Tissue factor pathway inhibitor on circulating microparticles in acute myocardial infarction

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Summary
In acute myocardial infarction (AMI), increased Tissue Factor (TF) expression on circulating monocytes and microparticles (MP) may contribute to thrombotic events. Because surface-bound Tissue Factor Pathway Inhibitor-1 (TFPI) inhibits TF activity on monocytes and endothelial cells decreased TFPI expression may reinforce the procoagulant activity of circulating MP. Aim of the study was to analyze TFPI expression and TF activity after stenting and thrombolysis in AMI. Thirty-nine patients of a randomized study comparing intravenous thrombolysis (n = 19) and stenting (n = 20) were included. Before and after therapy blood samples for analysis of MPs, TF antigen and activity, prothrombin fragment F1+2 and D-dimer were obtained. TFPI expression on TF positive MPs was decreased after thrombolysis but not after stenting. In contrast, TF plasma levels and TF positive MP remained unchanged in both treatment groups. After thrombolysis increased D-dimer and F1+2 plasma concentrations indicated activation of fibrinolysis and coagulation. Significance of MP TFPI for inhibition of TF activity was measured using inhibitory TFPI antibodies. Membrane-associated TFPI inhibited TF activity on circulating MPs. After thrombolysis inhibition of TF activity by TFPI was decreased as compared to stenting. Correlation of circulating TF with F1+2 only after thrombolysis, suggests a role for TF-induced activation of coagulation after thrombolysis. Enhanced TF activity on circulating MPs in AMI is inhibited by endogenous surface-bound TFPI. After thrombolysis but not after stenting MP TFPI is degraded and may induce thrombin generation due to unopposed tissue factor activity. Anti-TF therapies during thrombolysis may reduce thrombin generation in AMI.

Keywords
Tissue factor pathway inhibitor (TFPI), Ischaemic heart disease, hypercoagulability

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Introduction

In acute myocardial infarction (AMI) early reperfusion is the treatment of choice. The use of thrombolytic agents restores coronary artery patency only in about two thirds of patients with AMI. In contrast, mechanical reperfusion with primary angioplasty and stenting achieves higher patency rates (1).

Tissue Factor (TF)-mediated activation of the coagulation cascade plays a key role in intravascular thrombus formation and is inhibited by tissue factor pathway inhibitor-1 (TFPI). On cell membranes endogenous TFPI supports transient downregulation of the quaternary TF-VIIa-Xa-TFPI complex (2). In a previous study we have shown a decrease in the anticoagulant activity of TFPI-1 on circulating monocytes after thrombolysis (3). As TF expression on monocytes is increased not until 96 h after coronary intervention (4), decreased TFPI expression on monocytes may already be restored and, therefore, not alter thrombin formation.

Increased levels of circulating TF have been reported in patients with acute coronary syndromes and may contribute to subsequent thrombotic complications (5).

Independently of monocytic TF, blood-borne TF may play a role in the propagation of thrombosis (6). Circulating TF consists of soluble alternatively, spliced forms (7) and procoagulant microparticles (MP) (8) that may support thrombus formation. MPs are small membrane vesicles, released from blood cells or endothelial cells on activation or during apoptosis (8) and support coagulation by exposure of negatively charged phospholipids and TF. If inhibition of TF activity by endogenous TFPI occurs on procoagulant MP early in AMI, degradation of TFPI by

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thrombolysis might enhance TF activity, activate the coagulation cascade and, thereby, contribute to reocclusion after thrombolysis in AMI.

The aim of this study, therefore, was to analyze in AMI TF and TFPI expression on circulating MPs after thrombolysis and stenting and to investigate the role of circulating TF for thrombin formation *in vivo*.

Methods

Patient selection

We included 39 patients from a randomized study that compares fibrinolysis with alteplase with stenting plus abxicimab (STOPAMI I) (9). The study was approved by the institutional ethics committee for human subjects. Informed consent was obtained from all patients. All patients received 500 mg of aspirin and 5,000 U of heparin intravenously in the emergency room. After randomization patients received treatment with alteplase (n = 19) or stenting with abxicimab and ticlopidine (n = 20) as described (3). Serial venous blood samples were obtained before and 24–48 h after therapy. All blood samples were put on ice and processed immediately, as indicated below.

