

Rapid communication

# Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization

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## Abstract

**Objective:** Platelet activation is accompanied by the release of microparticles. However, little is known about the role of platelet-derived microparticles (PMP) in the regulation of angiogenesis and related clinical situations. The aim of our study was to evaluate the effect of PMP on angiogenesis and to analyze its mechanisms.

**Methods:** Both in vitro (rat aortic ring model, cell invasion test) and in vivo (agarose bead transplantation, artificial cardiac ischemia in Sabra rats) approaches were used in the study.

**Results:** A dose-dependent pro-angiogenic effect of PMP was observed in the rat aortic ring model. This effect could be eliminated by inhibition of VEGF, bFGF, and PDGF, but not heparanase. PMP exerted their effect via PI 3-kinase, Src kinase, and ERK, whereas protein kinase C and p38 were not involved. Moreover, PMP induced invasion of endothelial cells through a layer of matrigel. This effect was mediated by VEGF, heparanase, and PDGF, but not bFGF. Furthermore, PMP induced angiogenesis in an in vivo model in which agarose beads containing PMP were transplanted subcutaneously into mice. In addition, the effect of PMP on angiogenesis was evaluated in the model of in vivo chronic myocardial ischemia in rats. Ischemia induced a decrease in the number of functioning capillaries ( $34 \pm 21.5$  vs.  $157 \pm 42.0$  per view field), but their amount increased after injection of PMP into the myocardium ( $97 \pm 27.3$ ;  $p < 0.001$  vs. ischemia without PMP).

**Conclusions:** PMP induce angiogenesis both in vitro and in vivo. Injection of PMP into the ischemic myocardium might improve the process of revascularization after chronic ischemia.

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*This article is referred to in the Editorial by C.M. Boulanger and A. Tedgui (pages 1–3) in this issue.*

## 1. Introduction

Platelets accumulate at the sites of vascular injury or disturbances in blood flow, where they activate and release various granular mediators [1]. The platelet-derived bio-

logically active substances recruit additional platelets to the forming thrombus, thus contributing to its growth. However, recently a hypothesis has been put forward that platelets, in parallel with being an integral part of hemostasis, might participate in other processes, such as wound or ulcer healing, which is based on the development of blood vessels or angiogenesis [2,3].

Several years ago, the possible role of platelets in angiogenesis was proposed by Pinedo et al. [4]. In a recent study, we demonstrated stimulation of angiogenesis by platelet granular mediators [5]. It was shown that different stages of angiogenesis might be mediated by different intraplatelet compounds. VEGF, bFGF, PDGF, and heparanase

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were the basic mediators involved in the platelet effect, whereas platelet-derived PF-4 exerted an anti-angiogenic activity. However, its effect was insufficient to overcome the total pro-angiogenic effect of platelets.

Upon activation, various types of cells, including platelets, have been demonstrated to shed microvesicles, 0.05–1  $\mu\text{m}$  in size, usually referred to as microparticles [6]. Re-distribution of phospholipids between extrinsic and intrinsic outlets of the plasma membrane by floppase is considered the main, but not unique mechanism of microparticle formation and shedding [7]. In addition, platelets release exosomes from their  $\alpha$ -granules upon activation [8].

Platelets produce microparticles in response to various activatory stimuli [9]. Recently, different biological effects have been attributed to platelet microparticles (PMP), including stimulation of blood coagulation (with thromboplastin), possible participation in pathogenesis of atherosclerosis and vascular injury in inflammation [10,11], promotion of bone cell proliferation [12] and others. At present, at least one clinical syndrome (Scott's syndrome) has been described, in which pathogenesis is directly based on the lack of platelet ability to form microparticles in response to different activatory stimuli [13]. PMP were demonstrated to bind neutrophils, mediate neutrophil aggregation, and activate their phagocytic properties [14]. This could be important in immune and inflammatory responses in which neutrophils are involved. Binding of PMP to cells can modify cell functional properties. For example, it has been elegantly demonstrated that PMP can bind hematopoietic progenitors and stimulate their engraftment [15].

Recently, it has been observed that PMP affect endothelial cells (EC) protecting them from apoptosis and inducing proliferation and formation of tubule-like structures [16]. On the other hand, PMP can exert damaging effect on EC by inducing the inflammatory response and diminishing endothelium-dependent vessel dilation [17]. However, to the best of our knowledge, no data exist regarding the contribution of PMP in the regulation of angiogenesis in toto. In the present study, PMP were shown to substantially up-regulate blood vessel formation in the ex vivo model of rat aortic rings, and proved these findings in vivo. The contribution of different intra-particle mediators were analyzed.

## 2. Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.1. Reagents

Media and tissue culture supplements were from Biological Industries (Beit Haemek, Israel). Recombinant human VEGF and bFGF were from Pepro Tech, Inc (Rocky

Hill, NJ). Neutralizing mAbs against bFGF and PDGF were purchased from R&D systems, Inc (Minneapolis, MN). Calpostin C, SB203580, PD98059, PP2, LY294002 and VEGF receptor tyrosine kinase inhibitor (Cat. Number 676475) were purchased from Calbiochem–Novabiochem Corp (San Diego, California), mAbs to CD41 and von Willebrand factor (vWF) were from Dako A/S, Glostrup, Denmark. BSA-FITC and human thrombin were from Sigma (Rehovot, Israel).

