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Endothelial Progenitor Cells Bind and Inhibit Platelet Function and Thrombus Formation

Haissam Abou-Saleh, PhD; Daniel Yacoub, MSc; Jean-François Théorêt, PhD; Marc-Antoine Gillis, MSc; Paul-Eduard Neagoe, MSc; Benoit Labarte, PhD; Pierre Théroux, MD; Martin G. Sirois, PhD; Maryam Tabrizian, PhD; Eric Thorin, PhD; Yahye Merhi, PhD

**Background**—Interactions of endothelial progenitor cells (EPCs) with vascular and blood cells contribute to vascular homeostasis. Although platelets promote the homing of EPCs to sites of vascular injury and their differentiation into endothelial cells, the functional consequences of such interactions on platelets remain unknown. Herein, we addressed the interactions between EPCs and platelets and their impact on platelet function and thrombus formation.

**Methods and Results**—Cultured on fibronectin in conditioned media, human peripheral blood mononuclear cells differentiated, within 10 days of culture, into EPCs, which uptake acetylated low-density lipoprotein, bind ulex-lectin, lack monocyte/leukocyte markers (CD14, P-selectin glycoprotein ligand-1, L-selectin), express progenitor/endothelial markers (CD34, vascular endothelial growth factor receptor-2, von Willebrand factor, and vascular endothelial cadherin), and proliferate in culture. These EPCs bound activated platelets via CD62P and inhibited its translocation, glycoprotein Ib/IIa activation, aggregation, and adhesion to collagen, mainly via prostacyclin secretion. Indeed, this was associated with upregulation of cyclooxygenase-2 and inducible nitric oxide synthase. However, the effects on platelets in vitro were reversed by cyclooxygenase and cyclooxygenase-2 inhibition but not by nitric oxide or inducible nitric oxide synthase inhibition. Moreover, in a ferric chloride–induced murine arterial thrombosis model, injection of EPCs led to their incorporation into sites of injury and impaired thrombus formation, leading to an incomplete occlusion with 50% residual flow.

**Conclusions**—Peripheral blood mononuclear cell–derived EPCs bind platelets via CD62P and inhibit platelet activation, aggregation, adhesion to collagen, and thrombus formation, predominantly via upregulation of cyclooxygenase-2 and secretion of prostacyclin. These findings add new insights into the biology of EPCs and define their potential roles in regulating platelet function and thrombus. (Circulation. 2009;120:2230-2239.)

**Key Words:** progenitor cells ■ nitric oxide ■ platelets ■ prostaglandins ■ thrombosis
Platelet activation and adhesion to damaged blood vessels represent the first step in atherothrombosis. At sites of vascular injury, platelets roll and interact with various components of the subendothelial matrix via a number of adhesive receptors expressed on the platelet surface. This, in turn, induces signaling that leads to platelet aggregation through glycoprotein (GP) IIb/IIIa activation and binding to fibrinogen. Platelet activation is accompanied by the translocation of P-selectin (CD62P), which contributes to the recruitment of other blood leukocytes. In this regard, CD62P has also been involved in the interaction of platelets with EPCs.

Although platelets promote the homing of EPCs at sites of vascular injury and favor their differentiation into ECs, the functional consequences of such interactions on platelets remain unknown. In fact, EPCs secrete many vasoactive and angiogenic factors that may modulate vascular thrombosis and hemostasis. Accordingly, we hypothesized that they might influence platelet function. We therefore designed the present study to determine the impact and the mechanisms of action of EPCs on platelet function in vitro and in vivo. We found that human PBMC-derived EPCs bound platelets via CD62P and inhibited platelet activation, aggregation, and adhesion to collagen in vitro and thrombus formation in vivo, predominantly via upregulation of cyclooxygenase-2 (COX-2) and secretion of prostacyclin (prostaglandin I\(_2\) [PGI\(_2\)]).

**Methods**

Methods are available in the online-only Data Supplement. This study has been approved by the human and animal ethical committees of the Montreal Heart Institute. All human subjects were healthy volunteers of either sex aged from 20 to 55 years. They gave informed consent and were free from any drugs that interfere with platelet function. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Characterization of PBMC-Derived EPCs

We first characterized the differentiation of PBMCs into EPCs according to their time-dependent appearance in culture, morphology, surface markers, proliferation rate, and functional properties. As shown in Figure 1A, human PBMCs differentiated after 5 days of culture into an adherent population characterized by a central cluster of round cells with few sprouts of elongated cells at the periphery defined as a colony-forming unit–EPC (CFU-EPC). The number of CFU-EPCs that derive from 5 x 10^5 PBMCs averaged 8 x 10^4 colonies per well. After 10 days, the cells formed a monolayer of spindle-shaped flat cells. Cells at both 5 and 10 days of culture were positive for acetylated low-density lipoprotein uptake and ulex-lectin binding (Figure 1A). To gain more insights into the nature of these ex vivo expanded cells, we used flow cytometry to quantify the expression of typical markers on the surface of cells during their differentiation. As shown in Figure 1B, freshly isolated PBMCs expressed predominantly the monocyte/leukocyte markers CD14 (87%), PSGL-1 or CD162 (93%), and L-selectin or CD62L (46%), whereas the progenitor and endothelial markers were missing.