Microparticle isolation

MP were isolated by sequential centrifugation as described (10). Briefly, sodium citrate blood samples were centrifuged at 1,500 g for 15 min, followed by 1 min decantation at 13,000 g. Aliquots of 20 µl of the platelet-free plasma were diluted in 180 µl of filtered PBS containing 1 mM $CaCl_2$.

Flow cytometry

MP suspensions were incubated with the following antibodies: Fluorescein isothiocynate (FITC)-conjugated anti-TF, anti-TFPI mAb (American Diagnostica, Pfungstadt, Germany), washed and incubated with phycoerythrin (PE)-conjugated Fab fragments (Dako) for indirect TFPI-1 labeling. Phosphatidylserine probing was performed using annexin V Alexa 568 in appropriate buffer (Boehringer Mannheim). Incubation with irrelevant mAb was used as a control. To investigate the cellular origin of TF on MP costaining with anti-P1H PE (Chemicon, Marseille, France), anti-CD15 FITC, anti-CD14 PE and anti-CD62 FITC (Beckman Coulter, Krehfeld, Germany) was performed (n = 3). MP were identified according to their characteristic forward and side scatter and by their ability to bind annexin V and cell-specific antibodies. For quantification a known number of latex beads (LB30, Sigma-Aldrich, Schnelldorf, Germany) was added to the sample before analysis. The diameter of the beads (3 µm) discriminated them from the MP population. Analysis was stopped when 200,000 beads were counted and the amount of corresponding MP was analyzed. The number of TF-positive or TF-TFPI-1-double-positive MP in 1 µl of plasma was calculated according the following formula: (MP events/latex beads events) x (latex beads per tube/sample volume).

TFPI-1 and D-dimer immunoassay

Plasma concentrations of TF, TFPI-1, F1+2 and D-dimer were determined by sandwich type immunoassay (IMMUBIND TF and TFPI ELISA, American Diagnostica; Enzygnost F1+2, Enzygnost D-dimer, Dade Behring, Marburg, Germany). The inter-assay variability in the lower assay range were <14%. To inhibit endogenous TFPI samples were preincubated for 60 min with 100 µg/ml of a monoclonal inhibitory anti-TFPI antibody (W. Ruf, San Diego, CA).

TF activity was measured using a FXa generation assay in the absence and presence of 100 µg/ml rabbit anti-TFPI-1 IgG (kindly provided by Dr. W. Ruf) or nonimmune rabbit IgG (Sigma) as described (4). The inter-assay variability was 10%. TF activity in the presence of anti-TFPI mAbs represent the total capacity of TF activity in the plasma because endogenous inhibition is abolished. In the absence of TFPI antibodies TF activity is decreased according to the amount of endogenous TFPI. The TF activity that is regulated by endogenous TFPI is given as a percentage value relative to the total cellular TF, defined by the following formula: 100 x (TF activity in the presence of anti-TFPI mAbs–TF activity in the absence of anti-TFPI mAbs)/TF activity in the presence of anti-TFPI mAbs.

Statistical analysis

Differences between more than 2 matched samples were tested by Friedman's test followed by Wilcoxon's matched-pairs signed-ranks test, and differences between the groups by the Mann-Whitney-Wilcoxon rank sum test. A $p<0.05$ value in the two-tailed test was regarded as significant.

Results

Clinical and angiographic data

Patient groups did not differ significantly in sex, age, risk factor profile, medication or infarct size and location (Table 1). Stenting was successful in all patients restoring TIMI grade 3 flow.

Table 1: Baseline characteristics of study patients.

