

Platelet Microparticles Enhance the Vasoregenerative Potential of Angiogenic Early Outgrowth Cells After Vascular Injury

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Background—Angiogenic early outgrowth cells (EOCs) have been reported to contribute to endothelial regeneration and to limit neointima formation after vascular injury. Vascular pathologies comprise platelet activation and concomitant generation of platelet microparticles (PMPs). We hypothesized that PMPs may interact with EOCs in the context of vascular injury and modulate their regenerative potential.

Methods and Results—Using flow cytometry, confocal microscopy, and scanning electron microscopy, we demonstrated the binding of thrombin/collagen-induced PMPs to EOCs with subsequent membrane assimilation and incorporation. This interaction promoted phenotypic alterations of EOCs with increased expression of endothelial cell markers and transfer of the chemokine receptor CXCR4 to EOCs with enhanced responsiveness to its ligand CXCL12/SDF-1 α . In addition, PMPs augmented the adhesion of EOCs to extracellular matrix components and to the injured vessel wall and accelerated cytoskeletal reorganization and migration of EOCs. PMPs induced changes in the EOC secretome toward a more proangiogenic profile and amplified the EOC-mediated induction of proliferation, migration, and capillary tube formation by mature endothelial cells. Compared with untreated EOCs, the injection of PMP-treated EOCs resulted in accelerated reendothelialization after arterial denudation injury in athymic nude mice, whereas the EOC-mediated reduction of neointima formation remained unchanged.

Conclusions—Our data provide evidence that PMPs can boost the potential of EOCs to restore endothelial integrity after vascular injury. Major mechanisms involve the enhancement of EOC recruitment, migration, differentiation, and release of proangiogenic factors. (*Circulation*. 2010;122:495-506.)

Key Words: endothelium ■ inflammation ■ restenosis ■ platelets

Restenosis resulting from neointimal hyperplasia, negative remodeling, and elastic recoil is considered the Achilles heel of interventional cardiology.¹ In addition to the control of inflammatory reactions and smooth muscle cell (SMC) expansion, reendothelialization after vascular damage is a crucial mechanism limiting neointima formation.² Endothelial regeneration at sites of primary or iatrogenic vascular injury has been thought to rely mainly on the migration and proliferation of resident endothelial cells from the adjacent intact vasculature. However, recent studies revised this concept by demonstrating that angiogenic early outgrowth cells (EOCs), which exhibit phenotypic features of myeloid and endothelial cells and are known as endothelial progenitor cells, are recruited to sites of injury and accelerate reendothelialization.³ Notably, infusion of EOCs attenuates neointimal hyperplasia after arterial injury.⁴⁻⁶ Furthermore, EOCs may contribute to neovascularization in both ischemic hind limbs and acute myocardial infarction models.⁷

Clinical studies demonstrating a negative correlation of the number and functional capacity of circulating CD34⁺ vascular endothelial growth factor receptor-2 (VEGFR2)⁺ progenitor cells or ex vivo expanded EOCs with the extent or risk of cardiovascular disease have drawn further attention to the concept of cell-based therapy for cardiovascular diseases.⁷ Early clinical studies have produced conflicting findings with absent or moderate therapeutic effects, highlighting the need for a better understanding of underlying biological mechanisms.

Clinical Perspective on p 506

Relevant vascular stenosis and injury coincide with platelet activation, regularly accompanied by increased formation of platelet microparticles (PMPs).^{8,9} Elevated circulating PMP levels have been reported in patients with atherosclerosis acute vascular syndromes, or diabetes mellitus.¹⁰ These membrane vesicles with a size range of 0.1 to 1.0 μ m are shed by platelets after stimulation with physiological agonists (eg,

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thrombin and collagen) or in response to high shear stress in the vasculature.⁹ PMPs carry an array of platelet-derived products, including glycoprotein (GP) Ib, GPIIb/IIIa, P-selectin, CXCR4 receptor, chemokines, and various bioactive lipids, which they can transfer to recipient cells.^{11–13} For instance, PMPs may serve as a transcellular delivery system for CCL5/RANTES triggering monocyte recruitment to atherosclerotic endothelium.¹¹ PMPs can also directly bind to leukocytes and hematopoietic stem cells to promote adhesion.^{14,15} Besides procoagulant and inflammatory functions, PMPs may exhibit proangiogenic activity by promoting proliferation, migration, and formation of capillary-like structures of endothelial cells.¹⁵ Injection of PMPs into the myocardium stimulated postischemic neovascularization after chronic ischemia.¹⁶ Both growth factors (eg, vascular endothelial growth factor, basic fibroblast growth factor, or platelet-derived growth factor) and lipid components have been implicated in these effects.

Here, we evaluated whether PMPs can amplify the capacity of EOCs to mediate endothelial recovery after vascular injury. We demonstrated that PMPs modulate functional features of EOCs crucial for their regenerative potential, including recruitment and migration to sites of injury, differentiation, and release of angiogenic factors stimulating resident endothelial cells.

Methods

An expanded Materials and Methods section is available in the online-only Data Supplement.

Cell Isolation and Culture

Isolation and culture of EOCs and human umbilical vein endothelial cells (HUVECs) were performed according to established protocols.^{17,18} Platelets were isolated from platelet-rich plasma of healthy donors and activated with thrombin and collagen for 30 minutes.¹¹ After 1250g centrifugation, supernatants containing PMPs were gently passed through a 0.8- μ m filter and pelleted at 20 000g for 20 minutes. If not stated otherwise, EOCs were incubated with PMPs (30 μ g protein/mL, 27 230 \pm 7140 PMPs/ μ L) for 15 minutes and subsequently centrifuged at 500g for 7 minutes to remove unbound PMPs. For preparation of conditioned medium (CM), EOCs were washed and cultured in RPMI-1640 medium plus 1% FBS in the absence/presence of PMPs (CM-EOC versus CM-EOC/PMP). After 24 hours, the medium was collected, centrifuged at 20 000g, and passed through a 0.22- μ m filter.

Flow Cytometry

For characterization of surface molecules on EOCs and PMPs, analysis of AKT signaling (Cell Signaling Technology, Danvers, Mass), and determination of actin polymerization (F-actin staining, Sigma-Aldrich, Munich, Germany), we applied flow cytometry and followed the manufacturer's protocol for the respective antibodies. For flow cytometry-based PMP quantification, we used calibrated Trucount beads and standard-size beads. PMPs were then evaluated as a count of GPIb- and annexin V–positive particles with a size \leq 1.0 μ m.

Confocal Laser Scanning Microscopy

EOCs were prelabeled with lectin-FITC, reacted with CM-DiI–labeled PMPs for indicated time intervals, and subjected to analysis after fixation. Serial Z-stacked (0.5 μ m per section) confocal imaging was performed with a Zeiss LSM-510 Meta confocal microscope (Carl Zeiss, Jena, Germany), and image analysis was performed with LSM-510 software and Image-Pro Plus (Media Cybernetics, Bethesda, Md).

Scanning Electron Microscopy

Scanning electron microscopy (SEM) for analysis of untreated/PMP-treated EOCs was performed as described.¹¹ For determination of PMP binding to EOCs, we applied immunogold labeling detecting PMP-derived GPIb. For evaluation of CXCR4 transfer, PMPs were reacted beforehand with a CXCR4 monoclonal antibody (mAb) and secondary antibody conjugated with 20-nm gold particles, thoroughly washed, and subsequently incubated with EOCs.

In Vitro Adhesion Assays in Flow

Laminar flow assays analyzing the adhesion of EOCs to various substrates under flow conditions (1.5 dynes/cm²) were performed as described.¹¹

Intracellular Calcium Flux and Proliferation

Changes in the intracellular calcium levels of EOCs loaded with fluo-4AM were analyzed with a dual-excitation spectrofluorometer. Proliferation of EOCs and HUVECs was determined by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) according to the manufacturer's protocol (Merck, Darmstadt, Germany).

Cell Spreading, Migration, and Vascular Tube Formation on Matrigel

EOCs treated with/without PMPs were seeded on fibronectin-coated coverslips, and after various time periods, spreading was stopped by cell fixation. After staining for F-actin, cell spreading was analyzed with ImageJ software (NIH Image). Migration of EOCs treated with/without PMPs was evaluated by the use of modified Boyden chambers with 5- μ m-pore filters. In an integrative scratch wound assay, cell monolayers of EOCs or HUVECs were wounded linearly with a pipette tip and continuously cultured in unconditioned medium, in CM or with PMPs. The wounded area recovered with cells was analyzed with ImageJ software. Vascular tube formation of EOCs or HUVECs seeded on matrigel was examined by phase-contrast microscopy.

