Original Paper

Up-Regulated Expression of Matrix Metalloproteinases in Endothelial Cells Mediates Platelet Microvesicle-Induced Angiogenesis

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Key Words

Matrix metalloproteinase • Platelet microvesicle • Angiogenesis • Human umbilical vein endothelial cell

Abstract

Background/Aims: Platelet microvesicles (PMVs) contribute to angiogenesis and vasculogenesis, but the mechanisms underlying these contributions have not been fully elucidated. In the present study, we investigated whether PMVs regulate the angiogenic properties of endothelial cells (ECs) via mechanisms extending beyond the transport of angiogenic regulators from platelets. **Methods:** *In vitro* Matrigel tube formation assay and *in vivo* Matrigel plug assay were used to evaluate the pro-angiogenic activity of PMVs. The effects of PMVs on the migration of human umbilical vein endothelial cells (HUVECs) were detected by transwell assay and wound-healing assay. Real-time PCR and western blot were conducted to examine mRNA and protein expression of pro-angiogenic factors in HUVECs. Matrix metalloproteinase (MMP) activity was assayed by gelatin zymography. Moreover, the effects of specific MMP inhibitors were tested. **Results:** PMVs promoted HUVEC capillary-like network formation in a dose-dependent manner. Meanwhile, PMVs dose-dependently facilitated HUVEC migration. Levels of MMP-2 and MMP-9 expression and activity were up-regulated in HUVECs stimulated with PMVs. Inhibition of MMPs decreased their pro-angiogenic and pro-migratory effects on HUVECs. Moreover, we confirmed the pro-angiogenic activity of PMVs *in vivo* in mice with subcutaneous implantation of Matrigel, and demonstrated that blockade of MMPs attenuated PMV-induced angiogenesis. **Conclusion:** The findings of our study indicate that PMVs promote angiogenesis by up-regulating MMP expression in ECs via mechanism extending beyond the direct delivery of angiogenic factors.

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Introduction

Blood vessels play great roles in the processes of supplying tissues with oxygen and nutrients and disposing of waste [1]. An imbalance in blood vessel growth contributes to the development of numerous malignant, inflammatory, and ischaemic disorders [2]. Angiogenesis is one mode of vessel formation and entails the sprouting of pre-existing vasculature to form new vessels. Angiogenesis requires many coordinated activities, such as endothelial cell (EC) proliferation, migration, and alignment to form vessel-like tube [3]. Many regulators of angiogenesis both endogenous and pharmacological have been described and continue to be identified [4, 5].

Platelet microvesicles (PMVs), also known as platelet microparticles (PMPs), are shed from platelets upon activation or apoptosis and commonly defined as lipid membrane vesicles of 0.1–1 µm in size [6]. In recent years, it has become clear that PMVs, similar to platelets, possess important biological functions [7] and are thus involved in various physiological and pathophysiological responses. PMVs also play a great role in neovascularization [8–10] by serving as a tool or mediator through which platelets exert their pro-angiogenic effects, as various molecules that have traditionally been considered soluble and are released by platelets, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), are in fact bound to PMVs [11].

ECs are thought to release a cocktail of angiogenic factors [3, 12–14], which account for their beneficial effects on vascular homeostasis. Mounting evidences indicate that PMVs not only passively transport various proteins and receptors from platelets or megakaryocytes to recipient cells, but also act as circulating signaling modules by inducing specific responses in target cells. We previously demonstrated that SSL5-PMPs, staphylococcal superantigen-like protein 5 (SSL5)-induced PMPs, provoke inflammatory mediator release in monocytes [15]. Besides, PMVs induce changes in early outgrowth cells (EOCs) secretome towards a more pro-angiogenic profile [10], which plays a pivotal role in vasculogenesis. However, the effects of PMVs on EC secretion of pro-angiogenic factors and whether these effects are involved in angiogenesis are unclear. Thus, we studied the role of PMVs in angiogenesis and explored potential molecules released by ECs that are critical in mediating such action.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (mycoplasma negative, C2519AS). The cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 200mM L-glutamine (Gibco) and 1% (v/v) penicillin/streptomycin (Beyotime Biotechnology, Beijing, China) in a 5%CO₂ humidified atmosphere at 37°C.