	Thrombolysis (n=19)	Stent (n=20)	P
Gender (M/F)	15/4	16/4	0.7
Age, y, (range)	61±16	60±11	0.9
Active smokers, n(%)	9 (47)	10 (50)	0.7
Hypercholesterolemia, n(%)	12 (63)	14 (70)	0.7
Systemic hypertension, n(%)	11 (58)	14 (70)	0.5
Diabetes mellitus, n(%)	2 (11)	3 (25)	0.7
1-vessel disease	6 (32)	9 (45)	0.5
2-vessel disease	4 (21)	7 (35)	0.3
3-vessel disease	8 (42)	4 (20)	0.1
Target vessel:			
LAD	6 (32)	12 (60)	0.1
LCx	5 (16)	3 (15)	0.6
RCA	6 (32)	5 (25)	0.9
peak CK, U/L (range)	1140 (268–4630)	1383 (230–5962)	0.9

CK indicates creatine kinase. LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery; RCA, right coronary artery.

From the 19 patients that received thrombolysis 16 underwent coronary angiography within 2 weeks and in 5 patients occlusion of the coronary artery was found corresponding to the infarct area. Therefore, in these patients non-successful thrombolysis may be assumed.

TF and TFPI expression on circulating MP

After thrombolysis a significant decrease of surface-bound TFPI on TF positive MP was observed, whereas, no changes occurred after stenting. A representative FACS analysis is shown in figure 1: Figure 1A shows the characteristic light scatter properties of MP`s and latex beads that were used as an internal standard while figure 1B demonstrates an example of TF-positive MP. In figure 1C-D the TFPI expression on TF-positive MP`s after thrombolysis and stenting in AMI are shown in representative histograms.

The effect of thrombolysis on the number of TF-positive and the number of TF and TFPI-positive circulating MP`s was compared to the effect of stenting. After thrombolysis a significant decrease in TF and TFPI-positive MP was found, whereas after stenting no significant changes occurred (Fig. 2A). The amount of TF containing MP`s did not differ in both treatment groups either before nor after the treatment (Fig. 2B).

Thrombin generation and fibrin degradation *in vivo*

The extent of fibrinolysis in AMI was measured by analysis of D-dimer plasma levels, activation of the coagulation cascade by analysis of prothrombin fragment F1+2. As expected after thrombolysis a significant increase in circulating D-dimer plasma levels was found, whereas after stenting no changes occurred (Fig. 3A), documenting a strong activation of the fibrinolytic system following alteplase administration. Compared to stenting thrombolysis was associated with higher prothrombin fragment F1+2 plasma levels by trend (Fig. 3B). This, consequently, indicates thrombin generation and may reflect the prothrombotic effects of thrombolytic treatment (19).

Circulating TF antigen and activity

To investigate, whether the amount of circulating TF is altered during thrombolysis or stenting, we measured plasma concentrations of TF. No effect of either therapy was found (Fig. 4 A). Because endogenous TFPI associated with MPs inhibits TF activity, we analyzed total TF activity in the presence and the absence of anti-TFPI antibodies. We measured the residual TF activity as the TF activity that remains after inhibition of endogenous TFPI. The difference between total and the residual activity reflects the TF activity that is inhibited by TFPI and, therefore, reflects the functional significance of endogenous TFPI for the inhibition of TF activity. After thrombolysis the amount of TF that was inhibited by TFPI was significantly reduced, whereas after stenting no changes were found. These results demonstrate the functional significance of the TFPI decrease on circulating MP`s after thrombolysis.

Relationship of circulating TF and thrombin generation *in vivo*

To investigate, whether decreased TFPI on circulating MP`s indeed results in a imbalance between the TF and TFPI pathway potentially contributing to thrombin generation *in vivo* we ana-