### 2.2. Platelet microparticle isolation

This study conforms to the principles of the Declaration of Helsinki. Whole ACD-stabilized venous blood was obtained from healthy donors who had not taken any medication for at least 2 weeks. Platelet rich plasma (PRP) was isolated by centrifugation ( $120 \times g$ ). Platelets ( $20 \text{ ml PRP}$ ,  $6\text{--}12 \times 10^6/\mu\text{l}$ ) were washed twice at  $750 \times g$  in the presence of 5 mM citric acid, resuspended in 0.5 ml PBS containing calcium and magnesium, and 5 U/ml thrombin was added. After 5 min incubation with slow shaking, platelet aggregates were removed by 5 min centrifugation at  $1500 \times g$ . Then microparticles were isolated as described elsewhere [12]. Supernatant was collected, centrifuged at  $100\,000 \times g$  for 1 h at 4 °C, and the pellet was resuspended in 400  $\mu\text{l}$  of PBS. Upon isolation, PMP were characterized using a flow cytometer with mAb against CD41 and the presence of whole platelets in the suspension was ruled out. The amount of PMP applied in the experiments was expressed as total protein concentration [18,19] determined using the Bradford method.

### 2.3. Rat aortic ring model

All animals were housed and treated in accordance with protocols approved by the Institutional Care and Use Committee for Animal Research at the Hebrew University Hadassah Medical School, Jerusalem. The method described by Nicosia and Ottinetti [20] was used with some modifications as described elsewhere [5]. Stained samples were photographed with a Nikon (Coolpix 990, Japan) camera connected to the microscope (Olympus CK40, Japan; magnification  $\times 20$ ). The area covered by blood vessels (in  $\text{mm}^2$ ) was measured using the software ImagePro 4.5 (Media Cybernetics, USA).

### 2.4. Agarose plug transplantation

Agarose gel (4%, 30  $\mu\text{l}$ ) was mixed with PBS, VEGF/bFGF, or PMP (8  $\mu\text{l}$ ) and allowed to polymerize for 30 s. Sabra mice were narcotized with ketamine and xylazine. The dorsal skin was incised and the agarose bead introduced into the subcutaneous space. After 72 h, mice were re-opened and the beads with surrounding skin photographed. Beads with the underlining skin were dissected, fixed with 4% formalin, and histologic sections

(4  $\mu\text{m}$ ) prepared and stained for vWF, an antigen specific for EC [21].

### 2.5. Immunohistochemistry

Paraffin-embedded skin sections (4  $\mu\text{m}$ ) were deparaffinized by heating (30 min, 60 °C) and rinsing with xylene (3  $\times$  10 min), and dehydrated by immersing in increased concentrations of ethanol. Intrinsic peroxidase activity was blocked by H<sub>2</sub>O<sub>2</sub> (3% solution in methanol, 15 min). Samples were treated with pronase (Sigma; 0.1%, 30 min, at room temperature—RT) and incubated with anti-vWF antibody (1:200, 18 h, 4 °C). A Histostain-Plus Kit (Zymed Laboratories, San Francisco, CA) was used for staining, according to the manufacturer's instructions.

### 2.6. Endothelial cell culture and invasion assay

Bovine aortic EC were maintained at 37 °C, 8% CO<sub>2</sub>, humidified atmosphere, in DMEM low glucose, supplemented with 10% heat inactivated fetal calf serum, L-glutamine and 1% penicillin/streptomycin, for at least 3 days until an 80% confluent monolayer was formed. Cells were separated from the surface by trypsin, washed, and seeded into an upper compartment of blind well Boyden chambers in the medium without serum (210  $\mu\text{l}$ ,  $2 \times 10^5$  cells). The same medium, with or without PMP and the inhibitors, was placed into the lower compartment. A membrane (Whatman, pore size 8  $\mu\text{m}$ ) coated with matrigel (50  $\mu\text{l}$ , dissolved 1:3 in PBS), and allowed to dry in a hood, separated each compartment. The system was incubated for 18 h (37 °C, 8% CO<sub>2</sub>, humidified atmosphere). Membranes were fixed with ice-cold methanol (5 min) and stained with Diff-Quik Staining Kit (Dade Behring Inc, Newark, USA) according to the manufacturer's instructions. Cells from the upper side of the membrane were removed by gentle wiping with cotton. Endothelial cells that filtered to the lower outlet of the membrane were photographed and counted over the whole membrane.

