Original Article
Protein Z-deficiency is associated with enhanced neointima formation and inflammatory response after vascular injury in mice

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Abstract: Background: Protein Z (PZ) is a vitamin K-dependent coagulation factor without catalytic activity. Evidence points towards PZ as an independent risk factor for the occurrence of human atherosclerotic vascular diseases. The aim of this study was to investigate the role of PZ in vascular arterial disease. Material and methods: PZ-deficient (PZ$^{-/-}$) mice and their wild-type littermates (PZ$^{+/+}$) were subjected to unilateral carotid artery injury by using ferric chloride and dissected 21 days thereafter for histological analysis. Human aortic smooth muscle cells (SMC) were used for *in vitro* wound healing assay to assess the influence of PZ on SMC migration and for cell proliferation studies. Results: Morphometric analysis of neointima formation revealed a significantly increased area and thickness of the neointima and subsequently increased luminal stenosis in carotid arteries of PZ$^{-/-}$ mice compared to PZ$^{+/+}$ mice ($p < 0.05$, n = 9). Immunohistochemical analysis of neointima lesion composition revealed significantly higher numbers of PCNA-positive and α-SMA-positive cells in the neointima of PZ$^{-/-}$ mice. Furthermore, PZ showed an anti-migratory potency in *in vitro* wound healing assay with SMCs, while no effect of PZ on SMC proliferation was detectable. Conlusion: PZ contributes to a reduced neointima formation after vascular injury, underlining the modulatory role of the coagulation cascade in vascular homeostasis.

Keywords: Atherosclerosis, critical leg ischemia, thrombosis, coagulation, smooth muscle cells

Introduction

Despite major advances in vascular medicine over the past decades, cardiovascular diseases remain the main cause of morbidity and mortality in the Western world [1, 2]. Atherosclerosis constitutes the underlying pathology of coronary artery disease and poses the main contributor to death caused by cardiac diseases [3]. The important role of platelets in atherotrombotic disease has become evident from many studies investigating platelet inhibition. However, the impact of coagulation components and the crosstalk of coagulation and inflammation in atherosclerotic vascular disease are less well established [4]. Data from animal models investigating the effects of hypercoagulability on atherosclerotic disease revealed aggravated atherogenesis in different hypercoagulable genotypes such as factor V Leiden mutation and protein C (PC) deficiency [5].

Human Protein Z (PZ) is a 62 kDa vitamin K-dependent coagulation glycoprotein identified in human plasma in 1984 [6]. PZ is characterized by a structural homology with the other vitamin K-dependent proteins, factors VII, IX, X and PC [7], but in contrast to these zymogens, PZ has no catalytic activity [8]. PZ serves as a cofactor for the protein Z-dependent protease inhibitor (ZPI), a serpin of 72 kDa which inhibits factor Xa [9, 10].

In 2007, Sofi et al. observed an association between low PZ-levels and both the occurrence and the severity of peripheral arterial disease (PAD) in a case-control study, hypothesizing that PZ is linked to the atherosclerotic process apart from the acute thrombotic event with a

strict relationship to arterial risk factors [11]. In 2009 they could confirm these results by another case-control study, demonstrating again a significant association of low PZ-levels with the occurrence and severity of PAD [12].

Therefore, the aim of this study was to assess the role for PZ in arterial vascular diseases, by using an *in vivo* model of vascular injury in mice deficient for PZ and their wild-type littermates as well as established *in vitro* assays.

Material and methods

Mice

The experiments were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee (Rostock University Medical Center, Rostock, Germany; reference number: 7221.3-1-055/13). PZ-deficient mice (PZ$^{-/-}$) in a C57Bl/6x129 genetic background, as described by Yin et al. [13], were compared to their respective wild-type littermates (PZ$^{+/+}$). Male mice were used at an age of 3-6 months and a body weight of 25-30 g.

Genotyping of PZ mice

All animals were genotyped for presence or absence of PZ by PCR, as described by Yin et al. [13] using genomic DNA isolated from the tail tip.

