ORIGINAL PAPERS

Adv Clin Exp Med 2014, **23**, 2, 159–167
ISSN 1899-5276

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Expression of the PAR-1 Protein on the Surface of Platelets in Patients with Chronic Peripheral Arterial Insufficiency – Preliminary Report

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. The activation of pro-coagulation mechanisms associated with the vascular wall's immune and inflammatory responses wall to injury plays a crucial role in the mechanisms of the induction and progression of atherosclerosis.
Objectives. The aim of this study was to determine the role of protease activated receptors (PAR-1) expressed on the surface of blood platelets in the pathogenesis of chronic peripheral arterial obliterative disease (PAOD) in patients with obliterative atherosclerosis (n = 24) and diabetic macroangiopathy (n = 16), as well as in the controls (n = 12).
Material and Methods. In addition to the expression of PAR-1, serum/plasma concentrations of thrombin-antithrombin complex (TAT), the von Willebrand factor (vWF), the platelet-derived growth factor, monocyte chemotactic protein, the soluble form of the platelet endothelial cell adhesion molecule, thrombin activatable fibrinolysis inhibitor and interleukin 6 (IL-6) were determined.
Results. Compared to the controls, PAOD patients were characterized by significantly higher levels of PAR-1 expression, vWF, TAT and IL-6. Individuals with diabetic macroangiopathy did not differ significantly from individuals with obliterative atherosclerosis in terms of PAR-1 expression. Upon activation with thrombin receptor antagonist peptide (TRAP), the levels of PAR-1 were comparable in all analyzed groups. In patients with diabetic macroangiopathy, a significant association was observed between the expression of PAR-1 on the surface of the platelet and the serum TAT concentration, as well as between TAT and serum IL-6 concentration.
Conclusions. Enhanced expression of PAR-1 on the thrombocyte surface in chronic PAOD patients occurs equally in cases of diabetic macroangiopathy and in individuals free from this endocrine pathology (**Adv Clin Exp Med 2014, 23, 2, 159–167**).

Key words: adhesion molecules, diabetic macroangiopathy, obliterative atherosclerosis, PAR-1.

Advanced atherosclerotic process, manifested as ischemic heart disease, cerebral stroke or chronic ischemia of the lower limbs, is one of the leading causes of severe disability and mortality in developed countries.

Injury of the vascular endothelium is reflected by an intensified synthesis of tissue factor (TF) and general activation of the coagulation system. The activation of blood platelets constitutes a principal component in the pathogenesis of atherosclerosis, along with platelet adhesion to the vascular wall and their aggregation [1]. Activated thrombocytes are able to induce enhanced synthesis of chemokines, including PF-4, and as has been reported by many authors [2–4], this process is closely associated with increased expression of adhesion molecules, mostly E-selectin, the vascular cell adhesion molecule (VCAM-1), and the intercellular adhesion molecule (ICAM-1).

In recent years, the involvement of protease activated receptors (PAR) was postulated as a component of platelet activation. PAR proteins constitute a group of transmembrane receptors; they are activated by proteases and participate in cell-to-cell communication. The transmembrane domain of PAR proteins is composed of seven alpha-helices and it is their cytoplasmic part that facilitates binding to *G* proteins.

Four types of PAR (numbered from 1 to 4) have been identified thus far; they are characterized by 30% homology. The differences between particular types stem from the N-terminal (extracytoplasmic) sequence, which contains the site for the binding of protease and the hydrolysis of the peptide bond, determining this enzyme's ability to activate the receptor.

PAR-1 was the first receptor of PAR family to be identified and described. Its expression was detected on the surface of blood platelets, vascular endothelial cells and smooth muscle cells, among others [5].

Thrombin activates PAR-1 expressed on the surface of platelets, leading to changes in cellular shape and the release of Ca^{2+} and various compounds contained in the cytoplasmic granules. These processes are reflected by the higher aggregation properties of blood platelets. In turn, the activation of PAR-1 located within the endothelium and muscular membrane of a vessel results in vascular spasm and constriction [6].