### 2.7. Rat myocardial infarction model and effect of PMP on myocardial revascularization

Sabra rats were anesthetized with intraperitoneal ketamine and xylazine, intubated by venflon 18 GA and ventilated with positive pressure ventilation. A left thoracotomy was performed in the fourth intercostal space and the pericardium opened to expose the heart. A 7–0 silk suture was passed around a prominent branch of the left coronary artery with a taper needle and ligated. A cyanotic region was delineated on the surface of middle to apical portion of the left ventricle, corresponding to the area of severe ischemia. The development of ischemia was confirmed by ECG changes. Four injections of PMP (250  $\mu\text{g}/\text{ml}$  protein totally) in PBS (20  $\mu\text{l}$  per each injection) were performed at a distance of 2 mm from the center of the

cyanotic region in 4 directions oriented 90° apart from one another. Sham-operated rats that were injected only with PBS into the same region of the ischemic heart muscle, served as a control. Three weeks after the LAD ligation or sham operation, rats were re-anesthetized and ventilated and a left re-thoracotomy was performed. Bovine serum albumin conjugated with FITC (5 mg/ml, 100  $\mu\text{l}$  PBS solution) was injected into the left atrium. Rats were sacrificed in 1 min, pale region of the myocardium below the LAD ligation point, that corresponds to the ischemic zone, was excised and embedded into paraffin. Thereafter, slices in a plane parallel to the atrioventricular groove and through the center of the ischemic region were prepared. Samples were evaluated under a fluorescent microscope, an area of dead tissue was identified, and 5 occasional view fields were photographed with objective  $\times$  20 from the margin of the infarcted region (i.e., border zone). In this model, BSA binds immediately to the vascular endothelium. Therefore, vessels labeled with FITC may be considered as functioning. Stained vessels were counted in 5 random view fields and the average amount per view field calculated. Parallel assessment of angiogenesis in non-ischemic and non-infarcted regions of the left ventricle was performed.

### 2.8. Statistics

Statistical evaluation of the results was performed using one-way ANOVA test followed by pairwise multiple comparison procedure (post hoc test) with Holm–Sidak method.

## 3. Results

### 3.1. PMP induce angiogenesis in the aortic ring model

The first aim was to determine whether PMP affect vessel development in vitro. For this purpose, the aortic ring assay was used, in which an intensive angiogenic response to human cytokines (i.e., VEGF, FGF, endostatin) and platelets was previously shown [5]. Fig. 1 shows that PMP strongly induced sprouting as concentrations (1–100  $\mu\text{g}/\text{ml}$ ) increased. This was statistically significant starting from a 30  $\mu\text{g}/\text{ml}$  concentration (sprouts area of  $2.94 \pm 1.1 \text{ mm}^2$  vs.  $0.24 \pm 0.2 \text{ mm}^2$  in the control). The intensity of PMP-induced angiogenesis varied depending on a donor that was reflected by the standard deviation higher than that for the samples with VEGF/bFGF. Notably, the intensity of sprouting at PMP concentrations of 50  $\mu\text{g}/\text{ml}$  and higher was comparable with angiogenesis induced by VEGF and bFGF ( $4.21 \pm 2.4 \text{ mm}^2$  vs.  $5.24 \pm 1.4 \text{ mm}^2$ ,  $p > 0.5$ ).

### 3.2. Contribution of cytokines in PMP-induced angiogenesis

To identify factors mediating the effect of PMP, neutralizing mAbs against bFGF and PDGF were used, as