Vascular injury protocol

Mice were anaesthetized by intraperitoneal injection of ketamine (75 mg/kg bw) and xylazine (5 mg/kg bw) and subjected to carotid artery injury using 10% ferric chloride as previously described [14, 15]. Briefly, the left carotid artery was carefully separated from the accompanying nerve and vein and any adventitial tissue, which might prevent diffusion of the ferric chloride solution, was removed by forceps. The carotid was injured by placing a 0.5-1.0 mm strip of filter paper soaked in 10% ferric chloride solution onto the adventitia for 3 min. The wound was carefully sutured with prolene 6-0 (Ethicon Johnson & Johnson Medical GmbH, Norderstedt, Germany) and the mice returned to their cages.

Histology

Three weeks after injury, mice were anesthetized as described above and carefully perfused with physiological saline and fixed with phosphate buffered formalin (4%) through the left ventricle. Several 5 μm thick cross sections of the carotid artery were done in 200 μm intervals.

Morphometric analysis of neointima formation

Neointima formation was quantified per specimen in hematoxylin-eosin (HE) stained sections, in particular by assessing neointima area, thickness and luminal stenosis using computerized image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, Md., USA), as previously described [15]. Thickness of neointima was measured from the highest point of the area to the internal elastic lamina. Luminal stenosis was calculated by substraction of the neointima area from the area of the original lumen and is given in %. The results were averaged for each animal (n = 9 per group).

Immunhistochemical analysis of neointima lesion composition

Paraffin sections of carotid arteries at 3 weeks after arterial injury were analyzed for the presence of α-actin-positive smooth muscle cells (α-SMA; abcam ab5694) by analysis of the α-SMA-positive area in the neointima lesion. Proliferating cells were detected using anti-proliferating cell nuclear antigen (PCNA; abcam ab29 [PC10]) antibody. PCNA-positive cells were manually counted and expressed as the percentage of total cell nuclei within neointima lesion.

Cell culture

Human aortic smooth muscle cells (SMC) were purchased from Lonza (Basel, Switzerland). After thawing, the cells were seeded into 10 cm cell culture dishes and cultured according to the supplier's recommendations in SmGM™-2BulletKit™ (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS), 0.1% hEGF, 0.1% insulin, 0.2% hFGF-B and 1% penicillin/streptomycin. The cells were placed in a humidified incubator at 37°C and 5% CO_2 and used from passage 5 to 10.

In vitro wound healing assay

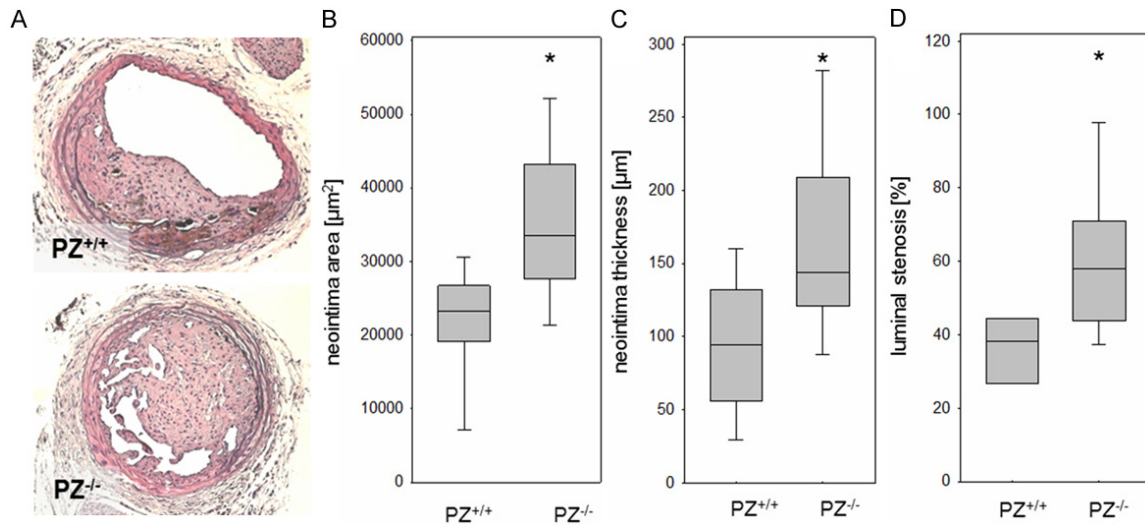SMC migration was analyzed employing the *in vitro* wound scratch assay [16]. The cells were

