

UROKINASE PLASMINOGEN ACTIVATOR SYSTEM IN HUMANS WITH STABLE CORONARY ARTERY DISEASE

TL Krasnikova,* YeV Parfyonova,* IA Alekseeva,* TI Arefieva,* SA Mukhina,* AB Dobrovolsky,*
YeV Titaeva,* AA Lyakishev,* TJ Resink,[†] P Erne[‡] and VA Tkachuk*

*Cardiology Research Centre of Ministry of Health, Moscow, Russia, [†]Department of Research, Basel University Hospital, Basel and [‡]Division of Cardiology, Kantonsspital Luzern, Luzern, Switzerland

SUMMARY

1. The present study compares plasma urokinase plasminogen activator (uPA) peptide levels, plasma plasminogen inhibitor (PAI-1) activity and urokinase receptors (uPAR) on peripheral blood monocytes of patients with stable coronary artery disease (SCAD) and healthy volunteers.

2. Urokinase plasminogen activator levels were analysed by ELISA and PAI-1 activity was determined by a plasmin generation method using the chromogenic substrate S2390. Relative uPAR numbers and the adhesion molecules CD11b/CD18 on peripheral blood monocytes were estimated using specific antibodies and flow cytometry.

3. Patients with SCAD were found to have higher plasma uPA peptide levels than age-matched healthy subjects (10.40 ± 0.99 vs 8.25 ± 0.53 pmol/L, respectively; $P < 0.05$).

4. Plasma PAI-1 activity was also higher in patients with SCAD than in healthy subjects (13.6 ± 2.5 vs 5.2 ± 1.0 IU/mL, respectively; $P < 0.05$).

5. Relative uPAR and CD11b/CD18 adhesion molecules were similar on peripheral blood monocytes of patients with SCAD and in healthy subjects.

6. The data indicate a pattern of expression/activity of uPA and PAI-1 in patients with SCAD suggestive of an impaired fibrinolytic ability.

Key words: coronary artery disease, monocyte, plasminogen activator inhibitor, urokinase, urokinase receptors.

INTRODUCTION

Ischaemic heart disease is frequently coupled with coronary atherosclerosis and the rupture of atherosclerotic plaques with superimposed thrombosis is a major cause for acute events in coronary artery disease.¹ The development of ischaemic heart disease is accompanied by a reduced fibrinolytic capacity.^{2,3} Reduced fibrinolysis may also be involved in the progression of atherosclerosis.^{4–6} Fibrinolytic capacity relates to the function of the plasminogen activator (PA) system. There are two types of PA, tissue plasminogen activator (tPA), which functions, in general, in circulating blood, and

plasminogen activator of the urokinase type (uPA), which potentiates pericellular fibrinolysis. The PA system converts plasminogen to plasmin, the latter not only affects the rate of fibrin degradation but can also activate metalloproteinases and latent forms of cytokines, such as transforming growth factor (TGF)- β , and even release basic fibroblast growth factor-2 from the extracellular matrix, two factors implicated in atherogenesis.^{7,8} Fibrinolysis can also be regulated by the plasminogen activator inhibitor-1 (PAI-1), the major physiological inhibitor of both tPA and, in humans, uPA.⁹ Urokinase plasminogen activator is a complex protein consisting of multiple domains and includes a serine protease site located within the C-terminal catalytic domain and an epidermal growth factor (EGF)-like domain within its N-terminal region.¹⁰ Its serine protease can actively participate in fibrinolysis as well as tissue remodelling, angiogenesis, tumour growth and even metastasis. It has been suggested that its EGF-like domain may be responsible for its mitogenic and chemotactic properties.¹¹

Endothelial, vascular smooth muscle cells and blood monocytes/macrophages produce uPA and uPA receptors (uPAR),^{12–15} in the latter cell type, uPA and uPAR production can be greatly increased during exposure to the inflammatory cytokines tumour necrosis factor (TNF)- α and interferon (IFN)- γ .¹⁴ Under these circumstances, when uPA binds to its cell surface receptor (uPAR), its proteolytic activity increases greatly.¹⁶ This complex is critical for monocyte chemotaxis¹⁷ and, during cell migration, the receptor–uPA complex is localized at its leading cell edge, participating in the degradation of extracellular matrix.^{16,17} Monocytes are also recognized to play an important role in thrombus formation, initiated by local increases in tissue factor expression.¹⁸ In this instance, their ability to produce uPA and uPAR suggests that they have a potential to regulate proteolysis within thrombi. Thus, it is possible that, depending on local environmental conditions, monocytes exert either procoagulative or fibrinolytic effects.