Secretome Profiling

The Human Angiogenesis Array Kit (R&D Systems, Minneapolis, Minn) was used according to the manufacturer's protocol to simultaneously evaluate angiogenic factors in the CM. Some factors were measured with flow cytometric bead-based multiplex assays (Bender MedSystems, Vienna, Austria) or ELISA (R&D Systems or RayBiotech, Cologne, Germany) according to the manufacturer's protocol.

Reverse-Transcription Polymerase Chain Reaction Analysis of CXCR4 and Transfection With Small Interfering RNA

Reverse-transcription polymerase chain reaction for analysis of CXCR4 on EOCs was performed as described.¹⁷ For downregulation of CXCR4, EOCs were transfected with a small interfering RNA (siRNA) sequence designed to specifically target messenger RNA–encoding CXCR4 (Qiagen, Valencia, Calif) or with control siRNA. Transfection of cells was enabled by the use of Fugene HD (Roche, Basel, Switzerland) according to the manufacturer's instruction.

Murine Model of Arterial Wire-Induced Injury

To evaluate EOC recruitment in situ, intravital microscopy of the carotid arteries from wild-type C57BL/6J mice was performed. CM-DiI–labeled EOCs, pretreated with/without PMPs, were injected intracardially 5 minutes after wire-induced carotid injury, and adherence of cells was subsequently examined by epifluorescence microscopy. For evaluation of reendothelialization and neointima formation, CM-DiI–labeled EOCs treated with/without PMPs were injected (5×10^5 cells) in athymic NMRI nude mice after carotid injury. After 5 days or 3 weeks, in situ perfusion fixation was performed, and neointimal and medial areas were quantified in serial sections by Movat pentachrome staining and planimetry. Reendothelialization was determined by luminal staining for CD31.

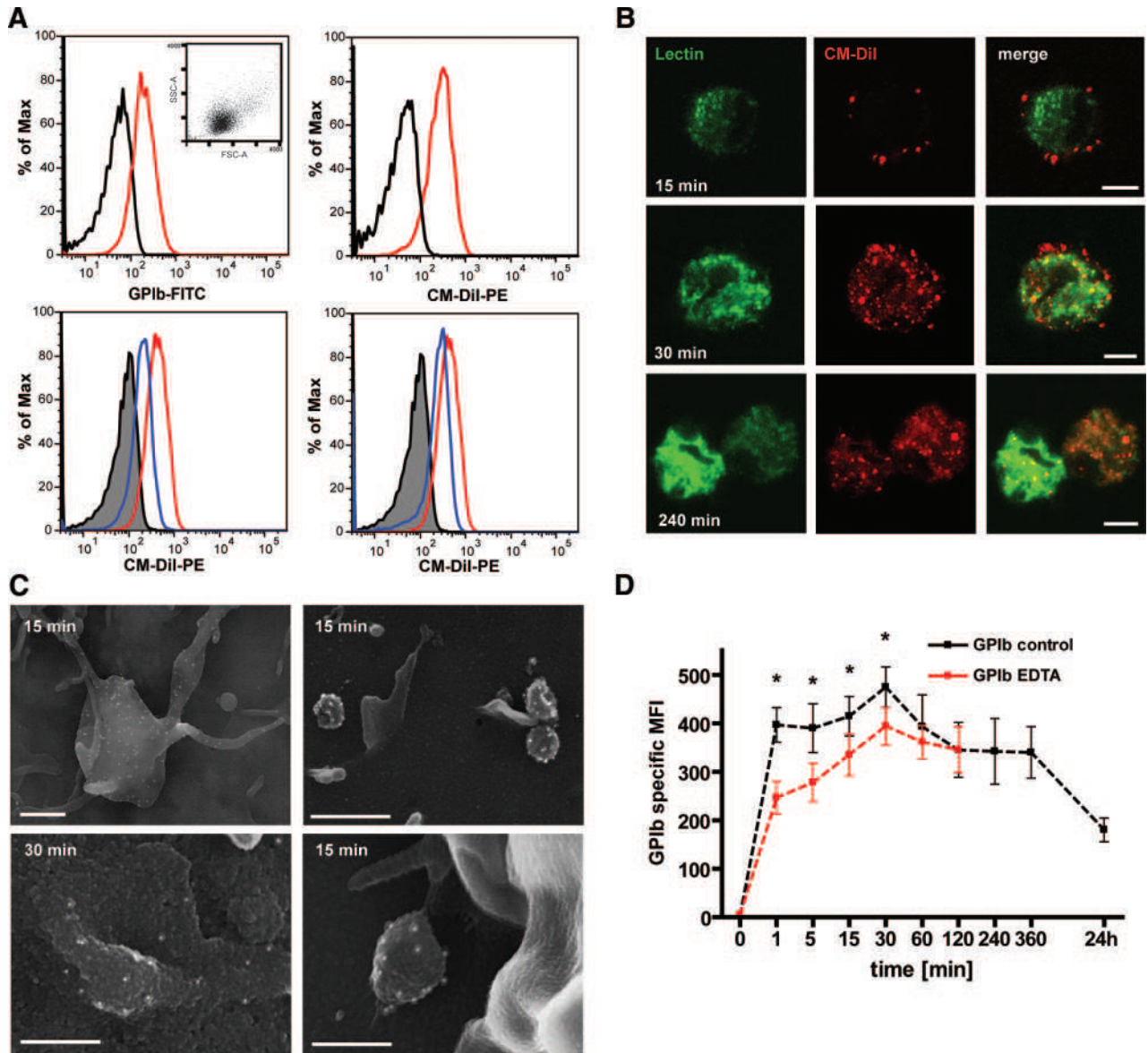


Figure 1. Interaction of PMPs with EOCs: binding, membrane assimilation, and incorporation. **A**, Flow cytometry analysis of EOCs left untreated (black line) or treated with PMPs for 15 minutes (red line). The presence of GPIb-specific fluorescence (top left) or red fluorescence detecting CM-Dil-labeled PMPs (others) was evaluated in an appropriate gate (inset). Coincubation was performed after pretreatment of PMPs with mAbs blocking P-selectin (bottom left, blue line) or GPIb (bottom right, blue line). **B**, Confocal laser scanning microscopy of FITC-lectin-stained EOCs reacted with CM-Dil-labeled PMPs. Scale bars=5 μ m. **C**, ScEM analysis of EOCs reacted with activated platelets (top left) or PMPs (others). Immunogold staining (white dots) was used to detect GPIb. Scale bars=1 μ m (top) and 500 nm (bottom). **D**, Flow cytometry-based time course analysis tracking GPIb on EOCs. EOCs were incubated with PMPs for indicated periods and posttreated with/without EDTA (red/black line). Data are expressed as the specific mean fluorescence intensity (MFI) for GPIb. Representative histograms and images are shown; n=3 to 8. * P <0.05 vs non-EDTA-treated EOCs, paired Student t test.

Statistical Analysis

Data represent mean \pm SD and were analyzed either by Student t test and 1-way ANOVA followed by Newman-Keuls posthoc test or by nonparametric Mann-Whitney test and Kruskal-Wallis test with posthoc Dunn test using the GraphPad Prism 4.00 as appropriate. Differences with values of P <0.05 were considered to be statistically significant.