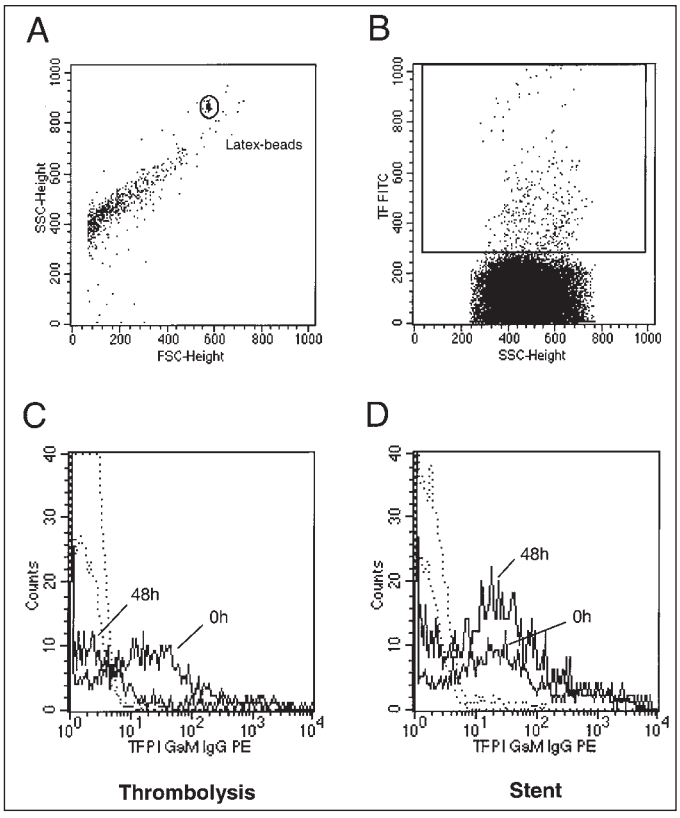

Figure 1: Flow cytometric quantification of TF positive MP. MP are discriminated by size according to the forward and side scatter. Quantification was performed using a defined number of latex beads (A). Detection of TF positive MP after staining with anti-TF FITC antibodies in relation to the side scatter properties (B). Representative histogram of TF positive MP from samples obtained before and after thrombolysis (C) and histogram of TF positive MP from samples obtained before and after stenting (D).

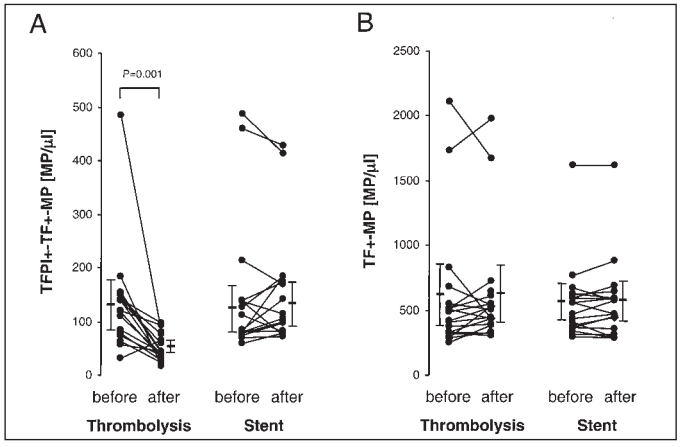

Figure 2: TFPI expression on circulating TF positive MP is decreased after thrombolysis in AMI. The number of circulating TF and TFPI positive MP (A) or TF positive MP (B) were measured before and after thrombolysis and stenting in AMI. Shown are the individual values from each patient. P values reveal statistical significance.

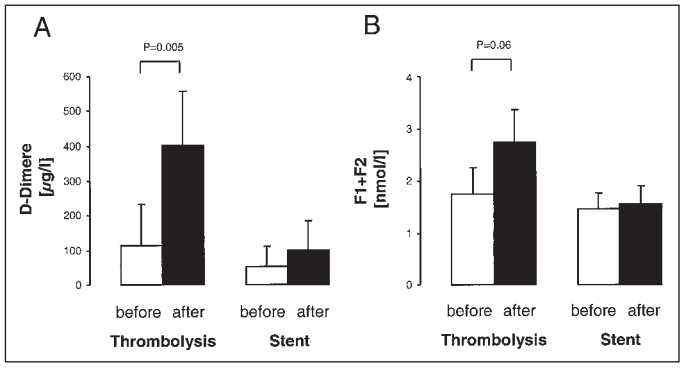

Figure 3: D-dimer and prothrombin fragment F1+2 are increased after thrombolysis in AMI. Plasma concentrations of D-dimer (A) or prothrombin fragment F1+2 (B) were measured before and after thrombolysis and stenting in AMI. Values are expressed as mean ± SEM. P values reveal statistical significance.

lyzed the association of circulating TF with plasma F1+2 concentrations. After thrombolysis there was a significant correlation with TF plasma levels and F1+2 concentrations (Fig. 5). No association was found in patients after stenting in AMI ($R = -0.4$, $P = 0.59$). Furthermore, no correlation was observed before therapy in both treatment groups. These data are an indication for an ongoing TF induced clotting activation following thrombolysis *in vivo*.