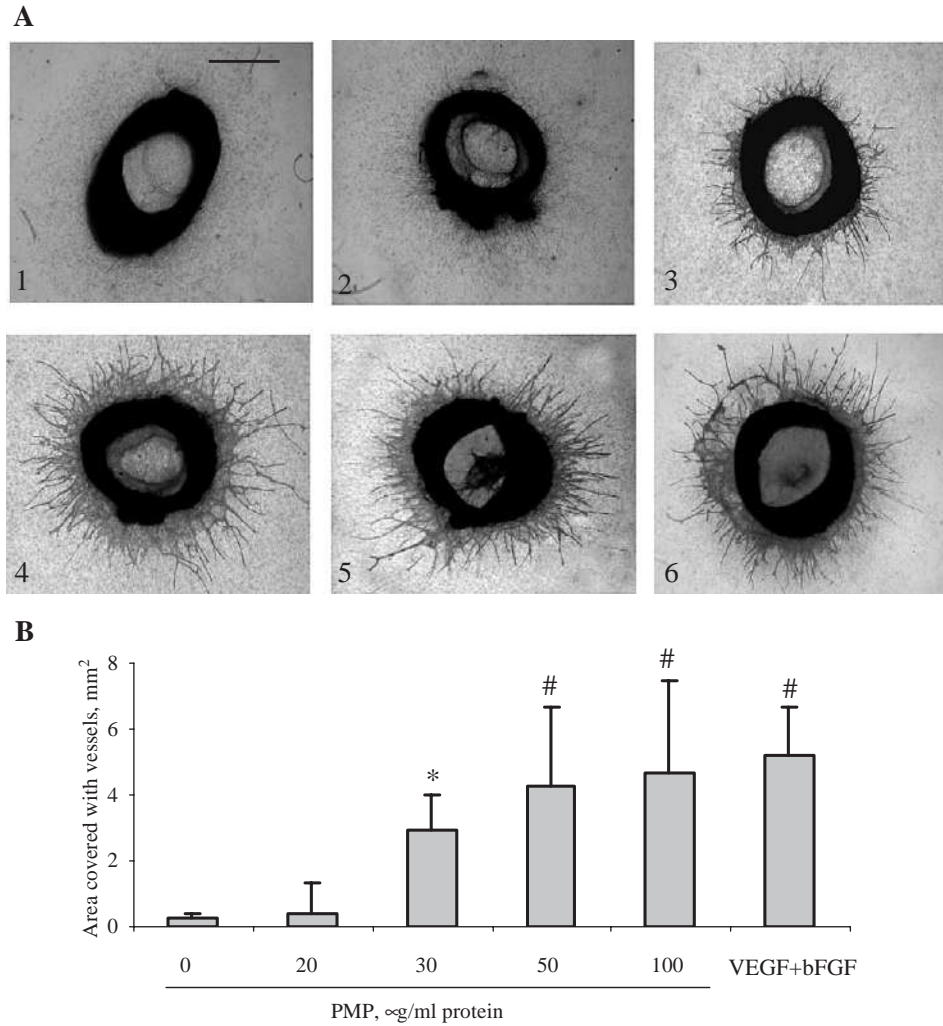


Fig. 1. PMP induce angiogenesis in the aortic ring model. (A) PMP in the indicated concentrations were added to the rings. After 7 days incubation, preparations were fixed, stained, and photographed. Samples shown: (1) negative control; (2) PMP 20 µg/ml; (3) PMP 30 µg/ml; (4) PMP 50 µg/ml; (5) PMP 100 µg/ml; (6) VEGF 50 ng/ml+bFGF 50 ng/ml. Representative experiment out of 7; bar 0.5 mm. (B) Quantitative analysis of the pro-angiogenic effect of PMP. The experimental series were compared using ANOVA followed by a post hoc test by Holm–Sidak method. \**p*<0.01 vs. negative control, #*p*<0.001.

well as VEGF receptor phosphorylation inhibitor and laminarin sulfate (an inhibitor of heparanase [22]). Inhibition of VEGF resulted in complete elimination of PMP-induced sprouting (0.7±0.5 mm<sup>2</sup> vs. 5.3±2.1 mm<sup>2</sup>; Fig. 2). At the same time, blocking of bFGF and PDGF led to a

partial, but statistically significant decrease in vessel formation (1.7±1.5 mm<sup>2</sup> and 2.4±1.2 mm<sup>2</sup>, respectively). Neutralized heparanase did not affect PMP-induced angiogenesis in this model (5.1±2 mm<sup>2</sup>). Irrelevant IgG added to the rings did not affect PMP-induced sprouting (data not

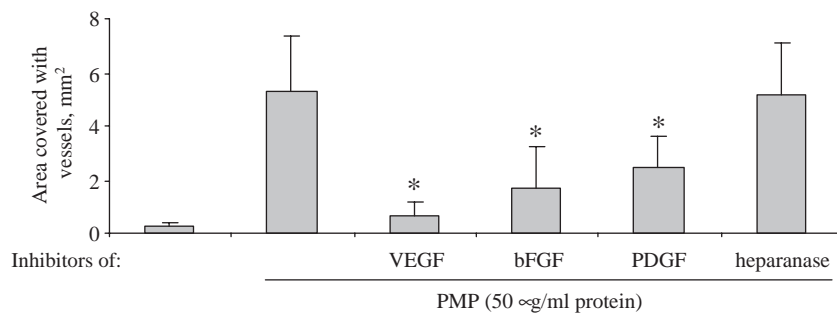


Fig. 2. Effect of cytokine inhibitors on PMP-induced angiogenesis. PMP were added to aortic rings with or without appropriate inhibitors. Doses of inhibitors used: VEGF receptor tyrosine phosphorylation inhibitor, 4 µM; anti-FGF mAb, 0.5 µg/ml; anti-PDGF mAb, 10 µg/ml; laminarin sulfate (heparanase inhibitor), 1 µg/ml. Mean±S.D. of 6 independent experiments. \**p*≤0.001 vs. PMP.