In atherosclerotic lesions within coronary arteries, monocytes also participate in lipid and cell debris removal as well as regulating inflammation.^{3,5} Here, their initial adhesion to endothelium and migration into the vessel wall is associated with the expression of the β 2-integrins CD11b/CD18;¹⁹ this complex (Mac-1) plays a pivotal role in monocyte adhesion to damaged endothelium.^{20,21}

The aim of the present study was to determine the extent to which expression of the uPA/PAI-1/uPAR system may be up-regulated in patients with stable coronary artery disease (SCAD). To this end, we compared plasma uPA peptide levels, plasma PAI-1 functional activity and monocyte surface uPAR and CD11b/CD18 expression in patients with SCAD and healthy subjects. Our results demonstrate that, compared with healthy subjects, patients with SCAD exhibit

Correspondence: Professor VA Tkachuk, Cardiology Research Centre of Ministry of Health, 3rd Cherepkovskaya Street 15a, Moscow 121552, Russian Federation. Email: <tkachuk@cardio.med.msu.su.>

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elevated plasma uPA peptide levels and plasma PAI-1 activity, but monocyte uPAR and CD11b/CD18 levels were similar.

METHODS

Study population

All patients gave informed consent to participate in the study and the protocol was approved by the Cardiology Research Centre's hospital ethics committee. Twenty-four patients with SCAD were recruited; SCAD was defined as the presence of effort angina with a stable pattern during the 6 months preceding the study, together with angiographically verified, clinically significant stenosis in the coronary artery disease, defined as reductions in lumen diameters of more than 70% (see Table 1). No patients had previous myocardial infarction and/or symptoms of heart failure. All medications, including aspirin, nitrates, calcium channel or β -adrenoceptor blocking agents and hypolipidaemic agents, were continued throughout the subjects' stay in hospital. Control subjects were 14 healthy volunteers from the hospital staff. They had no risk factors for cardiovascular disease and were not taking any medication. Neither the patients nor the healthy volunteers had any history of cancer or immunological disorders or, in the month preceding the study, had suffered from viral or bacterial infection or undergone any surgical or other invasive procedures.

Blood samples

Blood was obtained in the morning with minimal venous compression and was collected into the tubes containing appropriate anti-coagulants: 110 mmol/L sodium citrate, 15 mmol/L theophylline, 3 mmol/L adenosine, 0.2 mmol/L dipyridamole (pH 5.0) for plasma uPA antigen and PAI-1 activity determination; 130 mmol/L sodium citrate for the analysis of uPAR and CD11b/CD18 expression on monocytes (nine parts blood, one part anti-coagulant). To prevent *in vitro* leucocyte activation, blood samples for monocyte analysis were processed immediately (see later). Blood collected for uPA and PAI-1 estimation in plasma was centrifuged (1000 g, 20 min at 4°C) and 0.3 mL aliquots of plasma were collected and stored at -70°C until analysis (see later).

Plasma uPA peptides

Urokinase plasminogen activator was analysed using the UMUBIND uPA ELISA kit (894; American Diagnostica Inc., Greenwich, CT, USA) according to the manufacturer's protocol. Coefficients of variation (CV), both intra- and interassay, were 3.2 and 8.0%, respectively, according to our measurements. The assay kit detects single-chain uPA (pro-uPA) and high-molecular weight (HMW)-uPA forms as well as receptor-bound uPA and uPA complexed with PAI-1 in humans. All samples from SCAD patients and control subjects were processed in parallel in one batch.

Table 1 Characteristics of healthy volunteers and patients with stable coronary artery disease

	Healthy volunteers	SCAD patients
No. subjects	14	24
Age (years)	42.5 \pm 2.0	48.5 \pm 10.4
Gender (<i>n</i>)		
Male	10	18
Female	4	6
Coronary angiography (<i>n</i>)		
One vessel disease		19
Two vessel disease		5
Diabetes mellitus (%)	–	12
Hyperlipidaemia (%)	–	54
Smoking (%)	50	42

SCAD, stable coronary artery disease.