Figure 1. Morphometric analysis of neointima formation in PZ mice. (A) Representative images of HE-stained cross sections from carotids of PZ$^{+/+}$ and PZ$^{-/-}$ mice. (B) Quantification of neointima area, (C) neointima thickness and (D) luminal stenosis in PZ$^{+/+}$ mice and PZ$^{-/-}$ mice after carotid artery injury using 10% ferric chloride. Data are given as box plots indicating the median with the 25th and 75th percentiles. Mann-Whitney rank-sum test, *P value of less than 0.05 versus PZ$^{+/+}$ mice; n = 9.
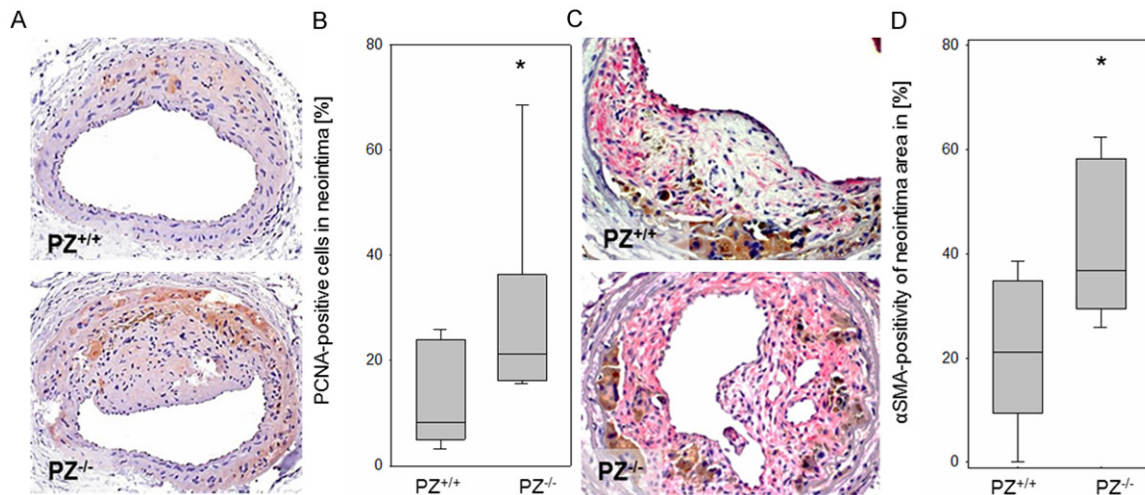

Figure 2. Cellular composition of neointima formation in PZ mice. A. Representative images of immunohistochemical detection of PCNA within vascular lesions of PZ$^{+/+}$ and PZ$^{-/-}$ mice (brown signal). B. Quantification of PCNA-positive cells in percent of all cells in the neointimal lesion. PCNA, proliferating cell nuclear antigen; Data are given as box plots indicating the median with the 25th and 75th percentiles. Mann-Whitney rank-sum test, *P value of less than 0.05 versus PZ$^{+/+}$ mice; n = 9. C. Representative images of immunohistochemical detection of α-SMA positive SMCs within the neointimal lesion (pink signal). D. Quantification of α-SMA positive SMCs within the neointima. α-SMA, α-smooth muscle actin; Data are given as box plots indicating the median with the 25th and 75th percentiles. Mann-Whitney rank-sum test, *P value of less than 0.05 versus PZ$^{+/+}$ mice; n = 9.