Furthermore, the activation of PAR-1 situated on the endothelial cell surface leads to the release of vWF, endothelin, nitric oxide (NO) and platelet factor (PGI2), and can be associated with an increase in cyclic adenosine monophosphate (cAMP) concentration [1, 7].

Additionally, the so-called microparticles (MP – small fragments cleaved from the platelets' cytoplasm during their activation) are postulated to be involved in the stimulation of the coagulation process. These particles are surrounded by the cell membrane and contain relatively high concentrations of phospholipids involved in the clotting process. It has been shown that MPs undergo adhesion to fibrin – perhaps promoting the development of the clot. However, the details of the role MPs play in this process and their potential involvement in the pathogenesis of impaired fibrinolysis is still the subject of ongoing research [8].

The aim of this study was to determine the role of PAR-1 expressed on the surface of blood platelets in the pathogenesis of chronic peripheral arterial obliterative disease in patients with obliterative atherosclerosis and diabetic macroangiopathy.

Material and Methods

The Participants

This study included 52 individuals: 16 patients with diabetic macroangiopathy associated with type 2 diabetes (T2DM, mean age 55.45 ± 2.54 years), 24 patients with obliterative atherosclerosis (mean age 53.79 ± 3.59 years), and 12 controls (mean age 51.50 ± 2.12 years). Thus, the youngest possible group of patients with peripheral arterial obliterative disease (PAOD), stage IIB according to Fontaine (ankle brachial pressure index [ABI] < 0.5), was qualified to participate in this study. Patients with T2DM received insulin therapy is association with oral hypoglycemic agents. Individuals with acute or chronic inflammation and particularly tissue necrosis were excluded from the study.

Ethics

All procedures were approved by the Ethics Committee of Wroclaw Medical University and the subjects gave their written informed consent before the start of any procedures.

Isolation of Blood Platelets

Analyses were performed on 5 mL samples of sodium citrate venous blood. The tube with the analyzed material was centrifuged for 5 min (150 × g) at room temperature to obtain platelet-rich plasma (PRP). In order to obtain platelet-poor plasma (PPP), the PRP was removed and the remaining material was centrifuged for another 15 min (3000 × g) at room temperature.

Activation of Platelets with TRAP

The platelets were activated by adding thrombin receptor antagonist peptide (TRAP, code no. T1573, Sigma-Aldrich) at a concentration of 20 μM per 200 μL of examined material (250,000 platelets/ /200 μL of plasma) and incubated for 4 min in darkness at room temperature. Upon activation, the platelets were stabilized with Cell Fix to inhibit the aggregation process.

Labeling Platelets with Anti-PAR-1 Antibody and Flow Cytometry

The material (5 μL of isolated platelets or TRAP-activated platelets) was distributed into plastic tubes. The appropriate antibody was added (5 μL) and incubated for 30 min in darkness

at room temperature. The following primary antibodies were used: Mouse monoclonal Thrombin Receptor IgG1 (2 μg per 5×10^5 of platelets, cat. no. ab48409; Abcam), and ready-to-use Mouse IgG1 (negative isotypic control, cat. no. X0931, DakoCytomation, Denmark). Subsequently, the samples were washed with 3 mL of 2% PBS-FCS (Sigma) and centrifuged for 5 min (1500 × g) at room temperature. Then a secondary antibody was applied (5 μL) and incubated for 20 min in darkness at room temperature. The following secondary antibodies were used: Polyclonal Goat Anti-Mouse Immunoglobulins/FITC Goat $F(ab')_2$ (1 : 10 dilution, cat. no. F0479, DakoCytomation, Denmark), and the ready-to-use platelet-specific marker Monoclonal Mouse Anti-Human CD61, Platelet Glycoprotein IIIa/FITC, Clone Y2/51CD61 (cat. no. F0803, DakoCytomation, Denmark). The samples were washed again with 3 mL of 2% PBS-FCS (Sigma) and centrifuged for 5 min (1500 × g) at room temperature. The obtained material was suspended in 0.5 mL of PBS without Ca^{2+} and Mg^{2+} and transferred into the FACS measurement tubes. The samples were analyzed on a BD FACS Canto flow cytometer.