At day 5, the cells started to express progenitor/endothelial markers, whereas leukocyte markers were downregulated. After 10 days of culture, the progenitor/endothelial markers were highly expressed (63% CD34, 40% P-selectin, 33% vascular endothelial growth factor receptor-2 [VEGFR2], 85% vascular endothelial cadherin [VE-cadherin], and 97% von Willebrand factor), whereas the monocyte/leukocyte markers CD14, PSGL-1, and L-selectin were absent. The platelet marker CD41 was absent in all cell populations, indicating that these cells were devoid of platelet contamination. Moreover, cells after 10 days of culture were found to be highly proliferative, as shown in Figure 1C.

EPCs Bind Platelets

The adhesive interactions between platelets and EPCs were determined by assessing the number of activated platelets that bound to 100 cells. As shown in Figure 2A, the binding averaged 157 platelets per 100 cells for freshly isolated PBMCs and decreased to 54 for EPCs at day 10 of culture. Inhibition of P-selectin significantly reduced platelet binding by 66% for PBMCs and 50% for EPCs, whereas the blockade of PSGL-1 was only effective in preventing the binding of
platelets to PBMCs but not to EPCs. As control, the binding of platelets to PBMCs or EPCs was not affected by L-selectin blockade.

EPCs Inhibit Platelet Activation and Aggregation

We then investigated the impact of EPCs on thrombin-induced platelet activation by assessing the translocation of CD62P and activation of GPIIb/IIIa by flow cytometry. As shown in Figure 2B through 2D, platelet activation was completely inhibited in the presence of the supernatant of EPCs, as revealed by the absence of CD62P translocation and GPIIb/IIIa activation, whereas the supernatant of PBMCs had no significant effect on platelet activation.

Given that EPCs impair platelet activation, we sought to examine their functional impact on platelet aggregation in response to 2 physiological agonists, thrombin and collagen (Figure 3). Platelet aggregation was not affected by PBMCs but was significantly decreased by washed EPCs in response to both agonists (Figure 3A, 3C, 3D, and 3F). Moreover, platelet aggregation was completely inhibited by the supernatant of EPCs in response to both platelet agonists, whereas the supernatant of PBMCs had no significant effect (Figure 3B through 3E). Thus, the predominant effects of EPCs seem to be related to the release of platelet inhibitory factors that downregulate platelet activation and aggregation.

EPCs Inhibit Platelet Function via PGI2 Secretion

To reveal the factor(s) responsible for the inhibitory effects of EPCs on platelet activation and aggregation, we preincubated differentiating cells with COX and nitric oxide (NO) inhibitors because prostaglandins and NO constitute potent modulators of platelet function. These treatments did not affect the differentiation of PBMCs into EPCs and did not alter their proliferative capacities (data not shown). The culture supernatant from treated cells was collected and assessed for PGI2 and NO secretion and tested on platelet aggregation. As shown in Figure 4A and 4B, EPCs released high levels of PGI2 and NO. In contrast to EPCs, the amount of PGI2 and NO released by PBMCs was negligible. Moreover, the release of PGI2 and NO was totally prevented when differentiated EPCs were cultured in the presence of indomethacin (a COX inhibitor) or 2-phenyl-4,4,5,5-tetramethylimidazoline-
1-oxyl 3-oxide (PTIO) (a NO chelator), respectively. To further validate the release of PGI2 and NO from EPCs, we assessed the expression of the constitutive and inducible forms of COX (COX-1 and COX-2) and NO synthase (NOS) (endothelial NOS [eNOS] and inducible NOS [iNOS] isoforms) in cultured EPCs, in comparison to PBMCs, by Western blot. As shown in Figure 4C, COX-1 was detectable in freshly isolated PBMCs (day 0) and increased in EPCs at day 10. However, COX-2 was specifically upregulated after the differentiation of PBMCs into EPCs. Similarly, iNOS was poorly expressed in PBMCs and profoundly increased in EPCs, whereas eNOS was barely detectable in both cell populations. Interestingly, the antiaggregatory or inhibitory effect of EPCs on platelets was reversed by incubating cultured EPCs with indomethacin (even in the presence of superoxide dismutase [SOD]) or NS-398 (a COX-2 inhibitor) but not with PTIO, N\(^\text{\text{\text{-}}}\)\(^\text{\text{\text{-}}}\)\(^\text{\text{n}}\)itro-L-arginine (L-NNA, a NOS inhibitor), or 1400W (an iNOS inhibitor) (Figure 4D and 4E).