Relationship between TFPI degradation and failure of thrombolysis

To analyze if degradation of TFPI is associated with successful thrombolysis patients that angiographically showed an occluded vessel 2 weeks after acute myocardial infarction were compared with patients that showed an open vessel (Table 2). No significant differences in TF positive MP, TF and TFPI positive MP, TF antigen or TF activity regulated by TFPI were detected in both groups after thrombolysis. Furthermore, activation of coagulation and fibrinolysis as assessed by prothrombin fragment F1+2 and D-dimer was similar.

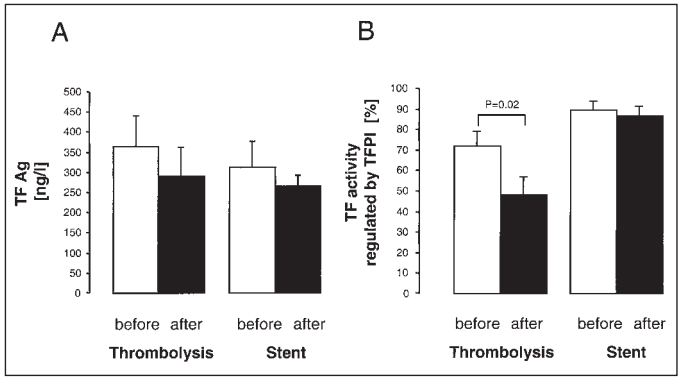

Figure 4: Decreased inhibition of TF activity by TFPI after thrombolysis in AMI. Plasma TF Antigen (A) and TFPI dependent inhibition of TF activity (B) were measured before and after thrombolysis and stenting in AMI. Values are expressed as mean ± SEM. P values reveal statistical significance.

Discussion

The major findings of our study are:

1. After thrombolysis in AMI TFPI expression on TF positive MP is decreased, whereas, after stenting no changes were observed.
2. Functional significance of this decreased TFPI expression was shown by a diminished inhibition of TF activity by TFPI after thrombolysis as compared to stenting.
3. The correlation of circulating TF with prothrombin fragment F1+2 after thrombolysis only, suggests a role for TF-induced activation of coagulation after thrombolysis.

Plasma TFPI circulates in various truncated forms mostly bound to lipoproteins. However, the majority of TFPI is produced by endothelial cells and remains associated with the endothelial surface (11). Cell-bound TFPI is believed to play a critical role in regulating surface TF-FVIIa and FXa activity by transient and reversible translocation of the quaternary complex TF-FVIIa-FXa-TFPI into glycosphingolipid-rich microdomains (2, 4). This mechanism occurs on the surface of endothelial cells as well as on monocytes. Among others, MP derive from monocytes and endothelial cells (10, 12). Thus, it seems conceivable that similar regulatory mechanisms occur on the MP surface as on intact cells. Recombinant TFPI has been shown to be proteolytically degraded by plasmin, elastase and matrix metalloproteinases (13–15). Moreover, in a cell-based system we and others have shown that TFPI is degraded on endothelial cells in vitro and on circulating monocytes after thrombolysis *in vivo* (3, 21, 22). Degradation of MP and cell associated TFPI after thrombolysis may, therefore, attenuate an important pathway of vascular surface thromboresistance. Whether diminished TFPI contributes to thrombin formation, or whether decreased TFPI occurs as an epiphenomenon has not been addressed so far. Monocytic TF is induced during the cause of AMI but occurs only 96 h after recanalization of the infarct-related coronary artery by stenting (4, 16). Thus, the early thrombin generation after thrombolysis, observed in this study, cannot be attributed to monocytic TF activity. Previous studies have shown an increased number of circulating procoagulant MP in acute coronary syndromes (8).