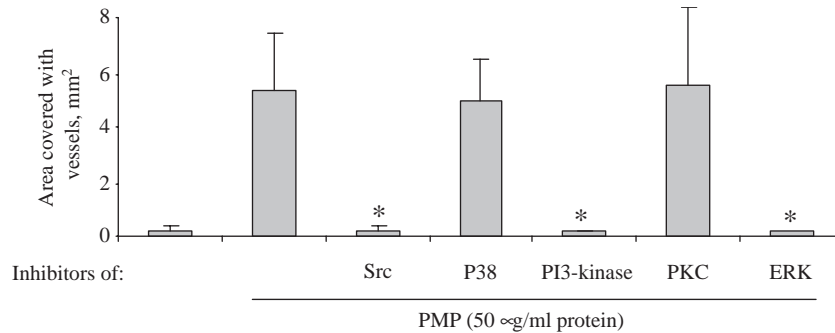


Fig. 3. Signaling pathways involved in the pro-angiogenic effect of PMP. PMP were seeded to the aortic rings in the presence or absence of signaling kinase inhibitors. Doses of inhibitors used: PP2 (Src kinase inhibitor), 10  $\mu$ M; SB203580 (inhibitor of p38 kinase, 20  $\mu$ M; LY294002 (PI3-kinase inhibitor), 20  $\mu$ M; Calphostin C (PKC inhibitor), 300 nM; PD98059 (ERK inhibitor), 50  $\mu$ M. Mean  $\pm$  S.D. of 6 independent experiments. \* $p$  < 0.001 vs. PMP.

shown). Thus, PMP exert their pro-angiogenic effect, at least partially, due to the mutual activity of cytokines located inside them.

### 3.3. Signaling pathways involved in PMP-induced sprouting

Angiogenesis is a multi-step process controlled by different signal transduction pathways. Inhibitors of the major signaling routes involved in vessel growth regulation by cytokines were applied. At least three signaling keypoints were essential for the PMP-induced angiogenesis: Src, PI3-kinase, and ERK since the corresponding inhibitors reversed the PMP-induced sprouting to  $0.22 \pm 0.08$  mm<sup>2</sup>,  $0.1 \pm 0.05$  mm<sup>2</sup>, and  $0.18 \pm 0.01$  mm<sup>2</sup>, respectively, as compared with  $5.3 \pm 2.1$  mm<sup>2</sup> in the control with PMP only (Fig. 3). At the same time, no effect on angiogenesis was observed following blocking p38 kinase and PKC ( $4.87 \pm 1.7$  mm<sup>2</sup> and  $5.6 \pm 2.7$  mm<sup>2</sup>, respectively). Thus, a mutual action of the cytokines inside PMP triggers specific pathways in EC leading to cell proliferation, movement, and angiogenesis, whereas other routes remained intact.

### 3.4. PMP promote EC invasion due to intrinsic cytokines

Since cell motility plays a pivotal role in angiogenesis, the PMP effect on EC invasion through a matrigel layer was examined. It was found that PMP strongly stimulated EC movement ( $640 \pm 254$  vs.  $52.6 \pm 50$  migrated cells per membrane; Fig. 4). Blocking of VEGF completely eliminated this effect, reducing the number of migrated cells to  $22.5 \pm 20$ . Additionally, inhibition of PDGF and heparanase markedly reduced EC invasion ( $261.4 \pm 170$  and  $144 \pm 170$ , respectively). Blocking of bFGF had no effect ( $700 \pm 495$ ).

### 3.5. PMP induce angiogenesis in vivo

The next aim was to determine whether PMP could also induce angiogenesis in vivo. Agarose beads were prepared and transplanted into mice as described in Methods. Beads that contained only PBS did not induce angiogenesis as judged by the absence of the “halo” of capillaries around the bead (capillary area of  $0.2 \pm 0.05$  mm<sup>2</sup>; Fig. 5A). This was supported by immunostaining of the skin in the bead vicinity

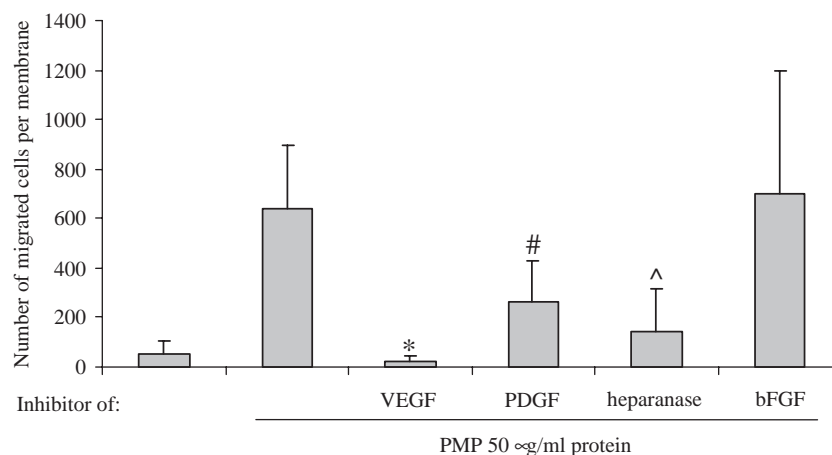


Fig. 4. PMP induce EC invasion through matrigel layer. Bovine EC ( $2 \times 10^5$  in 210  $\mu$ l medium) were seeded in the upper chamber of the Boyden system, and PMP, with or without inhibitors, placed in the lower chamber. Membrane coated with matrigel was located between the chambers. The system was incubated for 18 h at 37  $^{\circ}$ C, and cells on the membrane were fixed and stained. Cells on the upper side of the membrane were gently removed, and all cells on the lower side were photographed and counted. Presented are the mean  $\pm$  S.D. of 6 experiments performed in duplicate. Inhibitors were used in the same concentrations as in Fig. 2. \* $p$  < 0.001 vs. PMP; ^ $p$  = 0.002; # $p$  < 0.02.