Other Laboratory Tests

Standard biochemical parameters – lipid profile, hsCRP, and fibrinogen and uric acid concentrations – were measured in all participants. Additionally, serum/plasma concentrations of thrombin-antithrombin complex (TAT, Enzygnost* TAT micro, cat. no. OWMG G15E4141 S/CS, Dade Behring), the von Willebrand factor (vWF, Asserachrom vWF, cat. no. 11875396, Diagnostica Stago), platelet-derived growth factor (PDGF, Quantikine® Human PDGF-AB, cat. no. DHD00B, R&D Systems), monocyte chemotactic protein (MCP-1, Human MCP-1, cat. no. BMS281CE, Bender Med Systems), the soluble form of the platelet endothelial cell adhesion molecule (sPECAM-1, Human sPECAM-1, cat. no. BMS229, Bender Med Systems), thrombin activatable fibrinolysis inhibitor (TAFI, Imuclone®TAFI ELISA, cat. no. 873; American Diagnostics Inc.) and interleukin 6 (IL-6, Human Il-6, cat. no. BMS213/2CE; Bender Med Systems) were determined by means of ELISA.

Statistical Analysis

Continuous variables were presented as arithmetic means and their standard deviations (SD). The normality of variables was tested with the Shapiro-Wilk test and the homogeneity of variances was ascertained with Levene's test. The arithmetic means between two groups were compared with Student's t test. In comparisons among the three groups, ANOVA was used with Scheffé's post-hoc test. The direction and the strength of linear relationships between pairs of normally distributed variables were tested with Pearson's coefficient of linear correlation. All the calculations were performed using Statistica 8 (StatSoft®, Poland) software. Statistical significance was defined as $P \leq 0.05$.

Results

The clinical characteristics of the studied groups are summarized in Table 1. Patients with diabetic macroangiopathy and obliterative atherosclerosis were comparable in terms of age and the severity of peripheral ischemia. The analyzed groups did not differ significantly in terms of the degree of biochemical disorders (Table 2).

The results of the cytometric determination of PAR-1 protein expression on the surface of blood platelets are summarized in Table 3. The individuals with peripheral arterial occlusive disease were characterized by a nearly twofold increase in PAR-1 expression as compared to the controls ($P = 0.04$). Patients

Table 1. The clinical characteristics of the study participants

Parameter	Diabetic macroangiopathy (n = 16)	Obliterative atherosclerosis (n = 24)	Controls (n = 12)	P-value 1 vs. 2	P-value 1 vs. 3	P-value 2 vs. 3
	1	2	3			
BMI – kg/m^2	27.08 ± 2.06	24.36 ± 3.69	26.00 ± 2.83	0.150	0.801	0.545
Age – years	55.45 ± 2.54	53.79 ± 3.59	51.50 ± 2.12	0.388	0.304	0.640
Systolic pressure – mm Hg	141.36 ± 18.59	130.77 ± 11.20	124.65 ± 2.48	0.102	0.173	0.757
Diastolic pressure – mm Hg	80.45 ± 6.11	79.42 ± 6.53	87.02 ± 3.04	0.901	0.287	0.153
Distance of claudication – m	175.71 ± 38.81	167.70 ± 84.30	–	0.290	–	–
ABI	0.45 ± 0.19	0.48 ± 0.21	0.99±0.03	0.963	0.045	0.048

BMI – body mass index, ABI – ankle brachial index.