Taken together, these data indicate that, although EPCs specifically upregulate the expression of COX-2 and iNOS and release PGI2 and NO, their main effect on platelet activation and aggregation appears to be PGI2 dependent.

**EPCs Reduce Platelet Adhesion Under Flow**

Because EPCs inhibit activation and aggregation of washed platelets, we then assessed their impact on platelet adhesion to collagen in a whole blood perfusion system. As shown in Figure 5, perfusion of whole blood resulted in almost 25% coverage of the collagen surface with platelets, which was reduced to 7% by a GPIIb/IIIa antagonist, Reopro (positive control). Coincubation of blood with PBMCs or their respective supernatant had no significant effect on platelet adhesion. In contrast, the presence of EPCs or their supernatant reduced significantly platelet adhesion by 23% and 53%, respectively. The effects of EPCs and their supernatant were reversed, once again, by pretreating EPCs with the COX inhibitor indomethacin. Hence, the effects of EPCs on activation and aggregation of isolated platelets were also highlighted in a more physiological approach of platelet adhesion under flow in whole blood.

**EPCs Incorporate at the Sites of Mouse Carotid Injury and Inhibit Thrombus Formation**

Having shown the impact of EPCs on activation and aggregation of washed platelets and on adhesion in whole blood in...
vitro, we hypothesized that they may impair platelet function in vivo. Therefore, we examined the effect of EPCs on thrombus formation by injecting freshly isolated PBMCs and cultured EPCs or their supernatant intravenously into mice 15 minutes before FeCl₃-induced carotid arterial injury. As shown in Figure 6A, mice that received PBMCs had blood flows similar to those of control mice, which were characterized by complete occlusion of the arteries 10 minutes after FeCl₃-induced injury. In contrast, injection of EPCs or their supernatant significantly impaired thrombus formation, leading to an incomplete occlusion with 50% and 100% residual flow, respectively.

To further analyze the characteristics of the formed thrombi, the arteries were fixed immediately after blood flow measurements and were subjected to fluorescence confocal microscopy and histological and immunohistochemical analyses. The circumference of the arteries and thrombi was measured by computer-assisted planimetry, and the thrombus size was reported as percentage of total lumen area. Compared with control- and PBMC-treated arteries, which showed complete occlusion (Figure 6B and Figure 7A and 7E), the arterial thrombus formed in EPC-treated mice was partially occlusive (Figure 7I) and was significantly reduced by 80% and 33% by the supernatant of EPCs and EPCs, respectively (Figure 6B). In another set of experiments, PBMCs and EPCs were labeled with a fluorescent cell tracker before their intravenous injection and were identified in the luminal aspect of the thrombi as a cluster of red fluorescent clumps (Figure 7F and 7J). Injured arteries from control mice, used as negative control, were devoid from any staining (Figure 7B). This was also revealed in sections of injured arteries immunostained with an anti-CD34 antibody (Figure 7D, 7H, 7L). In control- and PBMC-treated mice, the injured carotids were negative for CD34 cells. In contrast, focal accumulation of CD34 cells, representing 10±2 cells per thrombus cross-sectional area, was observed within the thrombus and along the vascular wall in the carotids of EPC-treated mice.

Discussion
The present study provides novel evidence for the regulation of platelet function by EPCs. We found (1) that human PBMC-derived EPCs bound platelets via CD62P and (2) that PBMC-derived EPCs inhibited platelet activation, aggregation, and adhesion to collagen in vitro and thrombus formation in vivo, predominantly via upregulation of COX-2 and secretion of PGI₂. This study reveals a new biological role for EPCs in controlling platelet function, which may in turn limit thrombogenesis and maintain hemostasis at the sites of vascular injury.