PAI-1 functional activity

The activity of plasma PAI-1 was determined by the plasmin generation method using the chromogenic substrate S2390 (Kabi, Sweden), as described previously.²² One unit PAI-1 activity is defined as that amount that inhibits one international unit (IU) of tPA standard (Kabi). The intra- and interassay CV were 2.4 and 7.6%, respectively.

Flow cytometric analysis of monocyte uPAR and integrins

The binding of monoclonal anti-uPAR antibodies (#3936; American Diagnostica Inc.) and CD11b/CD18 antibodies (Diagnotech, Moscow, Russia) to the surface of monocytes was quantified using flow cytometry (Facsan, Becton Dickinson, San Jose, CA, USA). In brief, erythrocytes were lysed in buffer containing 154 mmol/L ammonium chloride, 12 mmol/L sodium bicarbonate and 1.4 mmol/L sodium-EDTA. Leucocytes were washed with phosphate-buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin (BSA) and 0.01% sodium azide, then incubated with saturating amounts of antibodies (30 min, 4°C). They were subsequently washed before incubation with fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibodies (Ortho, Rantzen, NJ, USA; 30 min, 4°C). After two additional washes, leucocytes were fixed with 1% paraformaldehyde in PBS containing 1% BSA and stored until analysis by flow cytometry. The monocyte population was separated by gating on forward and perpendicular light scatter and the degree of expression of surface uPAR and integrins was presented in relative fluorescence intensity units. In healthy volunteers, the day-to-day variability of immunofluorescence measurements ranged from 9 to 16%.

Statistical analysis

Data are presented as the mean \pm SEM. Differences between groups were analysed using unpaired Student's *t*-test. Possible relationships between plasma uPA and PAI-1 were examined using linear regression analysis. *P* < 0.05 was considered statistically significant.

RESULTS

Plasma uPA peptide levels

Urokinase plasma PA levels in healthy subjects and patients with SCAD are presented in Fig. 1. Urokinase plasminogen activator plasma levels in patients with SCAD ranged from 3.27 to 17.64 pmol/L, while in healthy subjects they ranged from 5.18 to 10.82 pmol/L (*P* < 0.05). In patients with SCAD, the mean value of 10.39 pmol/L uPA represented the upper level of values observed in the normal group. Fifty per cent of patients exhibited levels of uPA above the upper range (10.33 pmol/L) observed in normals.

Plasma PAI-1 activity

Plasma PAI-1 activities in healthy subjects and in patients with SCAD are shown in Fig. 2. Plasminogen activator inhibitor-1 activity ranged from 2.0 to 39.7 IU/mL in patients with SCAD compared with a range of 0.9–9.3 IU/mL in healthy volunteers (*P* < 0.05). Plasminogen activator inhibitor-1 activity in nine of 24 patients with SCAD exceeded the upper levels of those observed in the normal group. Linear regression analysis indicated no significant relationships between plasma uPA levels and PAI-1 activity (all *P* > 0.05). We did not find any differences in plasma uPA concentrations and PAI-1 activity between smokers and non-smokers in either group.

Monocyte uPAR and integrins CD11b/CD18

Monoclonal antibodies against uPAR detected all uPAR, both free and those complexed to ligands, on monocytes. Mean level of uPAR