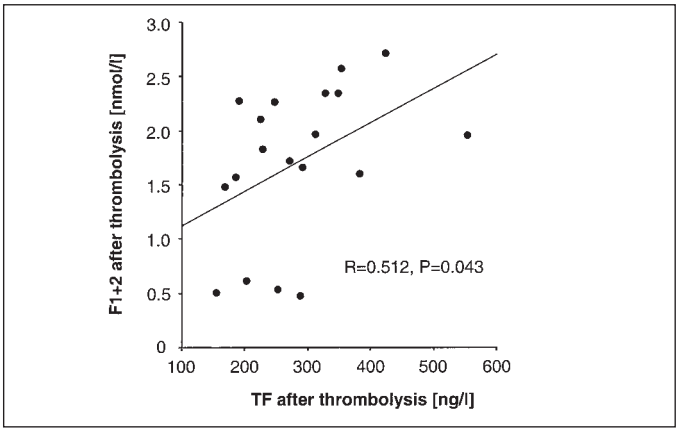

Figure 5: Association of circulating TF and plasma prothrombin fragment F1+2 after thrombolysis. Shown are the individual values from each patient.

Our results extend these observations showing that TF positive MP express TFPI. Moreover, degradation of TFPI on MP after thrombolysis contributes to thrombin formation due to unopposed TF activity. Thus, inhibition of TF activity by TFPI occurs on MP and impedes thrombin generation. Only degradation of TFPI during thrombolysis allows circulating TF to add to thrombin formation.

Recently, a pathway for the propagation of blood coagulation after endothelial injury has been proposed that involves the accumulation and localization of TF positive MP on the platelet thrombus by a mechanism involving CD15 and P-selectin (6, 17). Moreover, delivery of circulating TF into a developing thrombus occurred through interaction of platelet P-selectin and P-selectin glycoprotein ligand 1 on monocytic MP (18). These mechanisms provide a concept, how unopposed TF activity after degradation of TFPI on MPs may contribute to rethrombosis.

Procoagulant MP in AMI contribute only partially to circulating TF activity: A recently identified soluble, spliced variant of TF adds to circulating TF and may contribute to a procoagulant state (7).

Bolus or continuous administration of heparin has been shown not to alter surface TFPI expression or TFPI plasma levels (4, 20, 21). Therefore, continued heparin administration after thrombolysis may not contribute to the observed changes in TFPI expression. Furthermore, medication between the study groups differed in respect to GPIIb/IIIa antagonists and ticlopidine. Although ticlopidine has been shown to contribute to activation of TF in vitro (24), we did not find any changes in TF activity before and after ticlopidine or GPIIb/IIIa antagonists in the stented patients *in vivo*. In addition, surface TFPI-1 was not affected by the interventional procedure itself (4). These result suggest that changes in TFPI expression may be considered as a result of fibrinolysis.

Table 2: TF and TFPI positive MP, D-dimer, Prothrombin fragment F1+2, TF antigen and TF-dependent inhibition of TF activity and outcome of thrombolysis. Shown are the values after thrombolysis. Values represent mean ± SEM.

	Successful thrombolysis (n=11)	Non-successful thrombolysis (n=5)	P
TF positive MP /µl	627±04	657±40	0.3
TF and TFPI positive MP /µl	48±6	59±2	0.2
D-Dimer [µg/l]	393±73	484±170	0.9
Prothrombinfragment F1+2 [nmol/l]	2.4±0.25	3.2±0.6	0.6
TF antigen [ng/l]	300±73	261±56	0.11
TF-dependent inhibition of TF activity [%]	38±20	58±8	0.7

Although in our study with only a limited number of patients no relationship between TFPI degradation on MP and failure of thrombolysis was found, further studies with a larger study population are needed, to investigate the clinical relevance of the observed mechanism. Significance in clinical studies would provide the rationale for novel strategies to improve efficacy of thrombolytic therapy. Enhancement of endogenous TFPI activity, administration of exogenous excess TFPI or anti-TF therapies is then conceivable.

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