Table 2. The biochemical characteristics of the study participants

Parameter	Diabetic macroangiopathy (n = 16)	Obliterative atherosclerosis (n = 24)	Controls (n = 12)	P-value 1 vs. 2	P-value 1 vs. 3	P-value 2 vs. 3
	1	2	3			
Platelets – K/μL	268.55 ± 84.02	279.23 ± 76.22	247.00 ± 22.13	0.931	0.966	0.922
MPV – μm^3)	10.16 ± 1.58	9.48 ± 1.21	11.02 ± 1.10	0.356	0.344	0.114
Prothrombin ratio – %	110.05 ± 5.5	105.41 ± 8.41	105.7 ± 9.22	0.259	0.864	0.999
APTT – s	29.95 ± 2.84	30.23 ± 6.16	26.00 ± 6.08	0.890	0.117	0.270
AlAT – u/L	26.50 ± 13.87	27.50 ± 15.93	23.00 ± 7.07	0.876	0.746	0.700
AspAT – u/L	28.98 ± 13.38	27.47 ± 21.25	25.90 ± 2.69	0.853	0.765	0.919
GGTP – u/L	26.35 ± 13.81	32.34 ± 14.81	20.00 ± 4.24	0.198	0.554	0.295
Total cholesterol – mg/dL	221.09 ± 49.31	203.85 ± 59.29	236.5 ± 26.16	0.695	0.938	0.731
HDL – mg/dL	50.60 ± 8.43	44.90 ± 11.62	78.00 ± 9.90	0.366	0.008	< 0.001
LDL – mg/dL	122.99 ± 36.67	128.84 ± 50.8	130.50 ± 17.68	0.941	0.885	0.942
Triglycerides – mg/dL	164.64 ± 36.07	146.96 ± 64.95	88.50 ±7.78	0.224	0.405	0.855
Glucose – mg/dL	129.09 ± 34.1	91.42 ± 10.12	96.50 ± 12.02	< 0.001	0.119	0.941
Urea – mg/dL	36.72 ± 25.68	18.05 ± 12.89	30.06 ± 5.56	0.007	0.731	0.211
Serum creatinine – mg/dL	0.98 ± 0.17	1.09 ± 1.30	1.05 ± 0.07	0.350	0.586	0.718
Uric acid – mg %	5.63 ± 1.86	5.34 ± 1.40	5.11 ± 0.15	0.616	0.712	0.820
Fibrinogen – g/L	3.93 ± 0.46	4.80 ± 2.15	4.00 ± 0.06	0.323	0.830	0.690

Table 3. Percentage concentrations of PAR-1 protein in blood platelets before and after activation with TRAP

Parameter	Diabetic macroangiopathy (n = 16)	Obliterative atherosclerosis (n = 24)	Controls (n = 12)	P-value 1 vs. 2	P-value 1 vs. 3	P-value 2 vs. 3
	1	2	3			
PAR-1 prior to activation – %	18.30 ± 6.41	14.14 ± 6.13	9.18 ± 2.35	0.392	0.035	0.038
PAR-1 after activation – %	7.49 ± 4.82	6.31 ± 5.33	6.35 ± 2.05	0.829	0.998	0.960

with diabetic macroangiopathy did not differ significantly from individuals with obliterative atherosclerosis in terms of PAR-1 expression. Upon activation with TRAP, the levels of PAR-1 were comparable in all analyzed groups. Example scatter plots of blood platelets and microparticles determined by means of flow cytometry in the control group and in PAOD patients are presented in Fig.1.

The counts of microparticles in PAOD patients and in the controls are summarized in Table 4. The values are expressed as the number of positively-stained structures per 10,000 cytometer readings. Microparticles were characterized by a smaller size than normal blood platelets (forward scatter, FSC), but did not differ from normal platelets in terms of the number of internal granules (side scatter, SSC). They were distinguished from normal platelets on the basis of a lack of superficial expression of PAR-1. In both groups of patients, the number of microparticles determined before activation with TRAP was significantly higher than in the controls (P = 0.035 and P = 0.017, respectively). Patients with diabetic macroangiopathy and subjects with obliterative atherosclerosis did not differ significantly in terms of microparticle count.

The mean values and standard deviations of selected characteristics of endothelial injury and coagulation system activation, along with the levels of growth factors and pro-inflammatory cytokines, are presented in Table 5. Both groups of patients were characterized by significantly higher levels of vWF than in the controls (P = 0.042 and P = 0.038, respectively). Also, the levels of TAT in both groups of patients were higher than in the control group (P = 0.024 and P = 0.018). Although the level of TAFI in both groups of patients was higher than in the controls, these differences proved insignificant. Additionally, the levels of platelet activation markers (PDGF, sPECAM-1) in the studied patients did not differ significantly from the control values; nor did the MCP-1 and IL-6 concentrations.