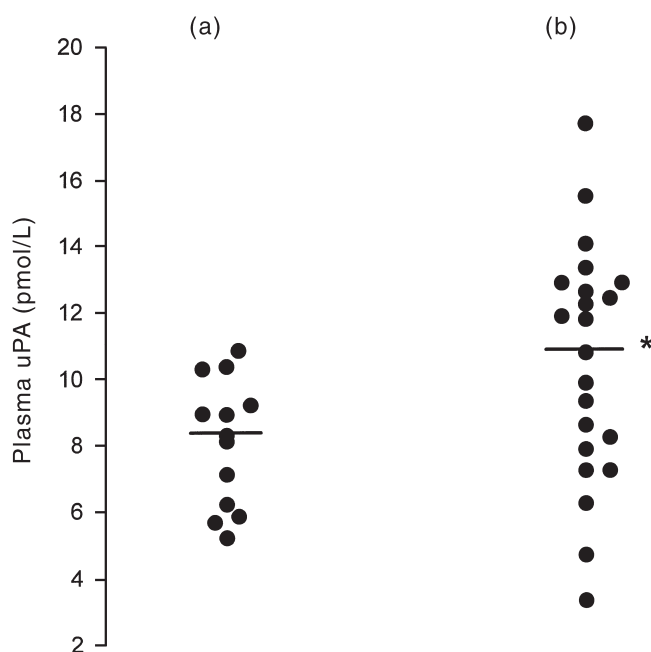


Fig. 1 Plasma urokinase plasminogen activator (uPA) antigen levels in (a) 14 healthy subjects (mean: 8.25 ± 0.53 pmol/L) and (b) 24 patients with stable coronary artery disease (mean: 10.39 ± 0.99 pmol/L). Bars indicate mean values. * $P < 0.05$ (two-tailed), determined using Student's *t*-test for unpaired data.

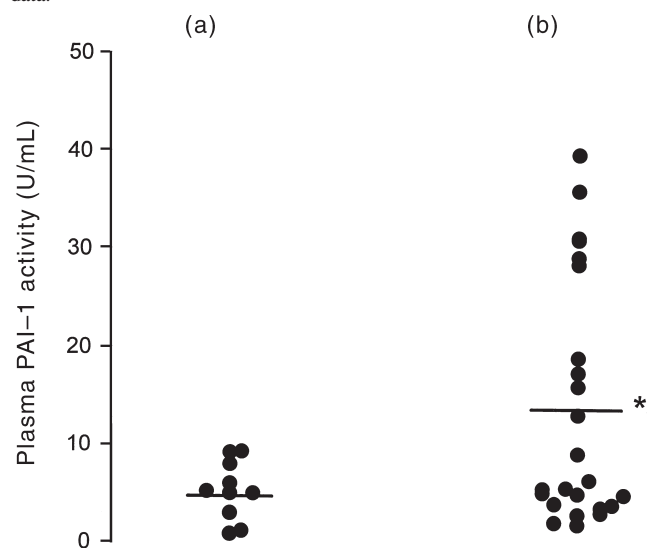


Fig. 2 Plasma plasminogen inhibitor (PAI-1) activity in (a) 10 healthy subjects (mean: 5.2 ± 1.0) and (b) 24 patients with stable coronary artery disease (mean: 13.6 ± 2.5). Bars indicate mean values. * $P < 0.05$ (two-tailed), was determined using Student's *t*-test for unpaired data.

on monocytes of patients with SCAD averaged 78 ± 24 relative fluorescence units ($n = 14$) and did not differ significantly ($P > 0.05$) from mean levels in healthy donors, which averaged 90 ± 5 relative fluorescence units ($n = 10$). There was also no significant difference in levels of CD11b/CD18 on monocytes of the healthy volunteers and patients with SCAD ($P > 0.05$; data not shown).

DISCUSSION

It is well known that coronary artery disease is linked with reduced blood fibrinolytic capacity.^{1,23,24} Increased levels of tPA immuno-

reactive peptides and elevations in plasma PAI-1 peptides have been associated with an increased risk of myocardial infarction in ischaemic heart disease.^{2,9} The present results extend these findings by also demonstrating significant elevations in plasma uPA peptide levels in patients with SCAD, as well as plasma PAI-1 activity. Monocyte uPAR levels in patients with SCAD were similar to those of healthy volunteers. The present study provides further evidence to indicate that blood fibrinolytic capacity is likely to be impaired in many patients with SCAD, which was not observed in the healthy donors with no overt signs of SCAD.

During clot formation, the presence of tPA, uPA and, probably, also uPAR-bound uPA on monocytes is essential to minimize the size of a thrombus or even to prevent its formation. It is known that tPA can be rapidly released, even from normal endothelium, when coagulation is stimulated.²⁵ Moreover, the fibrinolytic efficacy of tPA incorporated within a thrombus is 100–1000-fold greater than that of tPA located outside of the thrombus (i.e. in blood).²⁵ The data herein showing an elevation of plasma uPA in many patients with SCAD are likely to represent a capacity for increased fibrinolytic activity. Although patients with SCAD and healthy subjects had comparable uPAR on their monocytes, there is an increased potential for greater occupancy of uPAR on the monocytes of patients with SCAD because of their higher plasma uPA; this can result in an elevation in the proteolytic capacity of monocytes in such patients.