Preparation of Platelet Microvesicles

Human blood was collected by venipuncture with a 19-gauge needle into acid-citrate-dextrose (85mM trisodium citrate, 83mM dextrose, and 21mM citric acid) anticoagulant solution. Washed platelets were prepared as previously described [15]. Briefly, Platelet rich plasma (PRP) was obtained by centrifugation of the above sample at 110×g for 15 min to remove RBCs and leukocytes. Platelets were pelleted by centrifugation at 710×g for 15 min and washed three times with CGS buffer (120mM sodium chloride, 13mM sodium citrate, and 30mM dextrose, pH 7.0). The final pellet was resuspended in 0.22-µm filtered Tyrode's buffer (137mM NaCl, 2.8mM KCl, 12mM NaHCO₃, 1mM MgCl₂, 0.4mM Na₂HPO₄, 0.1% BSA, 10mM HEPES, 5.5mM glucose, pH 7.4) at 3×10⁸/ml. For generation of microvesicles, washed platelets in Tyrode's buffer containing 1 mM CaCl₂ were incubated with 30 µM thrombin receptor activator peptide 6 (TRAP6, Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37°C. Following the indicated incubation period, the sample was centrifuged at 710×g for 15 min to separate the platelets from microvesicles. The supernatants containing microvesicles were filtered through a 0.8-µm filter to remove residual platelets, and pelleted again at 25000×g for 60 min. Finally, the pellets containing microvesicles were resuspended in Tyrode's

buffer, which were directly used, or snap-frozen at -80°C to avoid repeated freezing and thawing. The amount of PMVs was quantified by both bicinchoninic acid (BCA) protein assay (Beyotime Biotechnology) and flow cytometry as we described previously [15]. We found that $50\text{ }\mu\text{g/ml}$ of protein concentration was equivalent to 6×10^6 /ml PMVs. Tyrode's buffer was used as a blank control ("Con" in the figures), while the supernatants ("Sup" in the figures) of centrifuged PMVs were used as a vehicle control in the experiments.

Antibody blocking

Neutralizing monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) were used to block PMVs and HUVECs interactions. CD40 ligand (CD40L)-mAb ($10\text{ }\mu\text{g/ml}$), CD40-mAb ($10\text{ }\mu\text{g/ml}$), or/and P-selectin glycoprotein ligand 1 (PSGL-1)-mAb ($50\text{ }\mu\text{g/ml}$) were preincubated with PMVs or HUVECs for 1 hour. Isotype monoclonal antibody of the same concentration was used for a control. Pan-MMP inhibitor GM6001 ($25\text{ }\mu\text{M}$, Tocris Bioscience, Bristol, UK), MMP2 inhibitor ARP101 ($25\text{ }\mu\text{M}$, Tocris Bioscience, Bristol, UK), and MMP9 inhibitor ab142180 ($25\text{ }\mu\text{M}$, Abcam, Cambridge, UK) were preincubated with HUVECs for 1 hours. Dimethylsulfoxide (DMSO) was used for a vehicle control.

In vitro Matrigel tube formation assay

Matrigel (Corning Costa Co., MA, USA) was thawed on ice at 4°C overnight. The solution was added into a 96-well plate ($60\text{ }\mu\text{L}$ per well) and incubated at 37°C for 1 h to allow matrix gel solidification. Afterwards, the HUVECs were trypsinized and seeded (1×10^4 cells per well) with Tyrode's buffer, supernatants, different concentrations (10 , 20 , or $50\text{ }\mu\text{g/ml}$) of PMVs, or VEGF (50 ng/ml , PeproTech Inc, Rocky Hill, USA) in serum-free DMEM. The plate was then incubated at 37°C for 6 h. Tube formation was observed under an inverted light microscope ($10\times$), and five representative fields from each well were photographed.

HUVEC proliferation assay

HUVEC proliferation was assessed by using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. Briefly, the cells were seeded into a 96-well plate (3×10^3 cells/well) and then treated with Tyrode's buffer, supernatants and different concentrations (10 , 20 , or $50\text{ }\mu\text{g/ml}$) of PMVs in serum-free DMEM for a period of 24 h and 48 h. After incubation, a CCK-8 solution was added to the medium (1:10 ratio) and incubated at 37°C for 2–4 h. The OD value was measured by the absorbance at 450 nm with an Infinite M200 Microplate Reader (Tecan, Austria).