It is well established that mobilization and recruitment of EPCs from bone marrow contribute to cell-based therapy and vascular repair. Thus far, therapeutic application of EPCs is still limited by the paucity of available cells. The “ex vivo” outgrowth of EPCs is, therefore, very important to ensure that an adequate cell number is available for their characterization and therapeutic application. Indeed, it has been shown that peripheral blood contains a complex assortment of progenitor...
cells that possess the ability to differentiate into EPCs in vitro. In this connection, we succeeded in adequately differentiating EPCs from PBMCs. The morphology of these cells changed from a population of irregular and small rounded cells to a flattened, spindle-shaped population that forms CFU-EPCs during the differentiation process, uptakes Dil-acetylated low-density lipoprotein, and binds ulex-lectin. Additionally, the expression pattern of surface markers of cultured cells changed during differentiation, as demonstrated by a decrease in pan-monocyte/leukocyte markers and an increase in progenitor/endothelial markers, as well as by their capacity to proliferate after 10 days of culture. Such a subtype of differentiated EPCs displayed some of the morphological, phenotypical, and functional characteristics of late EPCs that derived from the adherent population of PBMCs when plated on collagen or fibronectin in the presence of endothelial-specific growth media.\textsuperscript{9,10,30,31}

Accumulating evidence suggests that platelets play an important role not only in hemostasis and thrombosis but also in inflammation and tissue repair through paracrine mechanisms or direct interactions with other blood cells.\textsuperscript{25,32} Recently, it has been shown that platelets promote the homing and differentiation of EPCs at sites of vascular injury.\textsuperscript{17–24}

Conversely, EPCs may influence platelet function and modulate their thrombogenic properties during vascular repair. To analyze and characterize the action of EPCs on platelet function, we first showed that platelets can bind EPCs but to a lower extent than the binding to PBMCs. The decrease in binding during the differentiation process may be explained by the dynamic changes in the expression of cell surface markers, as well as by the upregulation of platelet inhibiting factors, throughout the process of PBMC differentiation into EPCs. Indeed, we observed a decrease in the expression of PSGL-1 and an increase in the secretion of PGI\textsubscript{2} and NO, thus providing a possible explanation for the significant decrease in the binding of activated platelets to EPCs. We also showed that PBMC-derived EPCs bind activated platelets in a CD62P-dependent manner, thus confirming previous findings demonstrating that the interaction between platelets and EPCs occurs, in part, via CD62P and its high-affinity receptor PSGL-1.\textsuperscript{17,19,21–24} In addition to PSGL-1, other mediators such as stromal cell–derived factor-1\textalpha, CXC chemokine receptor-2 and -4, and \(\beta_3\) and \(\beta_2\)-integrins participate in homing of EPCs at sites of vascular injury.\textsuperscript{17–20}

Moreover, incubation of platelets with the releasate of EPCs resulted in a profound inhibition of platelet activation,
as revealed by the inhibition of CD62P translocation and GPIIb/IIIa activation, both of which are involved in the formation and stabilization of platelet thrombus. The functional impact of EPCs on platelets was also highlighted by their inhibitory effects on thrombin- and collagen-induced aggregation of washed platelets and adhesion to collagen under flow in whole blood. The functional impact of EPCs on platelets was further depicted in a mouse arterial thrombosis model, in which injection of EPCs impaired thrombus mass by 33%, leading to an incomplete occlusion with 50% residual flow. Interestingly, the supernatant of the EPCs was more efficacious than EPCs in inhibiting thrombus formation in vivo, thus confirming the in vitro findings that the main immediate antithrombotic effect of EPCs is related to the release of platelet inhibitory factors. These antiplatelet properties may ultimately lead to the development of novel EPC-derived antithrombotic therapies.

These findings may be of important physiopathological relevance because the acute vascular response to injury involves the adhesion, activation, and aggregation of platelets, followed by the recruitment of other platelets, leukocytes, and possibly circulating EPCs. The recruitment of circulating EPCs at the site of injury may contribute (1) to attenuate the acute thrombotic response by inhibiting platelet function and (2) to promote reendothelialization and vascular repair. This may explain previous observations showing that ex vivo expanded EPCs seeded onto the lumen of a small-diameter vascular graft and implanted in vivo have a much lower chance of thrombosis and subsequent rejection compared with uncoated controls. Moreover, the use of EPC capture stents during percutaneous coronary intervention reduces the incidence of late stent thrombosis. Thus, EPCs modulate platelet function and appear to play an important role in the management of thrombotic reactions.