Results

Interaction of PMPs With EOCs: Binding, Membrane Assimilation, and Incorporation

To characterize interactions between PMPs and EOCs, we tracked the transfer of the platelet marker GPIb, which is

abundantly present on the surface of PMPs but negligible on EOCs (Figure II in the online-only Data Supplement). Incubation of EOCs with PMPs revealed significant surface staining for GPIb (Figure 1A). Similarly, CM-Dil-labeled PMPs were found to bind to EOCs. Pretreatment of PMPs with P-selectin or GPIb blocking antibodies significantly inhibited the EOC-PMP interactions (Figure 1A). To structurally investigate this interaction, we used confocal laser scanning microscopy and ScEM. The first approach revealed that CM-Dil-labeled PMPs formed rosettes around EOCs without signs of incorporation after 15 minutes of exposure (Figure 1B and Figure III and Movie I in the online-only Data

Supplement). After 30 minutes, PMPs were found both on the surface and within EOCs, and at later time points, the majority of PMPs were incorporated into EOCs (Figure 1B and Figure III and Movie II in the online-only Data Supplement). To discriminate PMPs from EOC-derived microparticles, membrane protrusions, or apoptotic bodies, we performed immunogold-labeling for GPIb in ScEM. Labeling was initially restricted to the surface of PMPs but not detectable on EOCs (Figure 1C). After 15 minutes of EOC-PMP interaction, some PMP-derived membrane protrusions extended on the EOC surface, and after 30 minutes, signs of membrane fusion were observed (Figure 1C). At later stages, focal areas of the EOC membrane displayed diffuse immunogold-labeling, indicating transfer of PMP-derived GPIb through membrane assimilation (not shown). Kinetic analysis by flow cytometry revealed that the association of PMP-derived GPIb with EOCs occurs within 1 minute of incubation, peaks at 15 to 30 minutes, subsequently declines, but remains detectable on the surface of EOCs even after 6 hours (Figure 1D). EDTA treatment after EOC-PMP incubation allowed a significant dissociation of PMPs during early time points but not after 30 minutes (Figure 1D), further supporting the concept of PMP membrane assimilation.

PMPs Modulate the Phenotype of EOCs

We next assessed whether PMPs affect the phenotype of EOCs by scrutinizing the surface expression of various endothelial, leukocytic, and hematopoietic cell markers. Presence of PMPs in the EOC culture for 24 hours was associated with significantly increased expression of the endothelial markers CD31 and VE-cadherin and of the CXCL12-receptor CXCR4 (Figure 2A and 2B and Figure IV in the online-only Data Supplement). Expression of the leukocyte markers CD14, CD45, and Mac-1 and the hematopoietic markers CD34 and CD133 was unaffected by the presence of PMPs. Prolonged incubation of EOCs with PMPs starting at day 1 of EOC culture did not further alter the expression profile of these receptors (not shown). Furthermore, expression of endothelial markers on the surface of PMP-incubated EOCs was comparable to angiogenic late outgrowth cells, which were cultured for 21 days in the absence of PMPs (not shown). Taken together, these data suggest that PMPs induce changes in the EOC phenotype with increased expression of mature endothelial markers.

PMPs Enhance Responsiveness of CXCR4 on EOCs

Given the relevance of CXCR4 in the homing, migration, and angiogenic activity of EOCs,^{17,19,20} we further investigated the expression pattern and kinetics of CXCR4 and GPIb in EOCs after exposure to PMPs to distinguish the mechanisms of CXCR4 regulation. CXCR4 is expressed on the surface of EOCs but also on PMPs and is found in intracellular compartments of EOCs (Figures I and V in the online-only Data Supplement). In contrast to GPIb, CXCR4 expression in EOCs declined after 1 minute of PMP exposure and then increased continuously after 30 minutes (Figure 3A). In comparison, permeabilized EOCs or EOCs treated with siRNA for knockdown of CXCR4 displayed an early and stable increase in CXCR4 over 24 hours after PMP addition

(Figure 3A and Figure VI in the online-only Data Supplement), indicating that PMPs are a contributing factor to the increased CXCR4 expression on EOCs. The transient decrease in CXCR4 in intact EOCs within 30 minutes may be due to internalization of CXCR4 followed by its recycling and recovery of surface expression. Notably, this phenomenon of transient reduction was not observed for CD31 (Figure VII in the online-only Data Supplement). When PMP-derived CXCR4 was pre-labeled by immunogold staining before incubation, ScEM analysis demonstrated focally accentuated immunogold staining on the surface of EOCs reacted with PMPs for >15 minutes (Figure VIII in the online-only Data Supplement). Because such immunogold staining exclusively detects PMP-derived but not EOC-derived CXCR4, this strongly indicates transfer of PMP-derived CXCR4 to the plasma membrane of EOCs. As evidenced by real-time polymerase chain reaction, CXCR4 messenger RNA expression was increased 4 to 6 hours after treatment with PMPs, likely contributing to elevated CXCR4 surface expression after 24 hours (Figure IX in the online-only Data Supplement).

As a measure of CXCR4 activation and signaling, we analyzed the effect of PMPs on the ability of CXCL12 to elicit an intracellular calcium flux in EOCs. At 100 ng/mL, CXCL12 induced a marked calcium transient, which was almost completely abrogated by a CXCR4-blocking mAb (Figure 3B). In response to PMPs, EOCs exhibited a robust calcium transient, which was independent of CXCR4 (Figure 3B). Importantly, pretreatment of EOCs with PMPs significantly enhanced the calcium flux induced by CXCL12 (Figure 3B), whereas pretreatment with CXCL12 caused a desensitization to a second CXCL12 stimulus (not shown).

Activation of AKT contributes to cell proliferation, migration, and angiogenesis.²¹ In untreated EOCs, CXCL12 (100 ng/mL) induced AKT phosphorylation at 15 and 30 but not 5 minutes. Treatment with PMPs alone resulted in a moderate but reproducible increase in AKT phosphorylation (Figure 3C). Notably, pretreatment with PMPs markedly accelerated and enhanced AKT activation in response to CXCL12, whereas a blocking CXCR4 mAb prevented AKT phosphorylation by CXCL12 (Figure 3C). Pretreatment of EOCs with PMPs for 2 hours further enhanced the effects of PMPs related to the increase in CXCR4 (not shown), whereas CXCL12 or PMP treatment did not alter unphosphorylated AKT levels (Figure X in the online-only Data Supplement).

PMPs Promote EOC Adhesion to Extracellular Matrix Components Under Flow Conditions

The ability of EOCs to mediate vascular regeneration is determined by their recruitment and interaction with sub-endothelial components of the extracellular matrix (ECM) exposed at sites of vascular injury. Hence, we scrutinized the effect of PMPs on EOC adhesion to ECM proteins under flow conditions. Marked adhesion of EOCs was observed on fibrinogen, fibronectin, vitronectin, and ECM produced by HUVECs but only rarely on resting HUVEC monolayers or collagen I (Figure 4A). Pretreatment with PMPs significantly increased EOC adhesion to all substrates, with the biggest relative effect on adhesion to HUVEC ECM (Figure 4A). After arterial injury, CXCL12 is lumenally