A summary of the analyzed parameters in PAOD patients and in the controls is presented in Table 6. Patients with PAOD were characterized by significantly

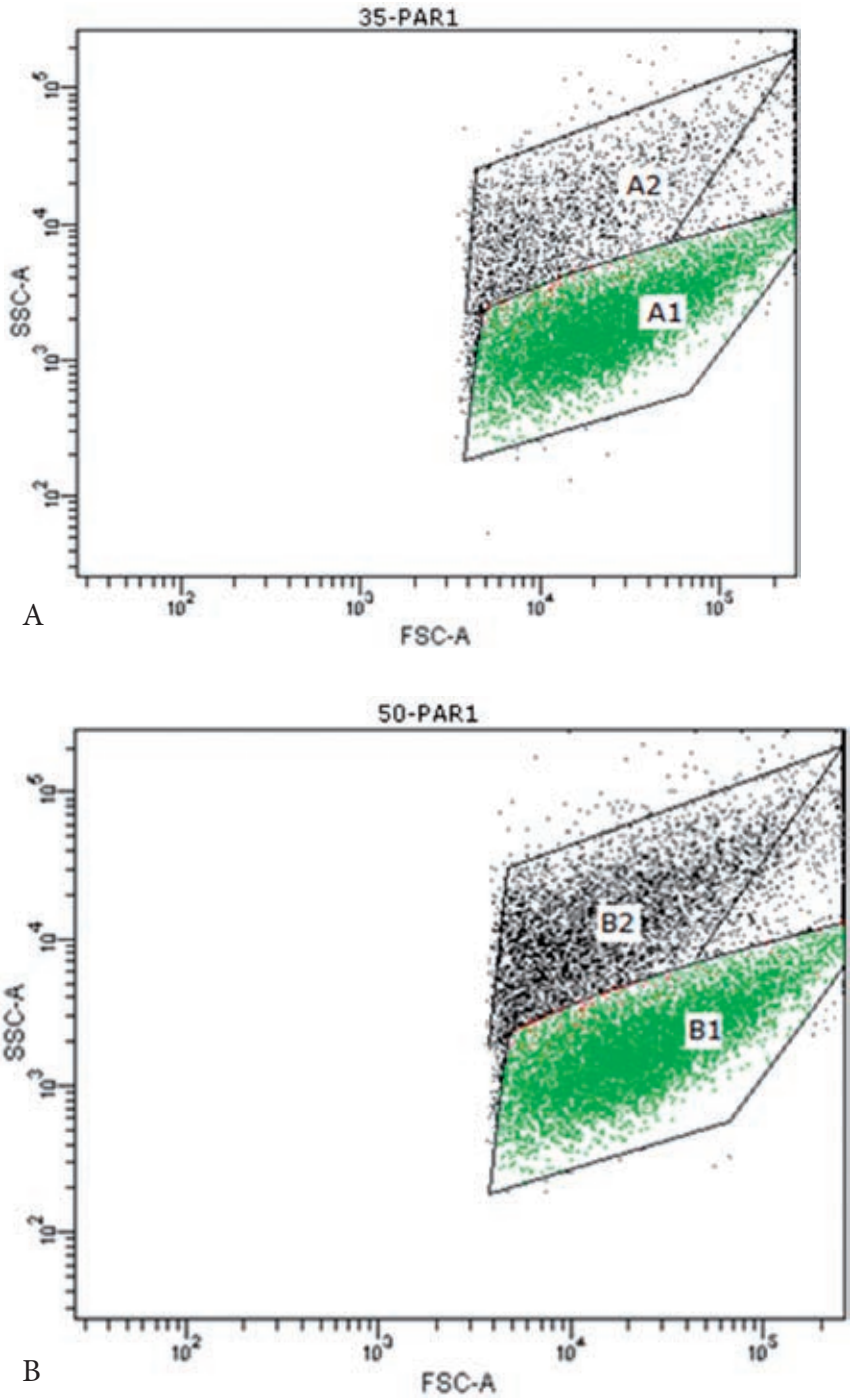

Fig.1. Scatter plot of blood platelets in a non-activated sample from a control (A) and a PAOD patient (B): normal, PAR-1-positive, platelets (A1 and B1) and PAR-1-negative microparticles (A2 and B2)

