

# Smooth Muscle Cell Migration Promoting Activity of Plasma Predicts Restenosis in Patients with Peripheral Arterial Occlusive Disease Undergoing Angioplasty

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## Key words

Peripheral arterial occlusive disease, angioplasty, restenosis, smooth muscle cells, growth factors

## Summary

**Background:** Efficacy of percutaneous transluminal angioplasty (PTA) is limited by restenosis occurring in a large proportion of patients. Smooth muscle cell (SMC) migration is a major pathomechanism of restenosis. We studied SMC migration inducing activity of plasma from patients with peripheral arterial occlusive disease (PAOD) undergoing PTA. **Methods and Results:** SMC migration was determined in a two-dimensional assay system after addition of 1/25 plasma dilutions. Mean increase in migration area was  $65.5 \pm 33.8\%$  in normal controls and  $67.7 \pm 53.2\%$  in patients with PAOD. 6 hours after PTA, plasmatic migration inducing activity was largely unchanged. In 19/30 patients with restenosis (6 months after PTA) migration promoting activity ( $82.7 \pm 60.0$ ) was significantly higher than in 11/30 patients with patent vessels ( $41.8 \pm 21.0$ ;  $p = 0.03$ ). No correlation of clinical risk factors with outcome was observed. A weak correlation was found between plasmatic migration promoting activity and levels of epidermal growth factor and transforming growth factor- $\beta$ . **Conclusion:** The capacity of human plasma to stimulate SMC migration in tissue culture can be used to assess the risk for restenosis after PTA in patients with PAOD.

## Introduction

Percutaneous transluminal angioplasty (PTA) has become routine therapy in patients suffering from peripheral arterial occlusive disease (PAOD). Primary success rates are high, but late outcome is limited by restenosis occurring in 30–50% of patients 6 months after PTA (1). Smooth muscle cell (SMC) proliferation and migration as well as deposition of extracellular matrix are major pathomechanisms of restenosis

(2). The endothelium is disrupted by the balloon catheter during PTA, platelets aggregate and leukocytes adhere to the disrupted layers (3). Activated platelets release adhesive molecules like fibrinogen, fibronectin, thrombospondin and von Willebrand factor, and the intramural thrombus formation is promoted (4). Platelets further release substances that induce vasoconstriction, mitosis and migration of cells (5), including growth factors such as platelet-derived growth factor (PDGF) (6), epidermal growth factor (EGF) (7), basic fibroblast growth factor (bFGF) (8), transforming growth factor- $\beta$  (TGF- $\beta$ ) (9), and insulin like growth factor-1 (IGF) (10).

We investigated whether balloon angioplasty leads to the generation of migration promoting activity in plasma, which might cause enhanced smooth muscle cell migration at the site of injury. Plasma samples of patients suffering from PAOD and undergoing PTA of a peripheral artery were tested in a two-dimensional in vitro migration assay (11) system for their ability to stimulate migration of cultured human umbilical artery SMC. We also determined the content of growth factors likely to be responsible for the migration promoting activity. In vitro data were correlated to individual patient's clinical outcome as determined by Color Duplex Sonography.

## Methods

### Clinical Trial

Thirty patients (17 male/13 female, mean age  $69.2 \pm 8.2$  years) undergoing PTA of a stenosis with 2–6 cm length, located in the femoral or popliteal artery were recruited. Twenty two patients were classified with PAOD I/3 (Fontaine II), and 8 with PAOD II/5 (Fontaine IV) according to the guidelines of the American Heart Association 1993. In Table 1 basic data of the patients are listed, for the group as a whole, and according to the late clinical outcome. Data on age, body mass index, risk factor distribution and periinterventional therapy are given in Table 1. Continuous drug therapy was as follows: Seven patients were on ACE inhibitors, 12 on calcium channel blockers, 9 on anti-diabetics, 6 on nitrates and 13 on vasodilators.

All patients were on continuous treatment with 100 mg acetyl-salicylic acid (ASA). This drug was given at a dosage of 300 mg orally the day before angioplasty, or intravenously 1 h before the intervention (12). Peri-interventional therapy was performed with unfractionated heparin (UH) ( $n = 17$ , aPTT adjusted for 48 h post-PTA) or low molecular weight heparin (LMWH, Fragmin, Pharmacia, Sweden) ( $n = 13$ , 2500 IU twice daily for 48 h post-PTA) (13). ASA treatment was resumed 48 h after PTA, again in a dosage of 100 mg.