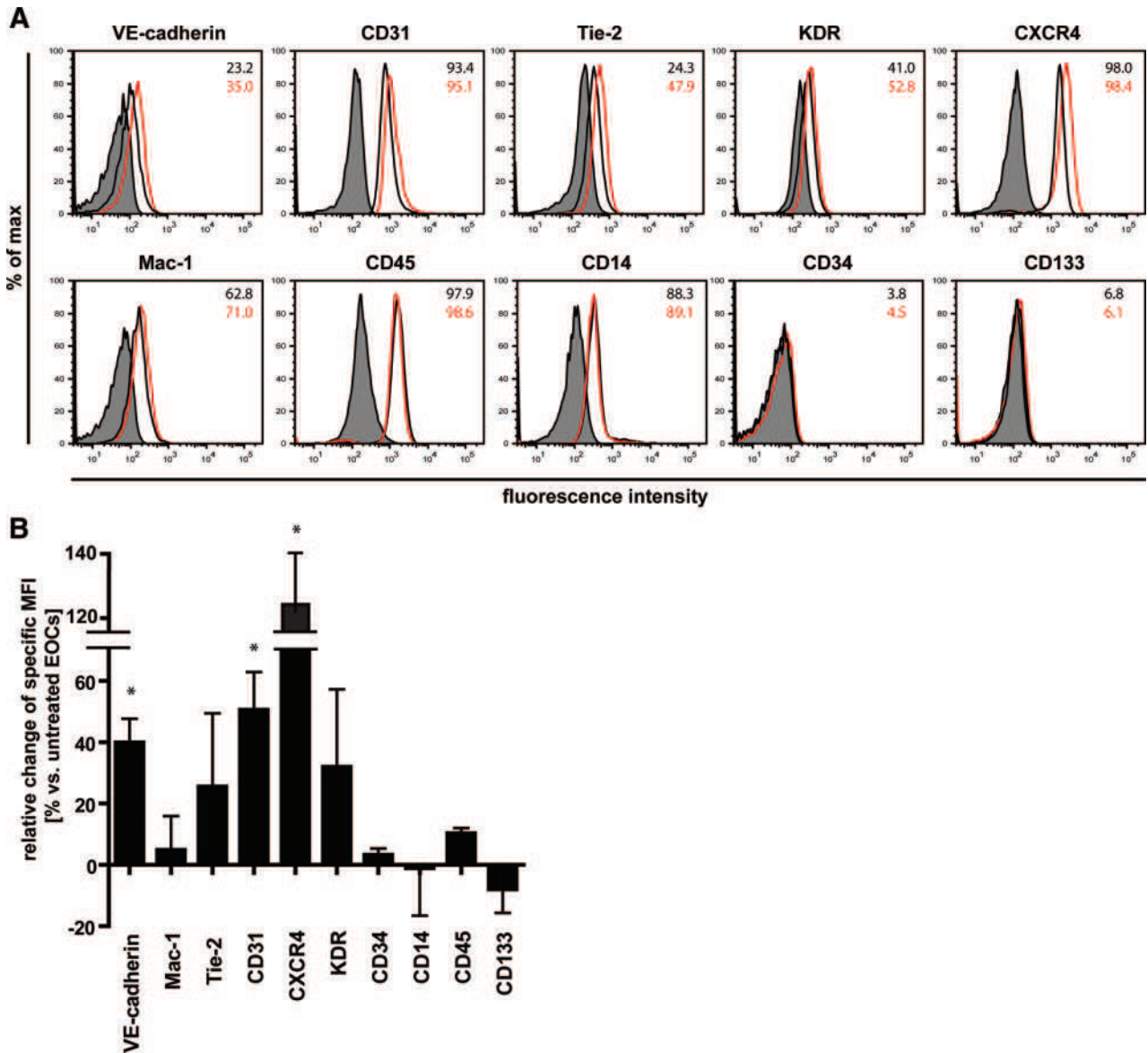


Figure 2. PMPs modulate receptor expression on EOCs. **A**, Analysis of endothelial, leukocytic, hematopoietic markers, and CXCR4 by flow cytometry. Representative histograms for staining with isotype controls (filled) and specific antibodies for untreated (black) vs PMP-treated EOCs (red). The percentage of positive cells is noted in the respective histograms. **B**, Synopsis of relative mean fluorescence intensity (MFI) changes in untreated vs PMP-treated EOCs. * $P < 0.05$ vs untreated EOCs, 1-way ANOVA and Newman-Keuls posthoc test; $n = 4$ to 8.

expressed and presented by platelets and medial SMCs, triggering the recruitment of progenitor cells.^{22,23} We thus tested the adhesion of EOCs to immobilized CXCL12. Surprisingly, EOC adhesion was comparable to that of fibrinogen, and pretreatment of EOCs with PMPs resulted in a strong enhancement of shear-resistant adhesion (Figure 4B). A blocking CXCR4 mAb but not a control immunoglobulin G reduced adhesion of both PMP-treated and untreated EOCs to immobilized CXCL12.

PMPs Induce Cytoskeletal Rearrangement and Migration of EOCs

Coordinated cell spreading and formation of membrane projections are essential for effective migration, requiring

cytoskeletal reorganization and actin polymerization. Flow cytometry analysis revealed that CXCL12 or PMPs stimulated rapid actin polymerization in EOCs, as evidenced by increased content of polymerized F-actin (Figure 5A). This effect was more sustained in PMP-stimulated than in CXCL12-stimulated cells. We next analyzed the extent and kinetic of EOC spreading. Pretreatment with PMPs significantly accelerated spreading on fibronectin-coated coverslips, as determined by an increase in cell area and a decline in circularity (Figure 5B and 5C). ScEM revealed that PMPs promoted pseudopodial activity of EOCs with augmented formation of elongated lamellipodia and filopodia (Figure 5D).

In a modified Boyden chamber assay, EOCs show migration toward a CXCL12 gradient, which was almost com-

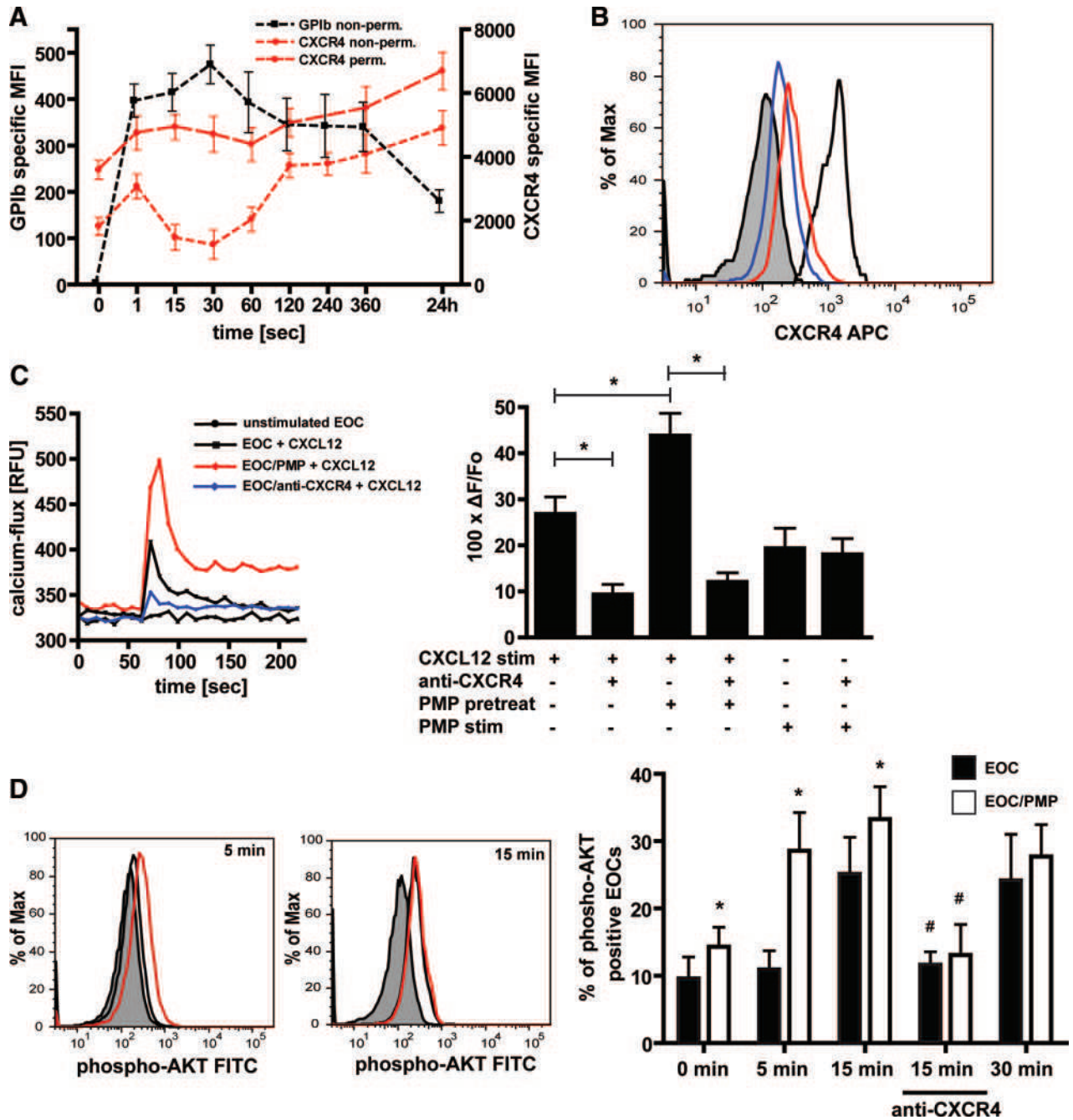


Figure 3. PMPs transfer CXCR4 and enhance its responsiveness in EOCs. **A**, Flow cytometry–based time course analysis of CXCR4 and GPIIb on EOCs. EOCs were incubated with/without PMPs for indicated periods and reacted with anti-CXCR4 (red line) or anti-GPIIb (black line). Total CXCR4 pool was evaluated in permeabilized EOCs. Results are expressed as specific mean fluorescence intensity (MFI) for GPIIb (left y axis) and CXCR4 (right y axis); n=4 to 8. **B**, Representative traces and quantification of calcium influx in EOCs labeled with fluo-4AM. EOCs were pretreated with/without PMPs and/or blocking CXCR4 mAb and subsequently stimulated with CXCL12 (100 ng/mL) or PMPs. Fluorescence was recorded in a spectrophotometric plate reader, starting 60 seconds before stimulation. ΔF/Fo indicates difference in peak and baseline fluorescence divided by baseline fluorescence. n=6, *P<0.05, Kruskal-Wallis test with posthoc Dunn test. **C**, Flow cytometry analysis of phosphorylated (phospho) AKT in EOCs. EOCs pretreated with/without PMPs (unfilled/filled bars and red/black lines) and/or anti-CXCR4 for 15 minutes were reacted with CXCL12 (100 ng/mL) for indicated periods. Shown are representative histograms (isotype controls as filled line) and data expressed as percentage of phospho-AKT–positive EOCs; n=6, *P<0.05 vs untreated EOCs, #P<0.05 vs EOCs without anti-CXCR4, Kruskal-Wallis test with posthoc Dunn test.