cultured in 12-well plates and a cross scratch wound was created in the center of the cellular monolayer by gentle removal of the attached cells with a sterile plastic pipette tip. The cells were then gently washed with PBS to remove single not-adherent cells and incubated with PZ (3 µg/ml; South Bend, IN, USA) or TNF-α (10 ng/ml; R&D Systems, Minneapolis, MN, USA) in serum- and growth-factor reduced medium (containing 1% FCS) in duplicate. Non-stimulated cells served as control (ctrl). Images of the cells were taken at 12 h after wound scratching. The images were captured using a fluorescence microscope (Leica, Germany) and wound closure was quantified employing imaging software (ImageProPlus Software, CA, USA)
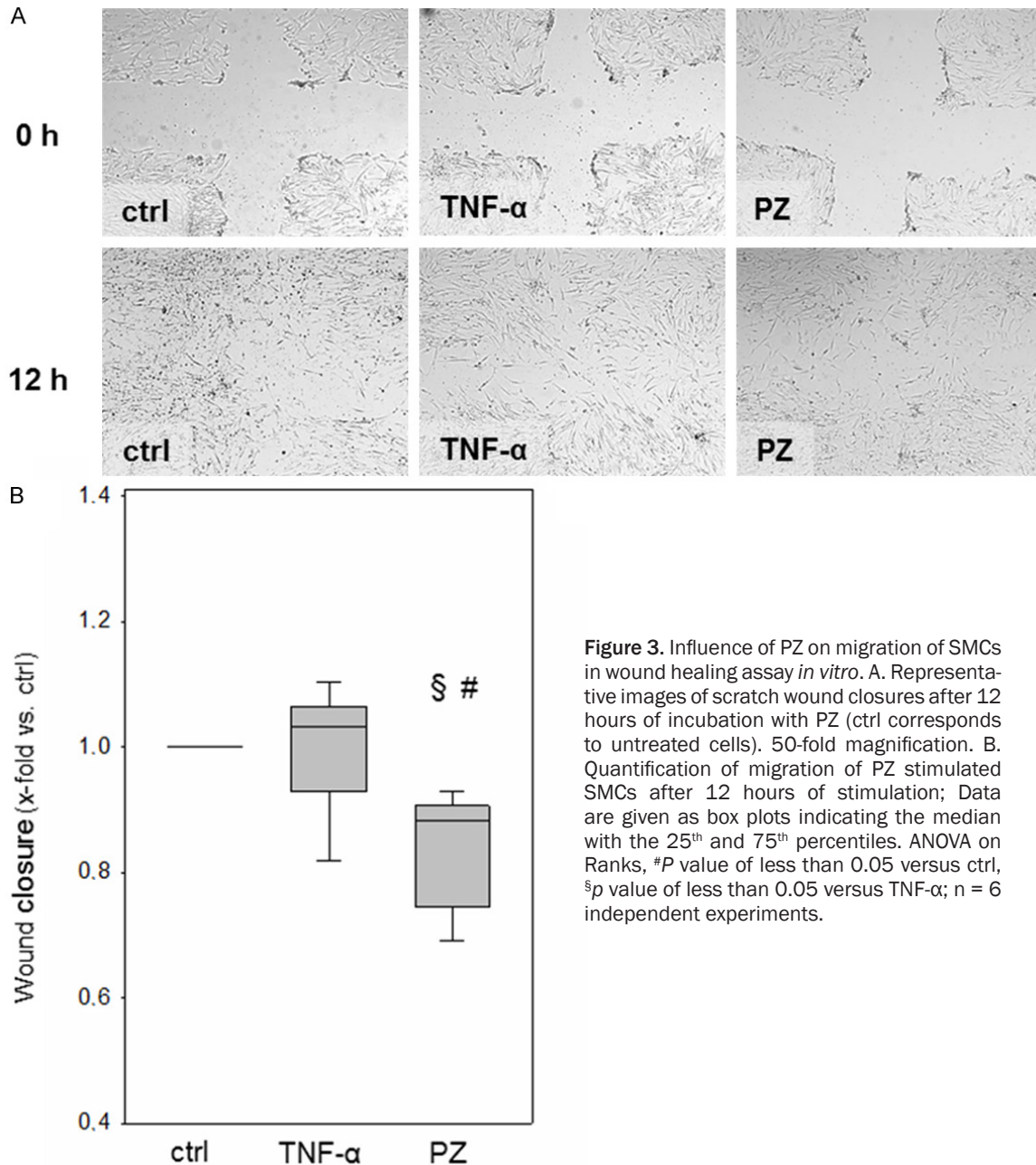
A

