.

Although mononuclear mRNA quantification may not directly reflect protein levels, a change in transcription may indicate a concurrent change in protein levels. Additionally, our results are not affected by dilutional changes common in patients undergoing CPB, as we extracted RNA from a predetermined number of mononuclear cells (5×10^6) isolated from each blood sample.

The 4G/5G polymorphism at position −675 in the promoter region of the PAI-1 gene has been reported to influence basal levels of PAI-1. Patients carrying the 4G allele have a 20% higher basal level of PAI-1 than those carrying the 5G allele (24). Our methods of measuring change in mRNA without an absolute measurement of mRNA concentrations does not permit a comparison of absolute PAI-1 mRNA levels with previously published PAI-1 protein concentrations. However, the association between carriage of the PAI-1 5G allele and change in PAI-1 mRNA, with greater reduction in PAI-1 mRNA in carriers of the 5G allele, may be an equivalent finding. This is in concordance with previous studies demonstrating lower PAI-1 levels in patients homozygous for 5G (24,1).

PAI-1 is a prothrombotic protein, with lower PAI-1 levels associated with a bleeding diathesis (25,26). Thus, the lesser PAI-1 gene expression observed in this study would seem more consistent with the propensity towards excessive bleeding commonly seen after cardiac surgery (27). We have also described homozygosity for the 5G variant and an increased tendency to receive procoagulant blood products. Although we did not demonstrate an association between genotype and the volume of perioperative blood loss, platelet, or hemoglobin levels, it is likely that patients demonstrating clinical bleeding were treated early and appropriately, therefore, avoiding larger blood loss or postoperative anemia. Of note, we were unable to link change in PAI-1 mRNA levels with blood coagulation transfusion or bleeding indices. Consistent with current practice, antifibrinolytic drugs were used in many patients in this study. These drugs, which inhibit plasminogen activation, do not

influence PAI-1 gene expression, but their administration may have obscured any linkage between PAI-1 gene expression and overt bleeding.

This study has limitations, including the fact that we only measured PAI-1 mRNA in mononuclear cells, which may not reflect gene expression in other tissues. Previous studies have shown that other cell types, in addition to monocytes/macrophages, produce PAI-1 (28,29). However, sequential sampling of peripheral blood is the only practical method available at present to serially test gene expression over a short interval in a dynamic environment such as exists after cardiac surgery. Further study investigating the role of other cell types in the PAI-1 gene expression may be warranted.

Postoperative bleeding is a common complication after cardiac surgery. It is often difficult to delineate the factors contributing to this condition. Our study describes a decrease in PAI-1 mRNA levels after CPB which may contribute to the increased tendency for bleeding perioperatively. In addition, this study also suggests an association between PAI-1 4G/5G genotype and a propensity to develop coagulopathy during early recovery after cardiac surgery.

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