pletely abrogated by a CXCR4-blocking mAb. Notably, migration toward CXCL12 was significantly enhanced by pretreatment with PMPs (Figure 5E). PMPs alone did not alter undirected random migration of EOCs in the absence of CXCL12. As a more integrative model for endothelial repair

after injury, we used a scratch wound assay. When PMPs were added to EOCs after induction of injury, an improved migratory response of EOCs resulting in accelerated repair of the wounded area was observed (Figure 5F). In contrast, pretreatment with PMPs for 15 minutes or 24 hours failed to

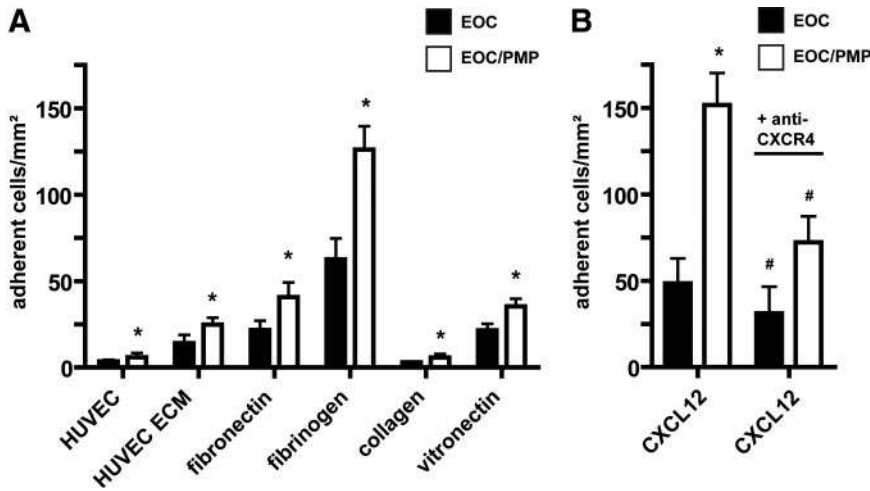


Figure 4. PMPs enhance adhesion of EOCs. A and B, EOCs pretreated with/without PMPs (unfilled/filled bars) and/or anti-CXCR4 were perfused at 1.5 dynes/cm² in a parallel flow chamber, and the number of cells firmly adherent to the indicated substrates was determined and expressed as adherent cells per 1 mm². * $P < 0.05$ vs untreated EOCs, # $P < 0.05$ vs EOCs in the absence of anti-CXCR4, Kruskal-Wallis test with posthoc Dunn test; $n = 4$ to 10.

induce vascular tube formation by EOCs on a matrigel matrix (not shown) and failed to enhance the proliferation rate of EOCs (Figure XI in the online-only Data Supplement).

PMPs Modulate the Secretome of EOCs and Support Their Regenerative Potential

EOCs are thought to release a cocktail of angiogenic factors, accounting for their beneficial effects on endothelial regeneration after arterial injury. We analyzed paracrine factors in CM-EOC or CM-EOC/PMP. A broad range of proangiogenic factors, including vascular endothelial growth factor, epidermal growth factor, hybridoma growth factor, and granulocyte-macrophage colony-stimulating factor, were considerably upregulated in CM-EOC/PMP (Figure 6A and 6B and Figure XII in the online-only Data Supplement). In contrast, CXCL8, CXCL16, and CXCL12 levels were significantly reduced in CM-EOC/PMP, and CCL2 levels remained unchanged (Figure 6A and 6C). Moreover, levels of the proinflammatory cytokines interleukin-1 β and tumor necrosis factor- α but also of the antiinflammatory cytokine interleukin-10 were elevated in CM-EOC/PMP (Figure 6C). Matrix metalloproteinase-9, its natural inhibitor (tissue inhibitor of metalloproteinase-1), and serpins like plasminogen activator inhibitor-1 were found at high concentrations in both CM-EOC and CM-EOC/PMP.

We scrutinized the functional effects of the altered secretome on key characteristics of mature endothelial cells. Compared with CM-EOC, CM-EOC/PMP promoted enhanced vascular tube formation by HUVECs on matrigel (Figure 6D and Figure XIII in the online-only Data Supplement). As judged by incorporation of BrdU and compared with control medium, HUVEC proliferation was increased on culture in CM-EOC and even more enhanced in CM-EOC/PMP (Figure 6E). Accordingly, culture in CM-EOC improved the restoration of HUVEC monolayers wounded in the scratch assay compared with control medium, an effect further accelerated in CM-EOC/PMP (Figure 6F).

PMPs Enhance EOC-Mediated Endothelial Regeneration After Injury In Vivo

To examine interactions of EOCs with the injured vessel wall in situ, we performed intravital microscopy in murine carotid arteries after wire injury. EOCs did not adhere to the

uninjured vessel wall (not shown) but showed substantial luminal adhesion after endothelial denudation. Pretreatment with PMPs significantly augmented EOC arrest (Figure 7A), corroborating our in vitro findings (Figure 4). Notably, EOC adhesion mostly occurred early after injury and was increased only slightly 2 or 4 hours after injury (not shown). Compared with vehicle-treated mice, neointimal hyperplasia but not medial area was reduced in EOC-treated mice 3 weeks after wire injury. The intima/media ratio was significantly reduced in EOC-treated carotid arteries (Figure 7B). Injection of PMP-treated EOCs resulted in a slightly more pronounced inhibition of neointima formation after 3 weeks (Figure 7B). Five days after injury, neointima formation was almost negligible in all groups. Accelerated reendothelialization is considered the major mechanism underlying attenuated neointima formation after treatment with EOCs.² As evaluated by luminal CD31 staining, endothelial recovery was improved in EOC-treated versus vehicle-treated mice 5 days after injury (Figure 7C). Notably, application of PMP-treated EOCs caused the most marked and almost complete reendothelialization 5 days after injury, demonstrating that PMPs amplify EOC-mediated endothelial regeneration. After 21 days, all arteries showed virtually complete endothelial recovery. Independently of EOC pretreatment, injected CM-Dil-labeled EOCs rarely integrated into the endothelial lining and were located in the neointima and adventitia (not shown).

Discussion

Endogenous or therapeutically transferred EOCs promote endothelial repair to limit neointima formation after vascular injury and support angiogenesis and postnatal vasculogenesis in ischemic diseases. Two major mechanisms are thought to account for the beneficial effect of EOCs on endothelial recovery.⁷ Some have implicated EOCs in reendothelialization by replacement or incorporation into regenerating endothelium and differentiation toward an endothelial phenotype. Given the low percentage of incorporated cells, it has been proposed, however, that EOCs promote endothelial regeneration and angiogenesis predominantly by exerting paracrine effects.^{2,6} This mechanism is effective when angiogenic factors are locally enriched as a result of sufficient recruitment of secretory active EOCs to the site of injury. Here, we