B

Figure 3. Influence of PZ on migration of SMCs in wound healing assay *in vitro*. A. Representative images of scratch wound closures after 12 hours of incubation with PZ (ctrl corresponds to untreated cells). 50-fold magnification. B. Quantification of migration of PZ stimulated SMCs after 12 hours of stimulation; Data are given as box plots indicating the median with the 25th and 75th percentiles. ANOVA on Ranks, #P value of less than 0.05 versus ctrl, §p value of less than 0.05 versus TNF-α; n = 6 independent experiments.

by counting migrated cells into the wound in 8 high power fields per wound.

Cell proliferation studies

Cell proliferation was analyzed by measuring DNA synthesis with a colorimetric bromodeoxyuridine (BrdU) enzyme-linked immunosorbent assay kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Briefly, 1×10^4 cells were seeded into a 96-well microplate and starved 5 hours before stimulation with PZ (3 µg/ml; South Bend, IN, USA) or TNF-α (50 ng/ml; R&D Systems, Minneapolis, MN, USA) in serum- and growth-factor reduced medium (containing 1% FCS). After 24 hours of stimulation the cells were then labelled with BrdU labeling reagent for 10 hours. After fixation, the cells were incubated with anti-BrdU antibody for 90 min. After washing, 100 µl of substrate (tetramethylbenzidine) was added to each well and the plates were incubated at room temperature for 30 min. The absorbance was measured at 450 nm

Table 1. Influence of PZ on SMC proliferation *in vitro*

	ctrl	PZ	TNF-α
	Median (IQR)	Median (IQR)	Median (IQR)
OD x-fold vs. ctrl	1.0 (1.0-1.0)	1.1 (1.1-1.2)	1.2 (1.2-1.3)

Data are given as median and IQR. ANOVA on Ranks; n = 3 independent experiments. Abbreviations: Ctrl, control; OD, optical density; PZ, protein Z; TNF-α, tumor necrosis factor alpha; IQR, interquartile range.

with a multilabel plate reader (VICTOR X, Perkin Elmer, Waltham, MA, USA).

Statistical analysis

All data are given as median and interquartile range (IQR; the 25% and 75% percentiles). Differences between groups were calculated using Mann-Whitney rank-sum test, followed by Bonferroni correction. Overall statistical significance was definded as a *P*-value of < 0.05. The statistical power was calculated for each significance at α = 0.05 with a level of 80%. Statistics, power calculation and graphics were performed using the software packages SigmaStat software version 3.5 and SigmaPlot software version 12.5 (Jandel Corporation, San Rafael, CA, USA).

Results

Murine PZ-deficiency is accompanied by increased neointima formation

Focal arterial inflammation that results in remodeling of the vascular wall is an initial step leading to arterial disease. Using the neointima lesion model in PZ+/+ and PZ-/- mice we morphometrically analyzed the developed lesions. Area of the neointima induced by ferric chloride was significantly increased in PZ-/- mice compared to their wild-type littermates (**Figure 1A**, **1B**), with the vascular lumen found almost occluded in PZ-/- mice. Also neointima thickness was significantly higher in PZ-/- mice with a median of 145 μm compared to 93 μm in PZ+/+ mice (**Figure 1A**, **1C**). As a consequence of increased neointima area and thickness, PZ-/- mice showed a significantly higher degree of luminal stenosis (57% in PZ-/- mice vs. 38% in PZ+/+ mice; *P* < 0.05; **Figure 1A**, **1D**). Five mice out of 14 PZ-/- mice died within a few days after induction of the neointima lesion, while no loss could be registered in the PZ+/+ mice group, which conceivably underlines the increased susceptibility

of PZ-/- mice to arterial vascular disease-related complications (data not shown).