PTA was done following a standardized intervention protocol (14). Immediately after the dilation control angiography was performed and, if necessary the dilation was repeated once. Color Duplex sonography was done before and

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	All Patients (n=30)	Restenotic Patients (n=19)	Non-Restenotic Patients (n=11)
Age	69.5±8.2; 68 (54-86)	71.7±8.1; 68 (60-86)	67.7±8; 65 (54-85)
Body Mass Index	26±3; 26 (20-36)	27±3; 26 (20-32)	26±4; 26 (20-32)
Fontain II	22/30	14/19	8/11
Fontain IV	8/30	5/19	3/11
Hypertension	19/30	12/19	7/11
Diabetes	11/30	7/19	4/11
Hyperlipidemia	16/30	10/19	6/11
Smoking	17/30	10/19	7/11
Heparin	17/30	12/19	5/11
LMWH	13/30	7/19	6/11
Re-Dilation	19/30	11/19	8/11

Table 1 Patients data for age, stage of disease, risk factor distribution and periinterventional drug therapy

after PTA. Success of PTA was documented measuring the total vessel diameter and the intraluminal diameter at the site of lesion. All patients had successful PTA (residual stenosis <30%) 48 h after intervention. Restenosis was defined as narrowing of the luminal diameter of more than 50% at the follow-up examination 6 months post-PTA (15).

The study protocol was approved by the Ethic Committee at the University of Graz and eligible patients provided written informed consent.

### Controls

32 healthy subjects (17 male/15 female; mean age 63.8 ± 4.8 years) without any clinical sign of arteriosclerosis were recruited as controls.

### Plasma Samples

Venous blood was drawn from the cubital vein (before and 6 hours after PTA) into citrate Vacutainer tubes (Becton-Dickenson, Meylan, France). Samples were centrifuged immediately at 1000g for 5 min at room temperature and were stored at -70° C until use. For 3 h 500 µl of plasma were mixed with 500 µl of 0.15M NaCl and 500 µl of 0.025M CaCl<sub>2</sub> in glass tubes at room temperature. Then the fibrin clot was removed with a Pasteur pipette and samples were sterile filtered using syringe filters with a pore size of 0.22 µm (Corning, Staffordshire, England).

### Tissue Culture and Migration Assay

Human umbilical artery SMC were isolated from pieces of human umbilical arteries of six different cords obtained after normal vaginal delivery by the explant technique (16). Tissue specimens were cut into several pieces of 1 to 2 mm in diameter, placed into Petri dishes (Corning) coated with 1% gelatine (Sigma, Saint Louis, USA) and covered with a drop of Medium 199 (M199, Sigma) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml

fungizone (Bio Whittaker, Walkersville, USA) and 20% supplemented calf serum (SCS, Hyclone, Logan, USA). After 3 to 5 days the explants became adherent, and the Petri dish was then filled with M199 containing SCS and antibiotics as described above. SMC from the explant were grown to confluence and cells were pooled, seeded into a 225 cm<sup>2</sup> gelatine precoated culture flask (Corning) and grown to confluence as described above. Cells were confirmed to be SMC by their typical "hill and valley" morphology and positive immunofluorescence staining with a monoclonal antibody against alpha SMC-actin (Boehringer Mannheim, Germany). Cells were subcultured using a 1:3 split ratio up to the 7<sup>th</sup> to 10<sup>th</sup> passage and used in the migration assay.

For the migration assay six well plastic plates (Corning) were precoated with sterile 1% gelatine for 4 h. Sterilized steel supports (11) were inserted into each well. SMC were seeded into the middle hole of each support (4000 cells in 200 µl M199) and 1200 µl M199 was applied into the outer chamber to obtain equal hydrostatic pressure. After 24 h the supports were removed and the cell layers were stimulated with 2 ml plasma dilution (1:25 in M199) for 72 h, supplementing the plasma dilution every 24 h. At the end of the incubation period the supernatant was removed, the cell layer rinsed once with phosphate buffered saline (PBS) and fixed with 3% Formaldehyde in PBS at room temperature for 30 min. Staining was performed with GEMSA solution for 10 min. Afterwards, plates were washed with distilled water and dried overnight.