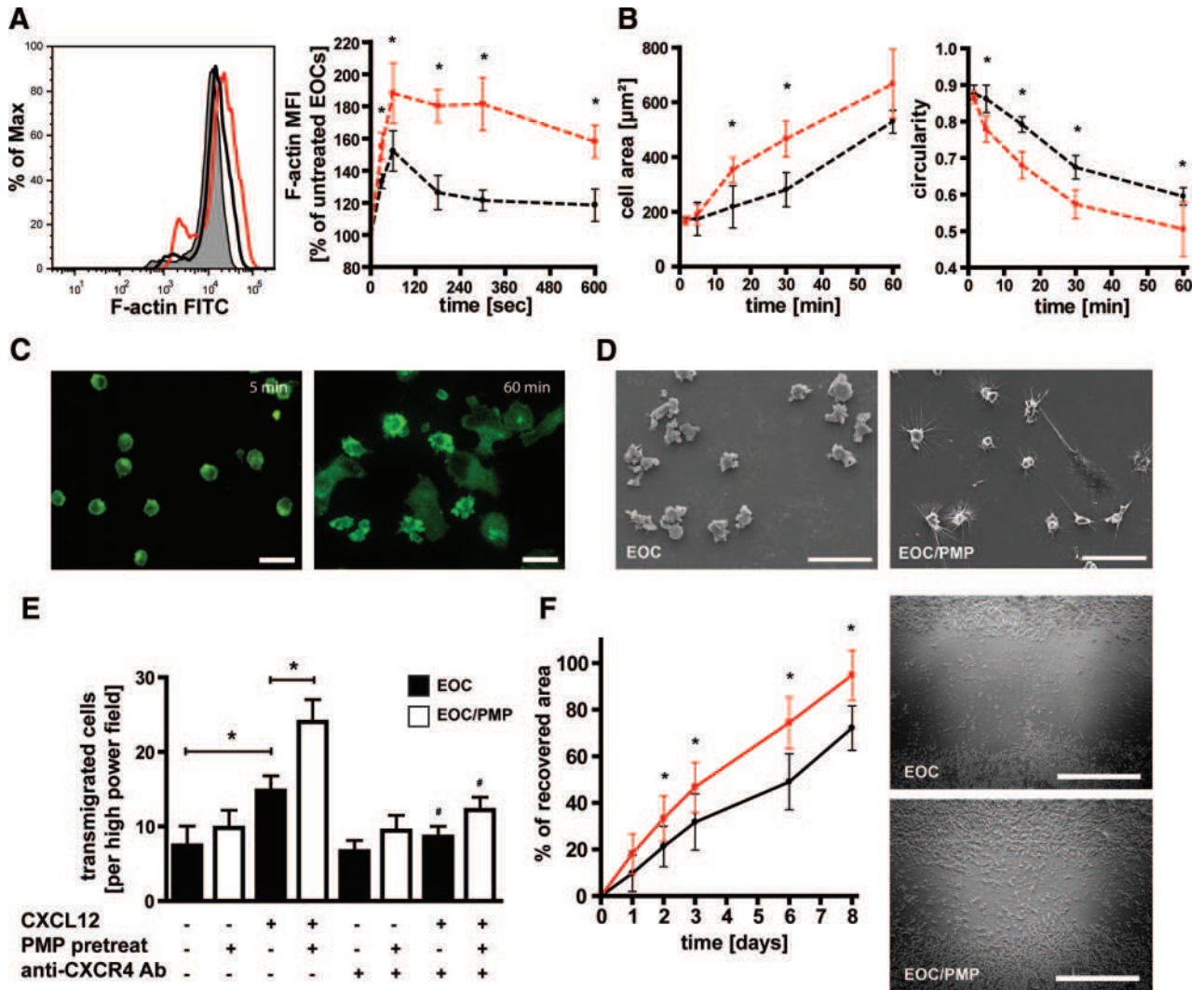


Figure 5. PMPs induce cytoskeletal reorganization and enhance migration of EOCs. A, Actin polymerization as judged by F-actin staining in permeabilized EOCs after addition of CXCL12 (100 ng/mL) or PMPs. Left, Representative histograms showing untreated (filled), CXCL12-treated (black line), or PMP-treated (red line) EOCs, each after 1 minute of treatment. Right, Time course analysis of actin polymerization expressed as specific mean fluorescence intensity (MFI) relative to untreated cells. $*P < 0.05$ vs CXCL12-treated EOCs, nonparametric Mann-Whitney test; $n = 4$. B, Analysis of spreading. EOCs treated with/without PMPs (red/black lines) were seeded on fibronectin-coated coverslips for indicated periods and stained for F-actin. Cell area and circularity were assessed. $*P < 0.05$ vs untreated EOCs, nonparametric Mann-Whitney test; $n = 4$. C, Representative images of F-actin-stained and PMP-treated EOCs seeded on coverslips for indicated periods. Scale bars = 20 μm . D, Representative ScEM images of untreated (left) or PMP-treated (right) EOCs seeded on fibronectin-coated coverslips for 15 minutes. Scale bars = 50 μm . E, EOC migration in a modified Boyden chamber assay. EOCs treated with/without PMPs were seeded in the absence/presence of anti-CXCR4 in the upper chamber, whereas CXCL12 (100 ng/mL) or control medium was placed in the lower chamber. $*P < 0.05$, $\#P < 0.05$ vs respective conditions without anti-CXCR4, Kruskal-Wallis test with posthoc Dunn test; $n = 4$. F, EOC scratch assay. Monolayers of EOCs, treated with/without PMPs (red/black line), were wounded linearly, and the area of the wound subsequently recovered by migrated EOCs was expressed as a percentage of the initial wound area. Quantified data and representative photomicrographs (time period, 2 days) are shown. Scale bars = 0.5 mm. $*P < 0.05$ vs untreated EOCs, nonparametric Mann-Whitney test; $n = 4$.

Figure 6. (Figure on next page). PMPs modulate the secretome of EOCs toward a more proangiogenic composition. Cell supernatants from CM-EOC or CM-EOC/PMP were collected 24 hours after culture in RPMI-1640 plus 1% FBS and defined as conditioned medium (CM). A, With the use of semiquantitative human protein arrays, the presence of paracrine factors was determined in CM-EOC (filled bars) and CM-EOC/PMP (unfilled bars). Results show mean pixel density of dots. $*P < 0.05$ vs CM-EOC, nonparametric Mann-Whitney test; $n = 3$. B and C, Concentration of cytokines/chemokines in CM-EOC and CM-EOC/PMP as measured by ELISA or flow cytometry bead assay. $*P < 0.05$ vs CM-EOC, Kruskal-Wallis test with posthoc Dunn test (B) and nonparametric Mann-Whitney test (C); $n \geq 4$. D, Representative images of vascular tube formation by HUVECs. HUVECs were suspended in CM-EOC or CM-EOC/PMP and seeded on matrigel for 6 hours; $n = 3$. E, Proliferation of HUVECs. HUVECs were cultured in control medium, CM-EOC, or CM-EOC/PMP for 24 hours, and BrdU incorporation was measured by spectrophotometry. $*P < 0.05$ vs control, $\#P < 0.05$ vs CM-EOC, Kruskal-Wallis test with posthoc Dunn test; $n = 4$. F, HUVEC scratch assay. The HUVEC monolayer cultured in control medium (dashed line), CM-EOC (black line), or CM-EOC/PMP (red line) was wounded, and the wound area recovered by migrating HUVECs was measured and expressed as a percentage of the initial wound area. Representative photomicrographs (time period, 36 hours) and quantification are shown. Scale bars = 0.5 mm. $*P < 0.05$ vs CM-EOC, nonparametric Mann-Whitney test; $n = 4$.