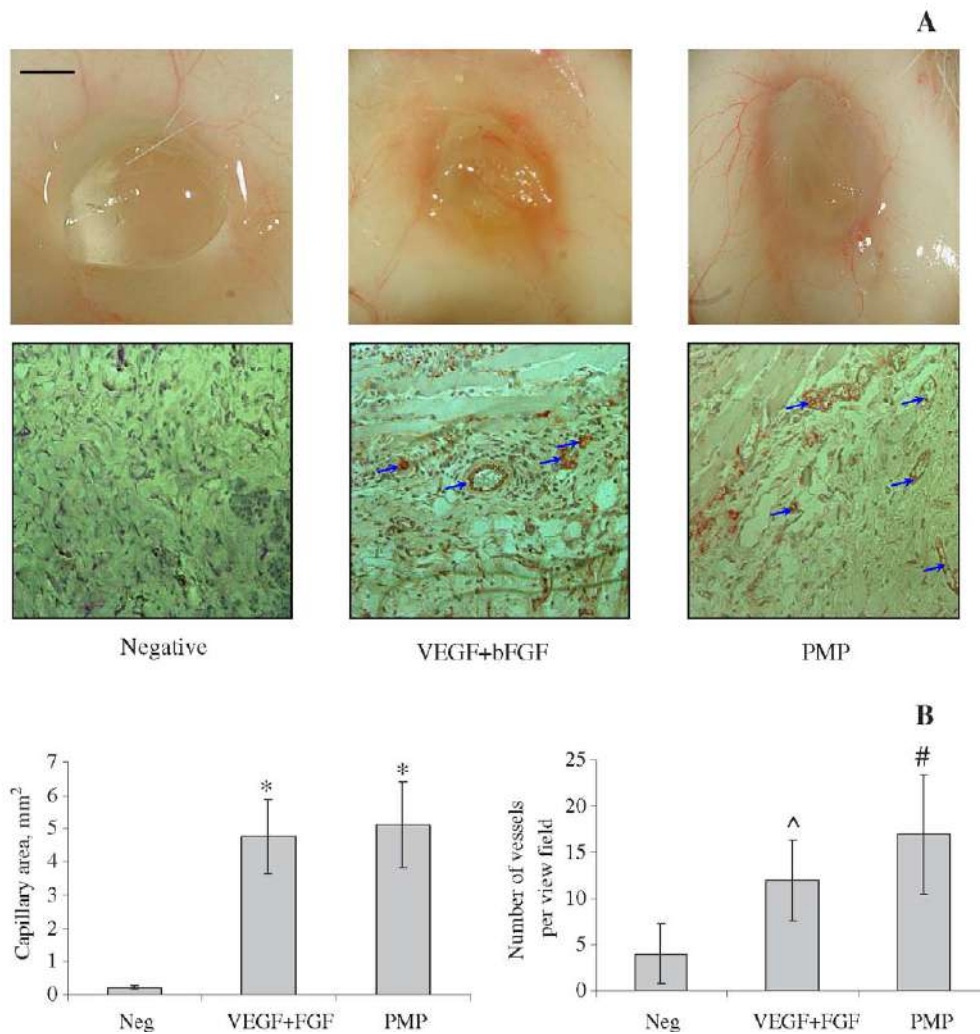


Fig. 5. PMP induce angiogenesis in vivo. (A) Agarose beads containing VEGF/bFGF (50 ng/ml each) or PMP (100  $\mu$ g/ml) with or without cytokine inhibitors were transplanted subcutaneously into Sabra mice. In 3 days, mice were sacrificed and beads photographed. To confirm the vascular nature of the “halo” in the beads’ vicinity, immunostaining with anti-vWF mAb was performed. Bar 1 mm. (B) Area of capillaries surrounding the beads was measured. Vessels were counted in 5 occasional view fields in each slide. Results are mean  $\pm$  S.D. of 4 independent experiments. The difference between the experimental series was evaluated by ANOVA followed by a post hoc test by Holm–Sidak method; \* $p \leq 0.001$  vs. control; # $p = 0.005$ ; ^ $p < 0.05$ . Magnification of the objective  $\times 100$ .

( $4 \pm 3.2$  capillaries per view field). In contrast, a substantial layer of capillaries developed around the beads that contained VEGF/bFGF ( $4.7 \pm 1.1$  mm<sup>2</sup>) or PMP ( $5.1 \pm 1.3$  mm<sup>2</sup>,  $p < 0.001$ ). Histologic staining confirmed the presence of vWF-positive small vessels in the skin in proximity to the bead ( $12 \pm 4.4$  and  $17 \pm 6.5$  for VEGF/bFGF and PMP, respectively,  $p < 0.05$ ). Fig. 5B represents a quantitative evaluation of the results obtained in three independent experiments performed in duplicate. Consequently, PMP can also induce vessel growth in in vivo conditions.