higher values of vWF and TAT as compared to the controls ($P = 0.041$ and $P = 0.002$, respectively). Furthermore, the patients and the controls differed significantly in terms of IL-6 concentrations ($P = 0.021$).

The study revealed a significant association between the expression of PAR-1 on the surface of the platelet and serum TAT concentration in patients with diabetic macroangiopathy (r = 0.74). Furthermore, an increase in TAT concentration in these patients was directly correlated with serum IL-6 concentration (r = 0.63).

The increase in microparticle count observed in PAOD patients was significantly correlated with serum MCP-1 levels (r = 0.42), although the mean level of the latter did not differ significantly from the control group.

Discussion

The activation of procoagulation mechanisms associated with the vascular wall's immune and inflammatory response to injury plays a crucial role in the mechanisms of atherosclerosis induction

Table 4. Platelet-derived microparticle count expressed per 10,000 of cytometer readings

Parameter	Diabetic macroangiopathy (n = 16)	Obliterative atherosclerosis (n = 24)	Controls (n = 12)	P-value 1 vs. 2	P-value 1 vs. 3	P-value 2 vs. 3
	1	2	3			
Platelet count prior to activation	8969.40 ± 183.26	9218.86 ± 438.80	8567.50 ± 128.43	0.263	0.193	0.218
Platelet-derived microparticle count prior to activation	1098.33 ± 346.42	1660.82 ± 876.52	709.33 ± 183.83	0.145	0.035	0.017
Platelet count after activation	8125.67 ± 532.08	7514.53 ± 905.86	5095.00 ± 1247.34	0.137	0.192	0.203
Platelet-derived microparticle count after activation	2432.43 ± 1654.55	2662.60 ± 1759.58	1519.00 ± 871.16	0.774	0.491	0.390

Table 5. Selected serum parameters of the study participants

Parameter	Diabetic macroangiopathy (n = 16)	Obliterative atherosclerosis (n = 24)	Controls (n = 12)	P-value 1 vs. 2	P-value 1 vs. 3	P-value 2 vs. 3
	1	2	3			
vWF – %	144.77 ± 11.09	152.58 ± 8.80	133.66 ± 6.08	0.599	0.042	0.038
TAT – ug/L	3.22 ± 1.12	3.11 ± 1.04	1.98 ± 0.43	0.953	0.024	0.018
TAFI – %	86.01 ± 14.2	76.83 ± 11.78	75.39 ± 9.01	0.141	0.153	0.952
MCP-1 – pg/mL	335.89 ± 43.57	344.81 ± 94.33	308.06 ± 78.25	0.956	0.749	0.521
PDGF – pg/mL	24160.2 ± 2961.05	25015.4 ± 3855.69	22742.9 ± 3753.68	0.809	0.690	0.283
sPECAM-1 – ng/mL	40.60 ± 5.51	42.41 ± 8.48	47.61 ± 6.34	0.812	0.141	0.216
IL-6 – pg/mL	1.83 ± 0.26	1.90 ± 0.41	1.56 ± 0.33	0.856	0.266	0.063

Table 6. Summary of the analyzed serum parameters in the PAOD patients and in the controls

Parameter	PAOD patients (n = 40)	Controls (n = 12)	P-value
	1	2	
vWF – %	150.26 ± 22.15	133.66 ± 6.08	0.041
TAT – ug/L	3.15 ± 1.05	1.98 ± 0.43	0.002
TAFI – %	79.56 ± 13.05	75.39 ± 9.01	0.371
MCP-1 – pg/mL	341.93 ± 80.78	308.06 ± 78.25	0.267
PDGF – pg/mL	24761.18 ± 3593.93	22742.9 ± 3753.68	0.141
sPECAM-1 – ng/mL	43.91 ± 7.73	47.61 ± 6.34	0.067
IL-6 – pg/mL	1.88 ± 0.36	1.56 ± 0.33	0.021

and progression. Blood platelets are the chief component of this process.