Transwell chamber migration assay

The capacity of HUVECs to migrate was determined using a Boyden transwell chamber (Corning Costar, Cambridge, MA, USA) with $8.0\text{-}\mu\text{m}$ pore-size polycarbonate filter inserts in 24-well plates. The cells (1×10^5 /ml, $100\text{ }\mu\text{L}$) were seeded in the upper compartment in the presence of Tyrode's buffer, supernatants, and different concentrations (10 , 20 , or $50\text{ }\mu\text{g/ml}$) of PMVs in serum-free DMEM. The lower compartment contained $600\text{ }\mu\text{L}$ of DMEM supplemented with 10% FBS to stimulate cell migration. After incubating for 8 h at 37°C , the medium in the upper chamber was aspirated, and the cells on the upper surface of the membrane were removed by gentle scraping with a cotton swab. Following fixation with 4% paraformaldehyde at room temperature for 20 min and staining with crystal violet stain solution for 1 h, the migratory cells on the lower surface of the membrane of five random non-overlapping fields were counted.

In vitro wound-healing migration assay

The HUVECs were seeded into 6-well plates at a density of 5×10^5 /well in DMEM containing 10% FBS, 200 mM L-glutamine (Gibco) and 1% (v/v) penicillin/streptomycin. After the cells had grown to 80% confluence, a vertical line was created by scraping the cells with a sterile disposable pipette tip to make a scratch wound. Then, the cells were washed twice with phosphate buffered saline (PBS) and incubated in FBS-free DMEM with Tyrode's buffer, supernatants, or different concentrations (10 , 20 , or $50\text{ }\mu\text{g/ml}$) of PMVs. After incubating for 12 h and 24 h, the cells that had migrated to the wound areas were photographed with an inverted light microscope.

RNA isolation and RT-qPCR

RNA was extracted using RNAiso Plus (Takara Bio Inc., Otsu, Japan) and cDNAs were synthesized by a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara). The primers used in the real-time PCR analyses

Table 1. Primers used in the quantitative real-time PCR analyses

Gene	Forward primer	Reverse primer
HIF-1 α	5'-TTTGGCAGCAACGACACAG-3'	5'-GCGGTGGGTAATGGAGACAT-3'
VEGF-A	5'-CGGCCTTCGCTTACTCTCAC-3'	5'-TCATGGGCTGCTTCTTCCAA-3'
PDGF	5'-CCATTCCCGAGGAGCTTATG-3'	5'-CAGCAGGCGTTGGAGATCAT-3'
IL-8	5'-AGTCCTTGTTCCACTGTGCC-3'	5'-AGTGCTTCCACATGCTCTCAC-3'
MMP-2	5'-GATACCCCTTTGACGGTAAGGA-3'	5'-CCTTCTCCCAAGGTCCATAGC-3'
MMP-7	5'-GGATGGTAGCAGTCTAGGGATTAAC-3'	5'-GGAATGTCCCATACCCAAAGAA-3'
MMP-9	5'-TATGACATCCTGCAGTGCC-3'	5'-TTGTATCCGGCAAACCTGGCT-3'
TIMP-1	5'-ACCACCTTATACCAGCGTTATGA-3'	5'-GGTGTAGACGAACCGGATGTC-3'
GAPDH	5'-TTTGGCTACAGCAACAGGGT-3'	5'-GGGAGATTCACTGTGGTGGG-3'

are listed in Table 1. The PCR amplification protocol involved 40 cycles of denaturation at 95°C for 10 seconds, primer annealing at 59°C for 20 seconds and primer extension at 72°C for 10 seconds. Each sample was analyzed at least in triplicates.

Western blot analysis

The cells were harvested and lysed in RIPA buffer (Beyotime Biotechnology) that contained a cocktail of both protease and phosphatase inhibitors (Roche, Basel, Switzerland). Protein concentrations were determined by BCA protein assay (Beyotime Biotechnology). The protein samples were subjected to 10% SDS-PAGE. After the proteins were transferred to PVDF membranes, the membranes were blocked and incubated with various primary antibodies at 4°C overnight. Anti-phospho-Erk1/2 (Thr185/Tyr187), anti-Erk1/2, anti-phospho-Akt (Ser473), and anti-Akt (Ser473) were purchased from Cell Signaling Technology, and anti-MMP2, anti-MMP7, anti-MMP9, and anti-TIMP-1 were purchased from Abcam. GAPDH (Sigma) was used as a loading control. The protein blots were visualized using an ECL Western Blotting Kit (Millipore, Boston, MA, USA).