### 3.6. PMP promote myocardial angiogenesis in chronic regional ischemia

To determine whether PMP augment revascularization of the ischemic myocardium, PMP were injected into the ischemic heart muscle after modelling of infarction by ligation of the left anterior descending coronary artery

(LAD) as described in Methods. LAD ligation induced a strong decrease in the amount of functioning blood vessels in the ischemic myocardium region ( $34 \pm 21.5$  vs.  $157 \pm 42.0$  per view field,  $p < 0.02$ ; Fig. 6). In contrast, injection of PMP resulted in a marked increase in the amount of capillaries in the ischemic myocardium compared to the ischemic myocardium injected with PBS only, without PMP ( $97 \pm 27.3$ ,  $p < 0.05$ ). Therefore, locally applied PMP strongly stimulate angiogenesis in the chronically ischemic myocardium.

## 4. Discussion

Platelets contain various angiogenesis-related substances that release into the environment upon platelet activation. Moreover, it was recently demonstrated that platelets, as a cellular system, could induce an angiogenic response [5,23].

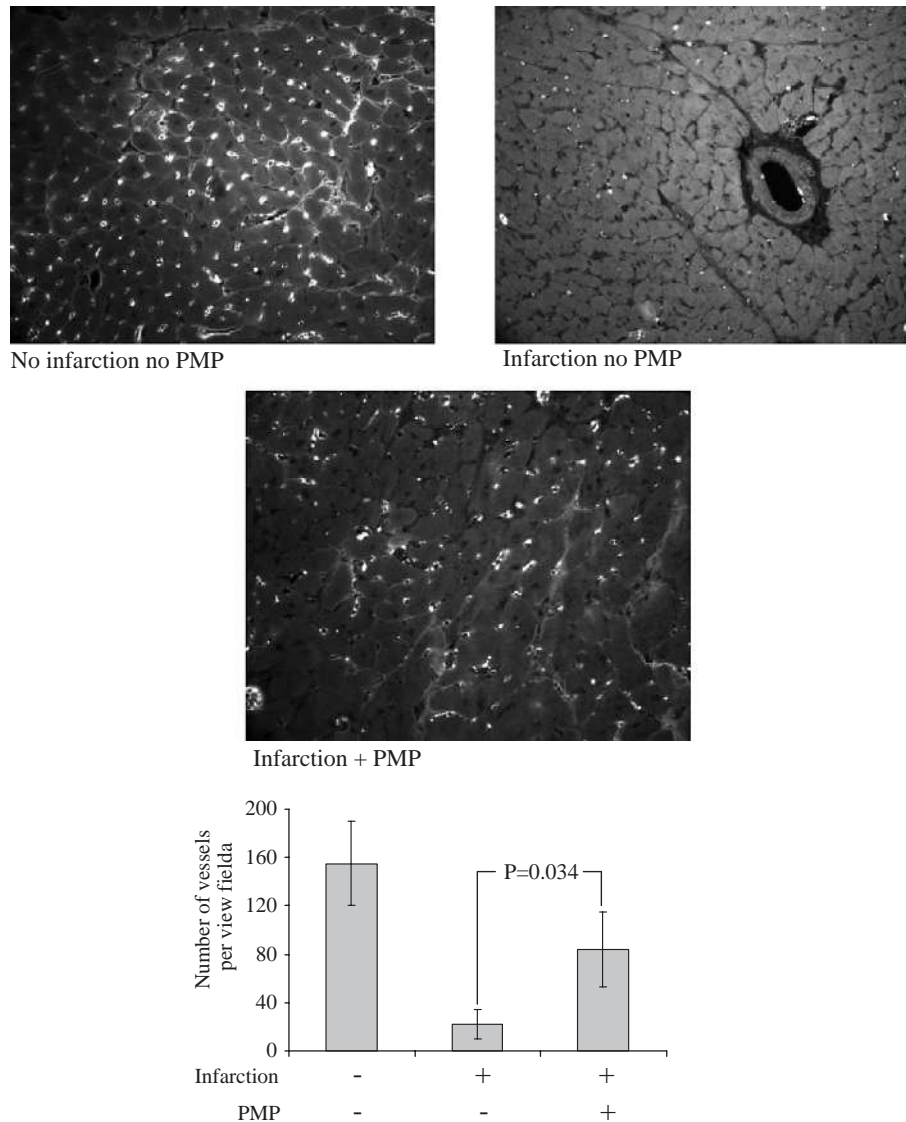


Fig. 6. PMP promote post-ischemic revascularization of myocardium. Sabra rats were anesthetized, intubated, and a left thoracotomy performed in the fourth intercostal space and the pericardium opened to expose the heart. Left coronary artery was ligated by a silk suture, and PMP (250  $\mu\text{g}/\text{ml}$  protein) in PBS (50  $\mu\text{l}$ ) were injected into the muscle of the left ventricle. Animals after LAD ligation injected with the same amount of PBS without PMP served as a control. Three weeks thereafter, rats were operated again and BSA conjugated with FITC was injected into left atrium. In 1 min animals were sacrificed and their hearts were used for preparation of paraffin-embedded sections. Samples were photographed with objective  $\times 20$ . Light grey color: blood vessels stained with BSA-FITC;  $n=3$  in each experimental group.