Stimulation of platelet surface situated PAR-1 by thrombin constitutes one of the main pathways of thrombocyte activation. Due to the proteolytic properties of thrombin, the N-terminal fragment of this receptor is cleaved and a hexapeptide forming the new N-terminus binds to the helix that forms the receptor's core and crosses cell membrane 7 times [9]. These changes in the receptor's conformation enable the signal to be transmitted into the platelet, the activation of the latter, and the release of biologically active compounds [10].

Many experimental studies have confirmed the importance of the aforementioned pathway of coagulation activation. It has been demonstrated that peptides and molecules acting antagonistically to PAR-1 (atopaxar-E5555, F16618, ER121958 and

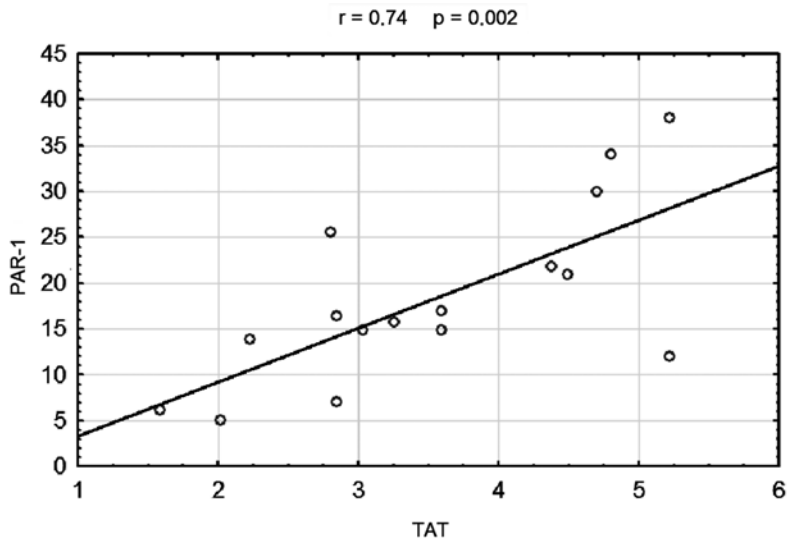

Fig. 2. Linear correlation between PAR-1 expression (%) on blood platelets and serum TAT concentration (μg/L) in patients with diabetic macroangiopathy

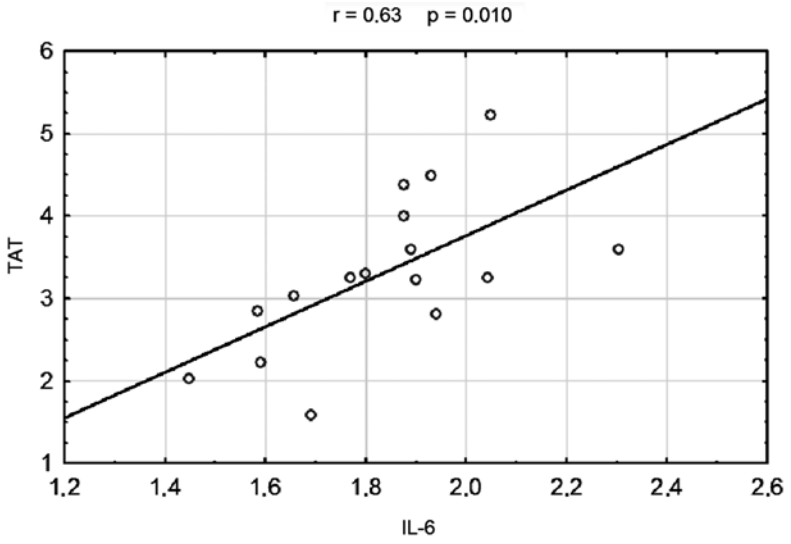

Fig. 3. Linear correlation between serum IL-6 (pg/mL) and TAT (μg/L) concentration in patients with diabetic macroangiopathy

SCH203099) protect the vessels against adhesion and the formation of intravascular clots [11–13].