Gelatin zymography

MMP-2/MMP-9 protein abundance and activation in conditioned medium were analyzed by gelatin zymography as described previously [15, 16]. Briefly, HUVECs were incubated in the presence of Tyrode's buffer, supernatants, or different concentrations (10, 20, or 50 μ g/ml) of PMVs in serum-free medium for 24 hours. Equivalent amounts (protein concentration) of cell culture supernatants quantified by BCA protein assay method were run on 10% SDS-polyacrylamide gels copolymerized with 1mg/ml of gelatin (Sigma) in Laemmli's sample buffer (Bio-Rad Laboratories Inc). After electrophoresis, gels were washed twice for 45 minutes in 2.5% Triton X-100, agitated in development buffer (0.05 M Tris-HCl pH 8.8, 200 mM NaCl, 5 mM CaCl_2 , 0.02% NaN_3) for 15 minutes at room temperature and then transferred to a 37°C incubator for 24 hours. After incubation, gels were immersed in fixing/destaining solution (methanol: acid: water, 4.5:1:4.5, v: v: v) at room temperature for 15 minutes and stained in Coomassie brilliant blue R250 solution (Beyotime Biotechnology) for 1 hours. Finally, gels were destained properly with fixing/destaining solution and were visualized using Molecular Imager® ChemiDoc™ XRS+ (Bio-Rad Laboratories Inc). Densitometric quantification of gelatinolytic activity of the band was measured in terms of pixel intensity by the Image Lab™ Software (Bio-Rad Laboratories Inc).

In vivo Matrigel plug assay

Matrigel plug assay was performed as previously described [17]. The *in vivo* angiogenesis protocol using a murine model of Matrigel plug subcutaneous implantation was approved by the Animal Ethics Committee of Third Military Medical University. Male C57BL/6 mice (6-8 weeks) were obtained from the Laboratory Animal Center of Third Military Medical University.

Mice were received subcutaneous injection using a 25-gauge needle in the right flanks of Matrigel gel (400 μ L) containing Tyrode's buffer, supernatants, PMVs (50 μ g/ml), PMVs (50 μ g/ml) plus different MMP inhibitors, VEGF (50 ng/ml) or VEGF with GM6001 (25 μ M), respectively. The Matrigel plugs were removed two weeks after implantation and photographed under a modular stereo microscope (Leica MZ10 F, Wetzlar, Germany), after which one portion of the plugs was fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned for hematoxylin and eosin (H&E)-staining.

Statistical analysis

Data analysis was performed using the GraphPad Prism 5.0 software package (San Diego, CA, USA). All experimental data are expressed as the mean $\pm$ standard error of the mean (SEM), and each experiment was repeated at least three times, unless otherwise stated. Data comparisons were performed using Student's t-test or one-way analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant.

Results

PMVs promote HUVEC tube formation *in vitro*

We examined the effect of PMVs on HUVEC tube formation with an *in vitro* Matrigel model. HUVECs were seeded on a Matrigel substrate supplemented with Tyrode's buffer, supernatants, or different concentrations (10, 20, or 50 $\mu\text{g}/\text{ml}$) of PMVs, and VEGF was set as a positive control. The results showed that PMVs enhanced HUVEC tube formation in a dose-dependent manner (Fig. 1 A and B), while the supernatants also induced a slight increase in capillary-like tube formation compared with the blank controls.

Effects of PMVs on HUVEC proliferation and migration

Proliferation and migration of ECs are involved in the process of angiogenesis. The CCK8 assay data showed that supernatants and PMVs promoted HUVEC proliferation and that the enhancements of HUVEC proliferation were similar compared with the blank controls (Fig. 2 A and B). In addition, we determined the effects of PMVs on HUVEC motility by performing a Boyden chamber chemotactic migration assay and found that PMVs treatment led to dose-dependent promotion of HUVEC migration (Fig. 2 C and D). Furthermore, our wound-healing assay also showed that HUVEC wound healing was gradually accelerated in a PMV concentration-dependent manner when determined at both 12 h and 24 h (Fig. 2 E and F).