Cellular composition of neointima formation reflects an increased inflammatory response in PZ-/- mice

In order to analyse arterial lesion composition carotids were stained for PCNA, which is an established marker for cell proliferation. Within the neointimal lesion PZ-/- mice exhibited significantly more PCNA-positive cells compared to their wild-type littermates PZ+/+ (21% PZ-/- mice vs. 9% in PZ+/+ mice; *P* < 0.05; **Figure 2A**, **2B**), indicating an accelerated proliferative phenotype of PZ-/- mice. Migration and proliferation of SMCs in response to endothelial activation are thought to be major pathomechanisms underlying intimal hyperplasia [17]. Immunohistochemical analysis revealed an increased area of α-actin-positive SMCs in neointimal lesions of PZ-/- mice (median 37%) vs. PZ+/+ mice (median 21%; **Figure 2C**, **2D**), indicating an increased proliferation of SMC in PZ-/- mice.

PZ diminishes SMC migration in vitro

To analyze the influence of PZ on SMC migration *in vitro,* a confluent monolayer of cells was scratched and incubated with PZ or TNF-α and were compared to unstimulated cells serving as control. Following 12 hours of incubation quantification of migrated cells revealed a trend towards increased migration of cells upon TNF-α stimulation. SMCs stimulated with PZ showed significantly diminished migration compared to TNF-α and ctrl (**Figure 3A**, **3B**).

PZ has no influence on SMC proliferation in vitro

Analyzing the proliferation of SMC by measuring BrdU incorporation, there was no significant influence of PZ on SMC proliferation compared to unstimulated control SMCs (**Table 1**). TNF-α stimulated SMCs showed a slight increase of proliferation.

Discussion

In the present model, placement of FeCl$_3$ soaked filter paper on a very small section of the carotid artery induced vascular injury with denudation of the inner vascular wall, resulting in vascular healing processes with an inflam-

matory component. Employing this model in PZ-deficient mice for the first time, our results contribute to the so far incompletely understood function of PZ in vascular homeostasis and disease.

The potential contribution of coagulation proteins to the process of atherosclerosis and thrombosis is so far incompletely understood [5]. Regarding animal studies analyzing the impact of mechanisms of the coagulation cascade on atherosclerosis, Loeffen et al. concluded that although there were variations in animal age, diet and atherosclerosis model in these studies, overall hypercoagulability on an atherogenic background increased the development and progression of atherosclerosis [5]. One of these studied coagulation factors is PC, a functional homologue of PZ [7]. PC$^{+/-}$ mice subjected to copper/silicona arterial cuff displayed enhanced focal arterial inflammation and thrombosis, leading to larger neointima formation and subsequent localized occlusion, as compared to their WT counterparts [18]. Zorio et al. found a significant association between low circulating APC levels and the extent and severity of coronary atherosclerosis, which might be related to the anticoagulatory and anti-inflammatory properties of APC [19]. It has also been reported that the presence of atherosclerotic plaques decreased the expression of thrombomodulin and endothelial PC receptor by endothelial cells [20]. Further, the activity of the PC anticoagulant pathway may be impaired in atherosclerotic arteries because of the combination of decreased HDL-cholesterol and increased LDL-cholesterol [21]. Thus, it is most likely that the beneficial effect of PC in vascular disease is due to a combination of anti-coagulant and anti-inflammatory properties. The strength of PC appears to be attributed to its capacity to restore the regulation of coagulation and inflammation at the endothelial site [22].