At the same time, platelet activation at sites of blood flow disturbances or endothelium injury results in formation of PMP. Since platelet activation frequently occurs at the sites where angiogenesis takes place (e.g., in the tumor vasculature, or in the proximity of thrombus in an ischemic site), a possible impact of PMP in blood vessel development would be of importance, either as a part of pathogenesis of the malicious processes, or as a counteracting factor.

In the present study, PMP triggered an angiogenic response, both in vitro and in vivo. This effect is mediated by intra-particle cytokines, i.e., VEGF, bFGF, and PDGF. Separate inhibition of each cytokine resulted in a significant suppression of the vessel sprouting, which suggests that a mutual action of pro-angiogenic compounds is needed for

the development of an angiogenic response. An absence of even one compound results in down-regulation of the whole process. These findings are generally in agreement with our previous report in which VEGF and bFGF mediated the pro-angiogenic effect of whole platelets [5]. However, in whole platelets, inhibition of PDGF did not result in sprouting inhibition in the aortic ring model. In contrast, PMP-induced vessel growth seems to be PDGF-dependent, which suggests a different cytokine ratio in PMP compared to platelets, where the absence of PDGF could be compensated by other agents operating at final stages of angiogenesis (possibly, bFGF).

In the present study, it was shown that PMP alone stimulated EC movement, which agrees with others [16].

However, in our experiments, EC invasion was examined through a layer of matrigel, which better mimics *in vivo* conditions. Additionally, agents which mediated EC invasion induced by PMP were identified. Their spectrum was similar to that described for whole platelets [5] (VEGF, PDGF, and heparanase), excluding bFGF, which is important for platelets, but is not involved in the experiments with PMP.

The angiogenic process is governed by a highly organized cascade of signaling events mediating basic steps of the vessel formation. PI3-kinase plays a pivotal role in mediating EC survival, proliferation, cytoskeleton reorganization, and cell motility, all critically important for vessel growth [24]. In our experiments, neutralization of PI3-kinase led to complete inhibition of sprouting, cell proliferation and movement in the ring vicinity. Moreover, the role of PI3-kinase in PMP-induced angiogenesis was confirmed in the invasion assay, in accordance with the previous findings by Kim et al. [16]. However, Kim et al. investigated the effects of PMP on basic properties of EC, such as proliferation, tube formation, and chemotaxis, whereas in the present study we have demonstrated the involvement of PI3-kinase in the process of PMP-induced angiogenesis *in toto*.

PI3-kinase is activated by angiogenesis-related cytokines, such as VEGF and bFGF [25]. Activation of PI3-kinase can occur by several mechanisms. VEGF receptor Flk-1/KDR on EC has been shown to be consistently associated with the regulatory subunit of PI3-kinase p85 [26] and to directly activate PI3-kinase upon binding to its ligand, VEGF. Furthermore, FGF receptor-1 activates PI3-kinase [27]. Additionally, activation of PI3-kinase may proceed via Src kinase family members [28]. The latter possibility was examined by inhibition of Src kinase using PP2, a potent inhibitor of Src activity. Application of the inhibitor robustly reversed the PMP-induced sprouting, which suggests that Src kinase was among the major pathways mediating the pro-angiogenic effect of PMP.

Stimulation of ERK by VEGF is a pathway mediating EC proliferation [29], whereas activated p38 contributes to vascular permeability and cell motility [30]. In our experiments, blocking the ERK kinase resulted in a significant inhibition of sprouting. In contrast, the p38 inhibitor did not affect PMP-induced vessel sprouting, which is in agreement with Zhu et al. [31], who report the role of p38 in recruitment of mural cells (vessel maturation), whereas premature “naked” vessels still developed even in the background of p38 inhibition.

Another signaling pathway involved in angiogenesis regulation is PKC [32]. In the present study, no effect of PKC blockage on PMP-induced angiogenesis was observed.

The pro-angiogenic activity of PMP was confirmed in the *in vivo* model of agarose beads transplantation. The intensities of PMP- and VEGF/FGF-induced angiogenesis were comparable, as judged by the capillary area in the skin in the bead vicinity. Histologic examination with immunostaining for vWF proved the presence of small vessels in the

skin around the beads. These results suggest that the stimulatory effect of PMP on angiogenesis is not restricted to artificial *in vitro* models but can also be exerted *in vivo*. This assumption was further proven by the observation that locally applied PMP induced a strong angiogenic response in the chronically ischemic myocardium. This finding expands the possible importance of PMP to the clinical situations where pathogenesis is dependent on the development of blood vessels *de novo*.

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