Fig. 1. PMVs promote HUVEC tube formation. (A) An *in vitro* angiogenesis assay of HUVECs was performed on Matrigel cast in a 96-well culture plate. Tube formation of HUVECs (1×10^4 per well) in the presence of Tyrode's buffer (Con), supernatants (Sup), and different concentrations (10, 20, or 50 $\mu\text{g}/\text{ml}$) of PMVs were observed after a 6 h-culture using an LEICA DMIRB microscope. VEGF (50 ng/ml) was set as a positive control. Images from five randomly selected fields were acquired (10 $\times$). Images shown are representatives of five independent experiments. (B) Tube number was counted in each field, and the tube number of each treatment was reported as the mean of the counts from five randomly fields ($n=5$). Data are presented as the mean $\pm$ SEM. ** $P < 0.01$ vs. Con. ## $P < 0.01$ vs. Sup. \$ $P < 0.05$ vs. PMVs (50 $\mu\text{g}/\text{ml}$). Scale bar represents 100 μm .

Fig. 2. Influences of PMVs on HUVEC proliferation and migration. After HUVECs were cultured in the presence of Tyrode's buffer (Con), supernatants (Sup), and different concentrations (10, 20, or 50 µg/ml) of PMVs for 24 h (A) or 48 h (B). CCK-8 assay was used to detect the proliferation of the cells (n=3). (C) Images of Boyden transwell assay of HUVECs treated with or without PMVs. (D) The number of HUVECs that migrated to the lower side of the membranes after incubation for 8 h is shown (n = 3). (E) Images of wound-healing assay of HUVECs at 0, 12 and 24 h after scratch wound placements. (F) The percent wound closure at 12 and 24 h after scratch wound placements is shown (n = 3). Data are presented as the mean ± SEM. **P < 0.01 vs. Con. ***P < 0.01 vs. Sup. Scale bar represents 100µm.

PMVs up-regulate MMP-2 and MMP-9 expression and activity in HUVECs

We investigated the mechanism underlying PMV-induced HUVEC tube formation and migration further. We first analyzed the effects of PMVs on the expression of some common pro-angiogenic factors. Interestingly, HIF-1α, VEGF-A, PDGF mRNA levels remained unchanged, while IL-8 levels were significantly reduced (Fig. 3A). Numerous studies have indicated that matrix metalloproteinases (MMPs), such as MMP-2, MMP-7, MMP-9 and TIMP-1, which mediate the process of extracellular matrix (ECM) remodeling, play a critical role in cell migration progress, and are a prerequisite for angiogenesis [18–22]. Thus, we next assessed the production of the four MMPs involved in angiogenesis. The quantitative real-time PCR and western blotting results showed that PMVs dose-dependently stimulated MMP-2 and MMP-9 expression in HUVECs, while there was no change in MMP-7 and TIMP-1 production (Fig. 3 A, B and C). Moreover, gelatin-zymography analysis showed that PMVs significantly stimulated synthesis and activation by HUVECs of MMP-2 and MMP-9 in a dose-dependent manner, while MMP-2 and MMP-9 derived from PMVs did not measurably contribute to gelatinolytic activity (Fig. 3 D and E).

Fig. 3. Effects of PMVs on the expression of angiogenesis-related molecules in HUVECs. (A) qPCR analysis of angiogenesis-related factors mRNA expression after treatment for 24 h as described in the Materials and Methods ($n = 3$). (B) MMPs and TIMP-1 protein expression were determined by western blot and normalized to the GAPDH levels. (C) The protein bands were quantified by densitometric analysis ($n = 3$). (D) Representative zymograms of cell supernatants subjected to gel electrophoresis. (E) The gelatinolytic activity of the transparent bands was quantified by densitometric analysis ($n = 3$). (F) HUVECs were treated with Tyrode's buffer (Con), supernatants (Sup), and different concentrations of PMVs (50 μ g/ml) for 0–60 min. The levels of phosphorylated and total of ERK1/2 and Akt were assessed by western blot analysis. (G) The protein bands were quantitated by densitometric analysis ($n = 3$). Data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. Con. # $P < 0.05$, ## $P < 0.01$ vs. Sup.