Our finding of elevated plasma PAI-1 activity is in accordance with other studies on patients with coronary artery disease, angina pectoris and survivors of myocardial infarction; in all these groups, fibrinolytic capacity has been reported to be impaired.^{1,9,24} Circulating plasma levels of PAI-1 can be regulated by many factors, a number of which increase endothelial or hepatic PAI-1 synthesis.²⁶ Plasminogen activator inhibitor-1 is also produced during platelet activation, which is increased in coronary atherosclerosis.²⁷ Of particular importance in the context of fibrinolytic capacity in SCAD is our observation that whereas uPA content was increased by 20%, PAI-1 activity increased by 200%. This differential between proteolytic capacity (i.e. uPA antigen level) and potential for inhibition of proteolytic activity (i.e. PAI-1 activity) strongly suggests that the fibrinolytic capacity in SCAD is significantly reduced. By analogy to the interpretation for concomitantly elevated tPA antigen and PAI-1 in angina pectoris, myocardial infarction and atherosclerosis,²⁸ concomitantly increased uPA antigen and PAI-1 in SCAD may reflect the inhibitory effect of PAI-1 on uPA activity. Moreover, because PAI-1 is the major physiological inhibitor of all plasminogen activator proteolytic activity in humans,⁹ we can also assume that SCAD patients exhibit an impairment of both uPA- and tPA-mediated fibrinolytic activity secondary to PAI-1 activity elevation.

In addition to its important protective function against stable thrombus formation, the PA system also plays a role in the development of atherosclerosis.^{3,5,28} Enhanced expression of PAI-1, tPA, uPA and uPAR has been reported in the endothelium, the media and the intima of human coronary atherosclerotic arteries.^{3,5,29} Of these four components of the PA system, the increase in PAI-1 expression is most predominant. Such overexpression of PAI-1 can be associated with thrombus formation and represents a risk for acute coronary thrombosis.^{3,5,28,29} Increased tPA, uPA and uPAR expression may also promote monocyte and smooth muscle cell migration, thereby facilitating proteolytic weakening of plaques.^{3,5,28,29}

Clinically, the expression of specific proteins on monocytes is frequently used as an 'inflammatory marker' of coronary artery

disease, especially of unstable angina and postangioplasty restenosis.^{20,21} Increased surface expression of Mac-1 (CD11b/CD18) has been found on monocytes and neutrophils in blood taken from coronary sinus of patients with unstable angina,²⁰ suggestive of an inflammatory reaction in the coronary vasculature in patients with unstable angina. In contrast, patients with stable angina have been reported not to have increased monocyte Mac-1 expression. We also found no changes in Mac-1 expression on monocytes of SCAD patients, compared with healthy volunteers. Mac-1 and uPAR can form a functional complex^{30,31} and this complex then has the potential to modulate uPAR-mediated signalling, despite it being a glycosylphosphatidylinositol (GPI)-anchored molecule without membrane and cytoplasmic domains, extrinsic signals being translated to the cell interior through the uPAR/Mac-1 complex. Because we did not find any differences in monocyte uPAR expression between patients with SCAD and normal subjects, it is unlikely that this signalling mechanism is relevant to patients with SCAD.

The regulation of systemic and local fibrinolysis depends on a balance between plasminogen activators and plasminogen activator inhibitors. Possibly, the elevation of plasma uPA antigen levels in coronary atherosclerosis reflects an impaired endothelial function in coronary vessels. Clearly, more work is required to substantiate this possibility. To conclude, plasma uPA antigen levels and PAI-1 activity are significantly increased in patients with SCAD compared with healthy volunteers. In future, it would also be important to determine the status of plasma uPA activity, PAI-1 activity and monocyte uPAR expression to gain insights into the importance of this system in unstable coronary artery disease as well as in patients susceptible to developing postangioplasty restenosis.

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