With respect to the structural homology of PZ and PC, comparable mechanisms may underlie the action profile of PZ, though not studied yet in full detail. In the current study PZ-deficiency is accompanied by increased neointima formation and addition of PZ to wound scratched SMCs limited cell migration. In an earlier study of our group it was shown, that murine PZ-deficiency in endotoxin-induced generalized Shwartzman reaction, displaying disseminated intravascular coagulation, is accompanied by an increased inflammatory response as reflected by excessive cytokine release and infiltration of inflammatory cells into tissue [23]. Cesari et al. reported a strong positive correlation between PZ and interleukin-6 at baseline and three months after an acute coronary artery event and concluded that PZ may be an acute phase marker with a longer latency time [24]. This strengthens a possible role of PZ in inflammation-related atherosclerotic diseases. Further on, PZ was found histologically in human macrovascular endothelial cells of arteries from healthy and atherosclerotic patients, where the proliferating subendothelial space in atherosclerotic vascular lesions showed significant immunopositivity for PZ [25]. It is not clear so far, whether PZ represents a causal role in these atherosclerotic lesions or is rather a contributor to the local wound healing response. As PZ was only immunologically detected, the positivity of PZ could be either due to PZ biosynthesis by endothelial cells, or due to PZ antigen deposition. Due to the fact that in the current study PZ revealed anti-migratory potency on SMCs *in vitro* and that PZ$^{-/-}$ mice showed increased neointima formation, it can be hypothesized that PZ has probably a compensatory function at the vascular wall in this model of vascular injury. Other groups also found PZ immunolocalized in the endothelium of arterial and venous vessel sections, which implies the binding of PZ to a postulated, but so far not identified endothelial receptor [26] and points towards a paracrine function of PZ independent of its function in the coagulation cascade.

Thus, based on the observations of this study, it is obvious that PZ activity is of importance in the development of neointimal lesions and contributes to vascular healing by coagulation-independent pathways. The further underlying mechanisms of the contribution of PZ in arterial vascular disease remain to be elucidated.

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Disclosure of conflict of interest

None.

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References

[1] Miniño AM, Murphy SL, Xu J, Kochanek KD. Deaths: final data for 2008. Natl Vital Stat Rep 2011; 59: 1-126.

[2] Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Magid D, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Nichol G, Paynter NP, Schreiner PJ, Sorlie PD, Stein J, Turan TN, Virani SS, Wong ND, Woo D, Turner MB; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics--2013 update: a report from the American Heart Association. Circulation 2013; 127: e6-e245.

[3] Krychtiuk KA, Kastl SP, Speidl WS, Wojta J. Inflammation and coagulation in atherosclerosis. Hamostaseologie 2013; 33: 269-282.

[4] Lipinski S, Bremer L, Lammers T, Thieme F, Schreiber S, Rosenstiel P. Coagulation and inflammation. Molecular insights and diagnostic implications. Hamostaseologie 2011; 31: 94-102, 104.

[5] Loeffen R, Spronk HM, ten Cate H. The impact of blood coagulability on atherosclerosis and cardiovascular disease. Thromb Haemost 2012; 10: 1207-1216.

[6] Broze GJ Jr, Miletich J. Human Protein Z. J Clin Invest 1984; 73: 933-938.

[7] Fujimaki K, Yamazaki T, Taniwaki M, Ichinose A. The gene for human protein Z is localized to chromosome 13 at band q34 and is coded by eight regular exons and one alternative exon. Biochemistry 1998; 37: 6838-6846.

[8] Broze GJ Jr. Protein Z-dependent regulation of coagulation. Thromb Haemost 2001; 86: 8-13.

[9] Han X, Fiehler R, Broze GJ Jr. Isolation of a protein Z-dependent plasma protease inhibitor. Proc Natl Acad Sci U S A 1998; 95: 9250-9255.

[10] Han X, Huang ZF, Fiehler R, Broze GJ Jr. The protein Z-dependent protease inhibitor is a serpin. Biochemistry 1999; 38: 11073-11078.