Fig. 4. Pan-MMP inhibitor weakens PMV promotion of HUVEC tube formation and migration. HUVECs were pretreated with the pan-MMP inhibitor GM6001 (25 μ M) for 1 hour, and co-cultured with Tyrode's buffer (Con), supernatants (Sup), and PMVs (50 μ g/ml) for 6 h (A), 8 h (C), and 24 h (E). Representative images of *in vitro* angiogenesis assays (VEGF (50 ng/ml) was set as a positive control), transwell assays and wound-healing assays are shown. (B) The tube number of each treatment is shown (n = 5). (D) The number of HUVECs per treatment that migrated to the lower side of the membranes is shown (n = 3). (E) The percent wound closure at 12 and 24 h after scratch wound placements is shown (n = 3). Data are presented as the mean $\pm$ SEM. * P < 0.05, ** P < 0.01 vs. Con, ## P < 0.01 vs. Sup, $^{\$}$ P < 0.05 vs. PMVs (50 μ g /ml), and † P < 0.05, †† P < 0.01 vs. Con, Sup, PMVs treatment without inhibitor. Scale bar represents 100 μ m.

PMVs promote HUVEC tube formation and migration by promoting MMP secretion

We sought to determine the role of MMPs in PMV pro-angiogenic activity using the MMP inhibitors. Firstly, HUVECs were pretreated with pan-MMP inhibitor GM6001 (25 μ M) for 1 h. As shown in Fig. 4 A and B, PMV-enhanced tube formation was markedly inhibited by GM6001, while there was no difference between the two groups with VEGF in the absence or presence of GM6001. In addition, the Boyden transwell migration assay and wound-healing assay results both showed that MMP inhibition by GM6001 significantly weakened the HUVEC migration enhancements stimulated by PMVs (Fig. 4 C, D, E and F). Besides, differential role of MMP2 and MMP9 were further defined by using specific inhibitors. As shown in Fig. 5, MMP-2 and MMP-9 inhibitors both weaken the PMV-enhanced tube formation and migration. Interestingly, the efficacy of MMP-2 inhibitor surpassed that of MMP-9 inhibitor. These results suggested that MMPs are pivotal in PMV-induced HUVEC tube formation and migration enhancements, and that MMP-2 plays a more important role than MMP-9.

Furthermore, we also explored the potential molecular basis of PMVs and HUVECs interactions resulting in angiogenesis. Platelet-derived P-selectin and CD40L are two

Fig. 5. Effects of specific MMP-2 and MMP-9 inhibitors on PMV-enhanced angiogenesis. HUVECs were treated PMVs (50 μ g/ml) in the presence of the MMP-2 inhibitor (MMP2i) ARP101 (25 μ M) and MMP-9 inhibitor (MMP9i) ab142180 (25 μ M) for 6 h (A), 8 h (C), and 24 h (E). Representative images of *in vitro* angiogenesis assays, transwell assays and wound-healing assays are shown. (B) The tube number of each treatment is shown (n = 5). (D) The number of HUVECs per treatment that migrated to the lower side of the membranes is shown (n = 3). (E) The percent wound closure at 12 and 24 h after scratch wound placements is shown (n = 3). Data are presented as the mean $\pm$ SEM. *P < 0.05, **P < 0.01 vs. Tyrode's buffer (Con), #P < 0.05, ##P < 0.01 vs. supernatants (Sup), and [†]P < 0.05, ^{††}P < 0.01 vs. Con, Sup, PMVs treatment without inhibitors. Scale bar represents 100 μ m.

important molecules involved in angiogenesis [23, 24]. P-selectin and CD40L are known to mediate platelet-endothelial cell (EC) interactions by binding to their respective receptors PSGL-1 and CD40 expressed on ECs [25]. We observed that CD40L or CD40 blocking antibodies had no effect on PMV-enhanced tube formation, nor for the use of PSGL-1 mAb to block P-selectin binding to PSGL-1 (Fig. 6).