### Computer Analysis

Migration areas were determined using an imaging program standardized by the National Institute of Health, USA. Plates were scanned by Macintosh Photoshop system (standard color 201 light and 17 dark greyscale, 100% imaging, 144 dpi). Borderlines of the migration areas were detected automatically by the program and the area obtained was visually compared with the original scanning picture. Individual migration areas were measured automatically with a standardized scale using a pixel system. Samples were tested in duplicates. Migration areas were given as percent change compared to the area obtained without plasma stimulation.

### Determination of Growth Factors

PDGF-AB, bFGF, TGF-β and EGF were determined in citrated recalcified plasma samples obtained 6 h after angioplasty, using commercially available ELISA systems (R&D Systems, Minneapolis, USA).

### Statistical Analysis

Statistical analysis was performed using the STATISTICA™ computer program. Values were expressed as mean ± standard deviation. Only non-parametric statistical models were used. Differences between two groups were calculated by Mann Whitney ranking test. Correlation analysis was done by Spearman test. Differences in the migratory activity before and after PTA were calculated using Friedman ANOVA. Multiple regression analysis models were employed to evaluate influence of clinical risk factors for restenosis, technical balloon catheter data and in vitro assay results. A p-value of less than 0.05 was considered as significant.

Table 2 Migration promoting activity according to late clinical outcome

	All patients (n=30)	Restenotic patients (n=19)	Non-Restenotic patients (n=11)	p-value*
Migration area before PTA	67.7±53.2	82.7±60.0	41.8±21.0	0.03
Migration area after PTA	72.6±67.2	89.6±76.1	43.2±33.9	0.1
p-value**	0.9	0.7	0.6	

\* p-values comparing migration areas in patients with late restenosis and patency

\*\*p-values comparing migration areas before and after PTA

Table 3 Growth factor values according to late clinical outcome

Growth Factor	All Patients (n=30)	Restenotic (n=19)	Non-Restenotic (n=11)	p-value
PDGF-AB pg/ml	112.3±127.8	123.0±153.4	92.0±55.1	0.47
BFGF ng/ml	14.6±15.3	16.1±17.6	11.2±8.7	0.67
EGF ng/ml	9.5±23.6	13.1±28.0	1.9±3.3	0.04
TGF-β ng/ml	3.5±1.4	3.6±1.2	3.2±1.8	0.05

\* p-values comparing growth-factor levels in patients with late restenosis and patency

## Results

Addition of human plasma to SMC in vitro resulted in a dose-dependent increase in the migration area observed. In 32 healthy controls the mean increase in migration area was  $65.5 \pm 33.8$  % (median 57; range 25-113). Patients with PAOD had a similar migration promoting activity ( $67.7 \pm 53.2$  %, Table 2) (median 49.5; range 9-228) prior to PTA.

Six hours after PTA, migration promoting activity was similar to pre-PTA (Fig. 1, Table 2).

Six months after PTA, restenosis was documented in 19/30 (63%) patients. The plasma of patients with restenosis had significantly higher migration promoting activity than plasma of patients with late patency (Fig. 2, Table 2).

Late clinical outcome was not associated with any of the following risk factors: obesity, hypertension, diabetes, hyperlipidemia and smoking, nor was the choice of peri-interventional heparin therapy (Table 1). Patients with late restenosis were insignificantly older than patients with patent vessels. No association of late outcome with drug therapy was found (data not shown). Redilation (in 19/30 patients) was not associated with a higher restenosis rate (Table 1).

Platelet counts were correlated with migration promoting activity ( $r = 0.4$ ,  $p = 0.04$ ) but not with late clinical outcome. Plasmatic migration promoting activity was weakly correlated with the content of EGF ( $r = 0.4$ ,  $p = 0.04$ ) and TGF-β ( $r = 0.38$ ,  $p = 0.04$ ). Furthermore, EGF and of TGF-β levels were significantly higher in patients with late restenosis than in patients with late patency (Table 3).

## Discussion

In our study we present evidence for the occurrence of plasma factors which promote the migration of SMC in tissue culture. This migration promoting activity occurs to a similar extent in the plasma of normal controls as well as in the plasma of patients with PAOD. The origin of these plasma factors remains unclear. Since several platelet released growth factors have been shown to induce SMC migration in culture (6), it seems probable that one or more of these are responsible for this phenomenon. Indeed, a weak correlation was found between the levels of EGF and TGF-β with SMC migration inducing activity. Although this activity was not higher in the plasma of patients with PAOD than in controls, a causal relation with the process of arteriosclerosis cannot be excluded: All PAOD patients studied had been treated with ASA, so the capacity of platelet activation might be suppressed. Platelet activation and growth factor release is most likely to occur in loco, at the site of an arteriosclerotic lesion. Indeed, platelets seem to play a

role in the induction of SMC migration in culture, since a – somewhat weak – correlation was obtained between platelet count and migration inducing activity.