Furthermore, Vane and Botting [14] revealed that besides inhibiting the activated thrombocytes, the blockade of PAR-1 receptors on endothelial cells and myocytes slows down the inflammatory processes and inhibits the proliferation of smooth muscles of the vascular wall.

The concentration of thrombin-antithrombin complex (TAT) is currently considered a sensitive indicator of coagulation activation, illustrating *in vivo* the generation of thrombin [15, 16]. Previous research performed in the current authors' center [17] as well as the current study have confirmed a significant increase in serum TAT concentration in patients with chronic peripheral arterial disease associated with atherosclerosis. Thrombin is involved in many well understood processes, most of them associated with PAR-1 activation [18]. In light of this fact, PAR-1 is frequently referred to in the literature as the first thrombin receptor [19].

The current study's investigation of patients with clinical signs of advanced atherosclerosis of the lower limbs (PAOD) confirmed a significant increase in PAR-1 expression on the surface of the platelet. A significant correlation between PAR-1 and serum TAT concentration confirmed the functional relationships between these two parameters in the process of atherogenesis.

Platelet activation at the site of a vascular wall injury is determined by the interactions between numerous glycoprotein platelet receptors and collagen fibers [1, 2]. Research performed earlier in our center by Gosk-Bierska et al. [20] confirmed a significant increase in GPIb-IX and GPIIb-IIIa glycoproteins in PAOD patients in two different platelet concentrations (125000/mm^3 and 250000/ /mm^3) as compared to the control group. These

glycoproteins bind to collagen via a common adhesion protein (vWF factor), but play different roles in the process of thrombocyte adhesion during the initial stage of primary hemostasis [21]. The GPIIb/IIIa complex is a functional receptor for fibrinogen, vWF and such intercellular matrix proteins as fibronectin or vitronectin on activated platelets [22]. Furthermore, vWF activates GPIIb-IIIa fibrinogen receptors determining the course of further stages of hemostasis [23]. Via interaction between GPIb-IX and vWF, platelet adhesion leads to massive reorganization of actin filaments in thrombocytes. In turn, interaction between the GPIIb-IIIa complex and vWF is vital for the translocation of signaling enzymes, the activation of the signal leading to morphological changes in the platelet cytoskeleton, the release of cytoplasmic granules, and platelet aggregation. Undoubtedly, the significant increase in vWF concentration observed in both groups of PAOD patients analyzed in this study promoted the activation of the glycoprotein platelet complexes, indirectly proving the involvement of PAR-1 in the induction of procoagulation mechanisms. Further stages of impaired hemostasis are associated with an ongoing immuno-inflammatory process as suggested by the increase in IL-6 concentration observed in this study and the significant correlation between the latter parameter and serum TAT [24].

Determining the role of microparticles in the pathogenesis of PAOD is an issue that still requires investigation. In this study, the microparticle count in PAOD patients was significantly higher than in the controls, and it correlated significantly with serum MCP-1 concentrations ($r = 0.4271$). However, PAOD patients did not differ significantly from the controls in terms of serum MCP-1 levels. Consequently, further research is needed to elucidate these questions in patients with chronic peripheral arterial disease.

In conclusion, the enhanced expression of PAR-1 protein on the surface of blood platelets in chronic PAOD patients occurs equally in cases of diabetic macroangiopathy and in individuals free from this endocrine pathology. However, patients with diabetic macroangiopathy are characterized by stronger correlations between PAR-1 expression and vWF and TAT levels.

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Conflict of interest: None declared

Received: 10.04.2013
Revised: 31.05.2013
Accepted: 7.04.2014