[11] Sofi F, Cesari F, Pratesi G, Cellai AP, Pulli R, Pratesi C, Gensini GF, Abbate R, Fedi S. Low protein Z levels in patients with peripheral arterial disease. Thromb Haemost 2007; 98: 1114-1117.

[12] Sofi F, Cesari F, Tu Y, Pratesi G, Pulli R, Pratesi C, Gensini GF, Abbate R, Fedi S, Broze GJ Jr. Protein Z-dependent protease inhibitor and protein Z in peripheral arterial disease patients. J Thromb Haemost 2009; 7: 731-735.

[13] Yin ZF, Huang ZF, Cui J, Fiehler R, Lasky N, Ginsburg D, Broze GJ Jr. Prothrombotic phenotype of protein Z deficiency. Proc Natl Acad Sci U S A 2000; 97: 6734-6738.

[14] Konstantinides S, Schäfer K, Thinnes T, Loskutoff DJ. Plasminogen activator inhibitor-1 and its cofactor vitronectin stabilize arterial thrombi after vascular injury in mice. Circulation 2001; 103: 576-583.

[15] Schäfer K, Konstantinides S, Riedel C, Thinnes T, Müller K, Dellas C, Hasenfuss G, Loskutoff DJ. Different mechanisms of increased luminal stenosis after arterial injury in mice deficient for urokinase- or tissue-type plasminogen activator. Circulation 2002; 106: 1847-1852.

[16] Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007; 2: 329-333.

[17] Schroeter MR, Eschholz N, Herzberg S, Jerchel I, Leifheit-Nestler M, Czepluch FS, Chalikias G, Konstantinides S, Schäfer K. Leptin-dependent and leptin-independent paracrine effects of perivascular adipose tissue on neointima formation. Arterioscler Thromb Vasc Biol 2013; 33: 980-987.

[18] Castellino FJ, Ganopolsky JG, Noria F, Sandoval-Cooper MJ, Ploplis VA. Focal arterial inflammation is augmented in mice with a deficiency of the protein C gene. Thromb Haemost 2006; 96: 794-801.

[19] Zorio E, Navarro S, Medina P, Estellés A, Osa A, Rueda J, Cubillo P, Aznar J, España F. Circulating activated protein C is reduced in young survivors of myocardial infarction and inversely correlates with the severity of coronary lesions. J Thromb Haemost 2006; 4: 1530-1536.

[20] Laszik ZG, Zhou XJ, Ferrell GL, Silva FG, Esmon CT. Down-regulation of endothelial expression of endothelial cell protein C receptor and thrombomodulin in coronary atherosclerosis. Am J Pathol 2001; 159: 797-802.

[21] Griffin JH, Kojima K, Banka CL, Curtiss LK, Fernández JA. High-density lipoprotein enhancement of anticoagulant activities of plasma protein S and activated protein C. J Clin Invest 1999; 103: 219-227.

[22] Levi M, Choi G, Schoots I, Schultz M, van der Poll T. Beyond sepsis: activated protein C and ischemia-reperfusion injury. Crit Care Med 2004; 32: S309-312.

[23] Butschkau A, Nagel P, Grambow E, Zechner D, Broze GJ Jr, Vollmar B. Contribution of protein Z and protein Z-dependent protease inhibitor in generalized Shwartzman reaction. Crit Care Med 2013; 41: e447-456.

[24] Cesari F, Gori AM, Fedi S, Abbate R, Gensini GF, Sofi F. Modifications of protein Z and interleukin-6 during the acute phase of coronary artery disease. Blood Coagul Fibrinolysis 2007; 18: 85-86.

[25] Greten J, Kreis I, Liliensiek B, Allenberg J, Amiral J, Ziegler R, Nawroth PP. Localisation of protein Z in vascular lesions of patients with atherosclerosis. Vasa 1998; 27: 144-148.

[26] Vasse M, Denoyelle C, Corbière C, Litzler PY, Legrand E, Vannier JP. Human endothelial cells synthesize protein Z, but not the protein Z dependent inhibitor. Thromb Haemost 2006; 95: 519-523.