The process of PTA itself does not influence plasmatic migration inducing activity. Thus, a major platelet activation process with liberation of platelet derived growth factors can be ruled out.

The major aspect of our investigation is the observation that migration inducing activity in tissue culture seems to be predictive for late clinical outcome. Patients with late restenosis were shown to have higher plasmatic migration inducing activity than those with patent vessels 6 months after the intervention. The conclusion from these findings is this: Some patients have factors in their plasma, which induce or facili-

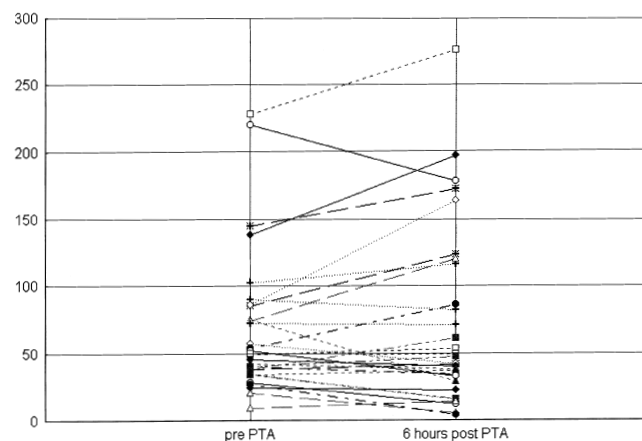


Fig. 1 Migration area increase after addition of plasma dilutions of 1:25 from 30 patients with PAOD, before and 6 h after PTA

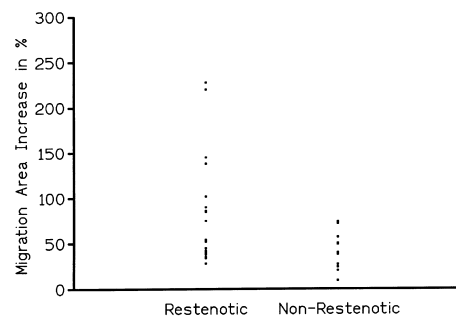


Fig. 2 Migration area increase after addition of plasma dilutions of 1:25 (pre-PTA) from patients with late restenosis and late patency

tate a repair process at the site of lesion, ultimately leading to unfavorable clinical outcome, e. g. restenosis. These factors are present also in the plasma of healthy controls. There is some evidence for the contribution of platelet factors, possibly EGF and TGF- $\beta$ .

Restenosis is a serious therapeutic problem after PTA (17). Strategies for the prevention of late restenosis include antiaggregatory and anticoagulatory drugs, aggressive lipid-lowering and others (18). As some of these therapeutic options are not without side effects, the identification of patients with an increased risk to develop restenosis has clinical impact. There are, however, only few recognized risk factors for restenosis after PTA in patients with PAOD. In a study of similar design as ours, Tschopl et al. (19) found the severity of arterial disease to be predictive of restenosis, as well as baseline levels of fibrinogen and C-reactive protein. Blood coagulation and fibrinolysis activation markers were increased in response to PTA, but this response was not predictive for outcome. Experimental data, however, highlight the importance early initiators of blood coagulation, especially factor VIIa and tissue factor, in the response to arterial injury (20). In a study by Maca et al. (21) increased lipoproteine (a) levels were predictive for adverse outcome. In the same study no association of restenosis with age, diabetes, hyperlipidemia, obesity and cigarette smoking was found. It is strongly to be suspected, however, that diabetes will be a risk factor for restenosis also in PAOD, since a negative influence on the outcome has been repeatedly reported in patients with coronary heart disease (22).

The number of patients enrolled in our study is relatively small. Various clinical risk factors of arteriosclerosis such as old age, obesity, hypertension, diabetes, hyperlipidemia and smoking were not predictive for unfavorable outcome, which the numbers given was to be expected. Plasmatic migration promoting activity, however, seems to be a fair predictor for restenosis, with a small intra-individual variability, as judged by the pre/post PTA values obtained. At the moment, this test system is probably too difficult for routine clinical use, but possibly similar in vitro models might allow the identification of patient groups at risk.

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