PMVs enhance angiogenesis mediated by MMPs in vivo

We exploited pro-angiogenic effect of PMVs *in vivo* in mice with Matrigel plug implants. As shown in Fig. 7A, a 2-week implantation of Matrigel plugs containing Tyrode's buffer and supernatants did not induce marked new vessel formation. However, Matrigel plugs containing PMVs induced significant angiogenesis, and the new vessels were much better developed, as evidenced by a more intense branching of the new vessels. In contrast, Matrigel plugs containing PMVs mixed with MMP inhibitors induced considerably less angiogenesis than their counterparts, and the new vessels were less well developed and

Fig. 6. Lack of effect of CD40L-CD40 and P-selectin-PSGL-1 interactions in PMV-enhanced angiogenesis. HUVECs were treated with PMVs (50 $\mu\text{g}/\text{ml}$) in the presence of anti-CD40L (10 $\mu\text{g}/\text{ml}$), anti-CD40 (10 $\mu\text{g}/\text{ml}$) monoclonal antibody (A) or anti-PSGL-1 (50 $\mu\text{g}/\text{ml}$) monoclonal antibody (B) for 6 hours. Representative images of *in vitro* angiogenesis assays are shown in the left panel. The tube number of each treatment is shown in the right panel ($n = 5$). Data are presented as Mean $\pm$ SEM. ** $P < 0.01$ vs. Tyrode's buffer (Con), ## $P < 0.01$ vs. supernatants (Sup), Scale bar represents 100 μm .

with limited branches than those induced by their counterparts. This is in contrast to the lack of effect of pan-MMP inhibitor GM6001 in VEGF-enhanced angiogenesis. Besides, the inhibitory effect of MMP-2 inhibitor also surpassed that of MMP-9 inhibitor *in vivo*. When branch numbers of newly formed vessels were counted, PMVs induced significantly more angiogenesis than tyrode's buffer and supernatants, MMP inhibitors could markedly weaken PMV enhancement of vessel formation, while MMP inhibition had no effect on VEGF-induced angiogenesis *in vivo* (Fig. 7B). The H&E staining of paraffin-embedded Matrigel plug sections also revealed that new vessel formation was rare in the two control plugs, most intense and well-developed in PMV plugs, and thinly scattered in the plugs supplemented with PMVs and MMP inhibitors (Fig. 7C).

Discussion

Of the microvesicles circulating in blood, 70% to 90% are derived from platelets [15, 26] in response to various stimuli, such as ADP, collagen, thrombin, or following complement activation [27]. Since platelet activation, characteristically occurs at the sites of blood flow disturbances or endothelial injury, where angiogenesis takes place (e.g., in tumor vasculature [28], or in proximity to thickened tunica intima [29]), the possible impact of PMVs on blood vessel development is an important issue.

Previous studies have demonstrated that PMVs, which serve as a source of cytokines and growth factors, may be involved in angiogenesis and vasculogenesis [8-10, 30]. Consistent with these findings, our experiments revealed that PMVs enhanced sprouting angiogenesis *in vitro* and *in vivo*. It is proposed that platelets exhibit pro-angiogenic activity via both membrane proteins and platelet releasates [11, 31, 32]. Indeed, we found that the supernatants of centrifuged PMVs, which are similar to platelet releasates but lack PMVs, slightly enhanced HUVEC tube formation, compared with Tyrode's buffer, findings in line with those of the above studies. Additionally, we showed that PMVs promoted HUVEC proliferation in a non-dose-dependent manner, similar to the supernatants. Kim et al. reported that PMVs concentration-dependently facilitate EC proliferation [8]. Such discrepancy may be due to the fact that they only used the blank control in their experiments. Our transwell assay and wound-healing assay results further indicated that PMVs increased HUVEC migration, compared with the both negative controls. Thus, the pro-angiogenic activity of

Fig. 7. PMVs promote angiogenesis mediated by MMPs *in vivo*. (A) Photographs of each Matrigel plug harvested from mice were taken under a modular stereo microscope (Leica MZ10 F, Wetzlar, Germany), 8 $\times$. (B) Angiogenesis intensity was assessed according to the numbers of new vessel branches that formed in the Matrigel plugs (n = 6); Data are presented as the mean $\pm$ SEM. ** P < 0.01, vs. Tyrode's buffer (Con); * P < 0.05, ** P < 0.01 vs. supernatants (Sup), ^{ss}P < 0.01 vs. PMVs (50 μ g/ml), ^{+}P < 0.05, ^{++}P < 0.01 vs. PMVs without inhibitor. (C) Paraffin-embedded implantation plug sections were stained with hematoxylin and eosin. New vessel formation (black arrowheads) was observed at 200 $\times$.