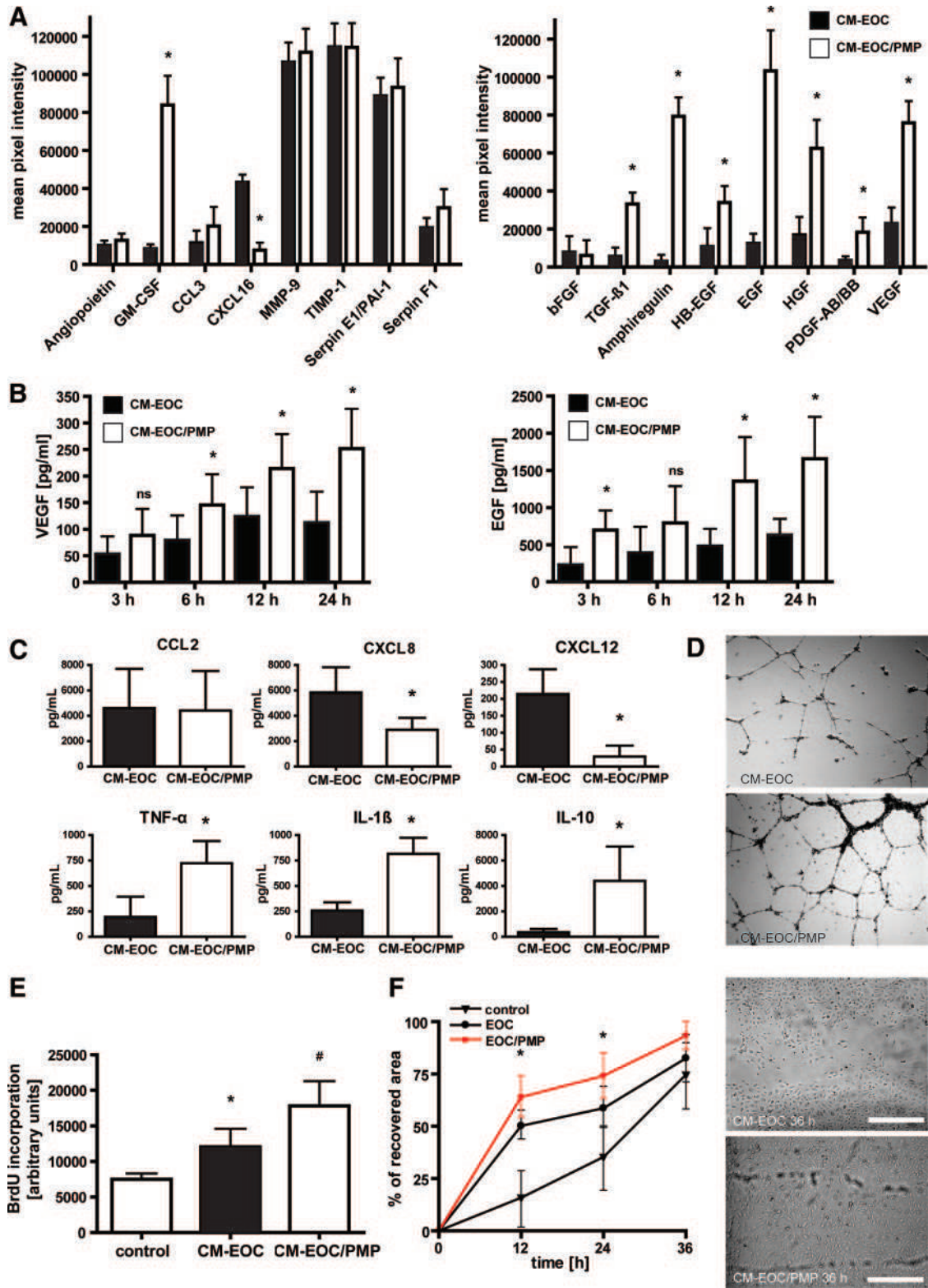


Figure 6. (Legend on preceding page).

provide evidence that thrombin/collagen-induced PMPs amplify key functions of EOCs, promoting both EOC adhesion and paracrine activity and leading to improved endothelial healing.

PMPs are rapidly and abundantly generated in response to vascular injury with platelet activation and may interact with circulating EOCs. Indeed, flow cytometry revealed P-selectin-

and GPIIb-dependent association of PMPs with EOCs. However, this method does not allow discrimination between PMP-EOC binding and membrane fusion or direct detection of PMP incorporation into EOCs. ScEM and confocal laser microscopy provided evidence for both membrane assimilation and progressive incorporation of PMPs. The kinetics of

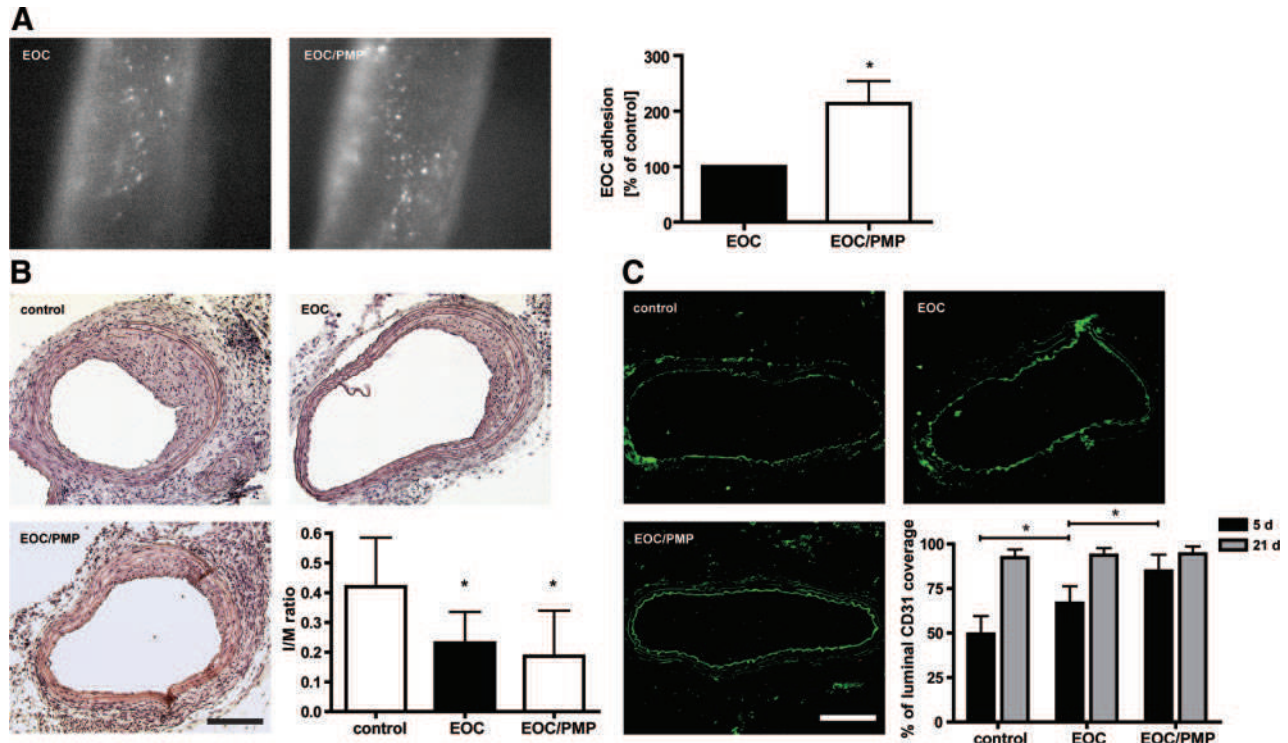


Figure 7. PMPs enhance the potential of EOCs to restore arterial integrity after injury in vivo. **A**, Adhesion of untreated and PMP-treated EOCs to carotid arteries of C57BL/6J mice was analyzed by intravital microscopy 15 minutes after wire injury. Left, Representative images show adhesion of CM-Dil-labeled EOCs to arteries. Right, Quantification of adhesion expressed as a percentage of untreated EOCs. * $P < 0.05$ vs EOCs, nonparametric Mann-Whitney test; $n = 7$. **B**, Neointima formation in carotid arteries of athymic nude mice 3 weeks after injury and injection of control medium, EOCs or PMP-treated EOCs. Shown are representative images of Movat pentachrome-stained sections and synopsis of intima/media (I/M) ratio. Scale bars = 100 μm . * $P < 0.05$ vs control, Kruskal-Wallis test with posthoc Dunn test; $n = 6$. **C**, Reendothelialization as evident by luminal CD31 staining and assessed 5 days (gray) and 3 weeks (black) after injury. Representative images are shown for CD31 staining at day 5. Scale bars = 100 μm . * $P < 0.05$, nonparametric Mann-Whitney test; $n \geq 4$.

GPIb detection on the EOC surface after PMP incubation (slower decline after peaking), relatively faster incorporation of PMPs into the EOCs, and time-restricted EDTA effects further substantiate the idea of PMP membrane assimilation. These results are in line with the concept of microparticles as an active transfer module for various factors, including platelet receptors, chemokines, bioactive lipids, and messenger RNA.^{11–13,24} Thus, PMPs may modify the phenotype of recipient cells, reprogram cellular functions, and render EOCs susceptible to novel interactions. Indeed, PMPs mediate increased expression of mature endothelial cell markers on EOCs. As a result, expression levels of these markers on EOCs treated with PMPs were comparable to angiogenic late outgrowth cells cultured for 21 days without PMPs. Prolonged presence of platelets is associated with a similar phenotypic switch in embryonic endothelial progenitor cells or human CD34⁺ cells.²⁵ Recently, Prokopi et al²⁶ proposed that characteristics used to monitor the endothelial properties of angiogenic EOCs (eg, CD31, von Willebrand factor, lectin staining) may rely on an uptake of PMPs by mononuclear cells during initial cell culture with residual platelet contamination. Our data support the idea that increased surface expression of endothelial markers, eg, CD31, may arise from PMP-related membrane transfer. However, the kinetics of CD31 expression on EOCs indicates that this is not the sole mechanism because transfer of CD31 by PMPs is a self-exhausting process likely to be completed within a few hours and does not contribute to the

further augmentation of CD31 expression during prolonged EOC-PMP coculture.