PMVs surpasses that of supernatants because of the superior ability of PMVs in promoting endothelial cell migration.

It's well known that PMVs, like cargo carriers, passively carry and transport an array of platelet-derived products, including adhesive molecules such as glycoprotein (GP)-Ib α , GPIIb/IIIa, P-selectin and CD40L; chemokines; bioactive lipids; inflammatory proteins and microRNAs to recipient cells, regulating intracellular signaling or gene transcription of target cells [33-35]. We and others have demonstrated that PMVs interact with monocytes and modulate their pro-inflammatory functionality [15, 36, 37]. Mause et al. showed that PMVs shift the multifaceted secretome of EOCs toward a more pro-angiogenic composition featuring elevated levels of angiogenic growth factors, such as VEGF and epidermal growth factor (EGF), but not MMPs [10]. In the current study, we showed that PMVs up-regulated expression and activity of MMP-2 and MMP-9 in HUVECs, whereas PMVs did not affect VEGF-A, PDGF, HIF-1 α , MMP-7, and TIMP-1 levels, and even decreased IL-8 mRNA level. These diverse actions of PMVs as revealed in the present study are in contrast to that reported by Mause et al. and may be due to different cell types used in the experiments. Further study is warranted to comprehensively and thoroughly explore the effects of PMVs on the modified endothelial cell secretome.

MMPs promote endothelial cell migration and tube formation by proteolytically remodelling the basement membrane [2, 38]. Among these, MMP-2 and MMP-9 have been emphasized because their type IV collagenase activity is essential during the initial phase of angiogenesis [20, 39]. In the current study, we found that MMP-2 might play a more important role than MMP-9 in PMV-enhanced angiogenesis.

Certain signaling pathways, such as the ERK and Akt signaling pathways, are known to be involved in the expression of MMPs associated with angiogenesis [40, 41]. As shown

in Fig. 3 F and G, phosphorylated ERK (P-ERK) levels were significantly increased after 10 min of treatment with PMVs, while Akt signaling pathway activity levels were decreased. Consistent with the above observations, Brill et al. found that ERK is essential for PMV-induced angiogenesis since ERK inhibitors can reverse the pro-angiogenic effect of PMVs [9]. Besides, other signaling pathways, such as PI3K and Src pathways, may also play great roles in angiogenesis. Furthermore, our results showed that CD40L-CD40 and P-selectin-PSGL-1 interactions were not involved in PMV-enhanced angiogenesis, which indicated that there might be other molecules carried by PMVs that relate to the pro-angiogenic activity. Therefore, it is necessary to identify the potential signaling pathways and molecules associated with PMV-enhanced angiogenesis in future studies.

We and others have confirmed the pro-angiogenic activity of PMVs *in vivo* [9]. Notably, PMVs may also exert adverse effects by promoting angiogenesis. We previously reported that plasma PMVs concentrations are higher in patients with acute coronary syndrome than in subjects with stable angina or healthy controls[42]. Moreover, several studies have demonstrated that platelet activation and high PMV levels are associated with atherosclerotic plaque progression [43, 44]. Recently, intraplaque angiogenesis, which is characterized by immature and leaky microvessels, has been regarded as an important risk factor contributing to the development of intraplaque hemorrhage and plaque destabilization [45, 46]. In addition, MMPs induce matrix degradation and cause fibrous cap thinning, which may also play great roles in atherosclerosis development. Therefore, further investigating whether and how PMVs are involved in atherosclerosis intraplaque angiogenesis is warranted.

In summary, we identified a novel mechanism by which PMVs promote EC angiogenic capacity and support vascular integrity maintenance after arterial injury. Specifically, we demonstrated for the first time that PMVs enhance endothelial cell pro-angiogenic activity by up-regulating MMP-2/-9 expression and gelatinolytic activity, possibly through the ERK signaling pathway activation, while MMP-2 might play a more important role than MMP-9. Our data have provided us with insights regarding the novel idea that PMVs may promote angiogenesis by up-regulating MMP expression in ECs, in addition to directly transporting angiogenic factors. Besides, this finding also expands the possible importance of PMVs in the clinical conditions where pathogenesis is related to angiogenesis, such as plaque angiogenesis and intraplaque hemorrhage in atherosclerotic plaque destabilization [45-47].

Disclosure Statement

The authors declare no conflict of interest.

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