CXCR4 and its ligand CXCL12 are essential for the mobilization, homing, migration, retention, and proliferation of (vascular) progenitor cells during steady-state homeostasis and injury and play a critical regulatory role in angiogenesis and postnatal vasculogenesis.^{17,19,20} Although functional transfer of CXCR4 to recipient cells after interaction with PMPs has been described,¹³ we demonstrate for the first time that PMPs influence the surface expression of CXCR4 through different sequential mechanisms. These include receptor transfer (within minutes), alteration of internalization and externalization (within minutes to hours), and modified gene regulation (within hours to days). Notably, we found that CXCR4-dependent functions, eg, CXCL12-triggered adhesion, migration, and signaling, were amplified in PMP-treated EOCs. These effects occurred when EOCs were allowed to interact with PMPs for 15 minutes, although at this time surface expression of CXCR4 was reduced. This suggests sensitization of CXCR4 present on EOCs on PMP exposure. Sphingosine-1-phosphate has been reported to stimulate and/or sensitize CXCR4-mediated signaling, thus improving the angiogenic activity of EOCs.²⁰ Platelets and PMPs harbor sphingosine-1-phosphate,¹⁵ giving rise to the notion that this bioactive lipid is responsible for an increased responsiveness of CXCR4 to CXCL12. By boosting CXCR4-dependent signaling, PMPs may stimulate a multitude of EOC

functions in a CXCL12-rich environment, eg, at sites of vascular injury with CXCL12-bearing platelets and SMCs.^{22,23}

Using intravital microscopy, we show that PMPs promote the adhesion of EOCs at sites of wire-induced injury. Adhesion assays in vitro revealed that PMPs increase EOC adhesion to a broad spectrum of ECM components, ECM produced by HUVECs, and immobilized CXCL12. Because PMPs themselves bind to sites of endothelial injury,²⁷ PMPs might enhance EOC arrest in part by bridging between EOCs and the denuded artery. Accordingly, we found that the presence of the GPIIb/IIIa inhibitor 7E3, known to inhibit PMP interaction with fibrinogen and fibronectin, diminished but did not completely inhibit the potentiating effect of PMPs on EOC adhesion, whereas adhesion of untreated EOCs was largely unaffected (S.F.M. and C.W., unpublished data, 2009). Together with adherent CXCL12-bearing platelets, PMPs may serve as an efficient capture system for circulating EOCs, directing them to sites of tissue damage.²⁸ Subsequently, PMPs promote spreading and migration of recruited EOCs to support interactions with resident vascular cells. Conversely, this imposes a caveat for antiplatelet therapy, which, despite its overall benefit, may adversely affect endothelial recovery.

The present study shows that PMPs shift the multifaceted secretome of EOCs toward a more proangiogenic composition with elevated levels of vascular endothelial growth factor, epidermal growth factor, and hybridoma growth factor. This entails increased proliferation, migration, and vascular tube formation of mature endothelial cells and may constitute a major mechanism responsible for the acceleration of endothelial regeneration after arterial injury and possibly relevant to EOC-mediated angiogenesis and vasculogenesis. The limited number of EOCs directly incorporated into the endothelial lining supports the notion of paracrine factors as crucial mediators of their vasoprotective function. However, further studies are warranted to elucidate functional consequences of the modified secretome in detail. By inducing granulocyte-macrophage colony-stimulating factor release, PMPs may foster mobilization and recruitment of progenitor cells.²⁹ Simultaneously, enhanced secretion of proinflammatory (tumor necrosis factor- α) and antiinflammatory (interleukin-10) cytokines may cause context-specific modulation of inflammatory reactions in the damaged vessel wall.³⁰ Interestingly, EOC-mediated reduction of intimal hyperplasia was not significantly altered by PMPs, implying that mechanisms other than accelerated endothelial recovery may be crucial for the effect of EOCs on neointima formation. Indeed, we found that the neointima of mice infused with PMP-treated EOCs displayed an increased cellularity and relative SMC content (S.F.M. and C.W., unpublished data, 2009). Mitogenic factors such as transforming growth factor- β 1 and platelet-derived growth factor-BB are elevated in CM-EOC/PMP and may facilitate SMC migration and proliferation.³¹ This may partly counteract the effect of improved reendothelialization and enhance the stability of the growing plaque. Nevertheless, the accelerated endothelial recovery can limit the risk of acute or late thrombosis after arterial injury, eg, by stent placement.

Notably, PMPs may also confer adverse effects by amplifying EOC functions. Recent studies draw attention to the functional ambivalence of EOCs in the context of neointimal hyperplasia as opposed to primary atherosclerosis because infusion of EOCs was found to increase plaque size and to decrease plaque

stability in apolipoprotein E-deficient mice.^{32,33} This may reflect the ambivalent role of angiogenesis in cardiovascular disease. Whereas EOC-derived paracrine factors may support endothelial recovery after injury to limit neointima formation, they may promote neoangiogenesis with the formation of immature and leaky microvessels in the primary atherosclerosis. These nascent microvessels play a crucial role in atheroprogession and plaque destabilization by constituting key entry points for cellular and soluble lesion components.³⁴ Accordingly, viral transfer or direct administration of vascular endothelial growth factor, which is strongly secreted by PMP-treated EOCs, has been shown to promote primary atherosclerosis and lesion expansion in apolipoprotein E/B-100-deficient mice.³⁵ Moreover, it remains to be elucidated whether PMPs may also enhance homing of progenitor cells giving rise to SMCs, which have been related to contribute to neointima formation.³³

Collectively, our findings identify a role of PMPs, specifically when induced by thrombin/collagen, in amplifying the vasoregenerative capacity of EOCs and supporting the maintenance of vascular integrity after arterial injury. The underlying mechanisms include increased recruitment and migration of EOCs to the site of injury, promotion of EOC differentiation, sensitization of CXCR4, and increased supply of angiogenic growth factors stimulating resident mature endothelial cells. Our data provide insights into a novel concept by which platelets, through their PMPs, might indirectly support angiogenesis and postnatal vasculogenesis beyond a direct involvement of their angiogenic factors.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Restenosis caused by neointimal hyperplasia has remained the Achilles heel of interventional cardiology. Animal studies demonstrated that angiogenic early outgrowth cells (EOCs) accelerate reendothelialization and attenuate neointimal hyperplasia after arterial injury. However, clinical studies testing the concept of cell-based therapy for cardiovascular diseases have produced conflicting findings with absent or moderate therapeutic effects, emphasizing the need for a better understanding of underlying biological mechanisms. Vascular pathologies are accompanied by platelet activation with concomitant generation of platelet microparticles (PMPs). We have observed abundant binding of PMPs to EOCs with subsequent PMP membrane assimilation and engulfment, allowing transfer of PMP-derived components to EOCs. Additionally, PMPs augmented recruitment of EOCs to the injured vessel wall and mediated increased responsiveness of CXCR4 toward CXCL12/SDF-1 α , which is abundantly presented by exposed smooth muscle cells and adherent platelets at the site of injury and takes center stage in regulating cellular functions of EOCs. EOCs promote endothelial regeneration in particular by exerting paracrine effects on resident endothelial cells. Profiling of the secretome of EOCs revealed that PMPs stimulate the release of proangiogenic factors, thus enhancing proliferation and migration of endothelial cells. Finally, we show that PMPs amplify the potential of EOCs to restore endothelial integrity after vascular injury, possibly limiting the risk of acute or late thrombosis after arterial injury, eg, stent placement. Mediated amplification of EOC functions also shed new light on the role of PMPs in angiogenesis and native atherosclerosis because EOCs are known to induce angiogenesis and postnatal vasculogenesis but may have adverse effects on atheroprogession.