The fundamental role of EPCs on platelets may be facilitated primarily by the secretion of thromboreistant factors such as PGI$_2$ and NO, which are well-known antiplatelet mediators. In fact, we have shown that PBMC-derived EPCs

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**Figure 7.** EPCs are recruited into arterial thrombi in vivo and reduce thrombus formation. Representative histological transverse sections of FeCl$_3$-injured mouse carotid arteries, stained with hematoxylin-eosin (H&E) and observed by optical microscopy, are shown (left). Arterial thrombus was completely occlusive in control- and PBMC-treated mice (A and E), whereas it was partially occlusive in arteries from EPC-treated mice (I) (magnification ×10). Middle panels show confocal imaging of mouse carotid arteries after vascular injury. PBMCs and EPCs were labeled with intracellular fluorescence marker (DiI), injected before arterial injury, and assessed by confocal fluorescence on cryostat cross sections (F and J). Injured arteries from untreated mice were used as negative control (B). The corresponding differential interference contrast (D.I.C.) of the identical sections is shown on the right (C, G, K) (bar=50 μm). Right panels show CD34 immunostaining of the injured arteries. CD34-positive cells appeared only in the EPC-treated mice, confirming the recruitment of EPCs to the luminal aspect of arterial thrombi in vivo (arrows in L). The cells in D and H are CD34 negative (magnification ×100).
specifically upregulate COX-2 and iNOS during their differentiation and produce PGI₂ and NO. In the present study, however, we identify PGI₂ as the principal mediator involved in the inhibitory action of EPCs on platelet function. This is based on our findings that, in contrast to PBMCs, which constitutively expressed COX-1 but did not produce PGI₂, EPCs specifically upregulated COX-2 and produced PGI₂. Additionally, the effects of EPCs on platelet function were reversed by a pan-COX inhibitor (indomethacin) even in the presence of superoxide dismutase, which ruled out an implication of NO in this phenomenon. Indeed, the effects of EPCs were also reversed by a specific COX-2 inhibitor (NS-398), whereas NO scavenging with PTIO, NOS inhibition with N²-nitro-L-arginine, or iNOS inhibition with 1400W was without any significant effects. Thus, PGI₂ most likely represents the major EPC-derived platelet inhibitor. The high levels of NO released from EPCs could be correlated with a high expression of iNOS, whereas eNOS expression was negligible in EPCs. Our findings are in accordance with a recent study showing that iNOS, but not eNOS, is detectable in peripheral blood–derived EPCs. The low expression of eNOS in EPCs derived from peripheral blood may be explained by the fact that they are not fully matured ECs and are preferentially prone to produce NO in a monocytic/macrophage fashion through iNOS upregulation. This may suggest that the level of NO production in EPCs via iNOS is not sufficient to affect platelet function. Recently, Marjanovic et al. have shown that NO synthesis during platelet activation not only involves eNOS but is also mediated by the constitutively expressed platelet iNOS. iNOS-derived NO is important in promoting platelet activation, both ex vivo and in vivo, via a cGMP-dependent mechanism. Although our data point to the lack of effects of EPC-derived NO on platelet aggregation, the contribution of platelet-derived NO and its signaling pathways, which are believed to play biphasic roles in platelet function, on the effects of EPCs remained to be explored. Moreover, the role of iNOS in EPCs appears to be important in angiogenesis, perhaps by promoting the recruitment of EPCs near the damaged endothelium. Taken together, this may explain, in part, our observation that the inhibitory effects of EPCs on platelet function in vitro were predominantly PGI₂ dependent but not NO dependent. This does not negate, however, the role of EPC-derived NO as a regulatory factor involved in the secondary phase of vascular repair, including angiogenesis.

Our data demonstrate that EPCs regulate platelet function via upregulation of COX-2 and a PGI₂-dependent inhibition of platelet activation, aggregation, adhesion, and thrombus formation. In addition to the well-documented roles of EPCs in angiogenesis and vascular repair, our findings highlight a new biological role for EPCs in regulating platelet function, which may, in turn, limit thrombogenesis and maintain hemostasis at sites of vascular injury.

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**Disclosures**

None.

**References**

Endothelial progenitor cells (EPCs) represent a promising therapeutic approach for the treatment of cardiovascular diseases. However, comprehensive delineation of the biology of these cells and the manner in which they interact with other blood and vascular cells is critical to fully understand their potential therapeutic properties. Herein, we addressed the interactions between EPCs and platelets and their impact on platelet function and thrombus formation, which may be relevant to the management of atherothrombosis during acute coronary syndromes and after percutaneous coronary interventions. We found that human peripheral blood monocyte-derived EPCs in culture bound platelets via CD62P and secreted prostacyclin. Moreover, in a murine arterial thrombosis model, injection of EPCs led to their incorporation into sites of injury and impaired thrombus formation, leading to an incomplete occlusion. In addition to the well-documented roles of EPCs in angiogenesis and vascular repair, our findings highlight a new biological role for EPCs in regulating platelet function, which may, in turn, limit thrombogenesis while maintaining hemostasis at sites of vascular injury. These antiplatelet properties may ultimately lead to the development of novel EPC-derived antithrombotic therapies.
SUPPLEMENTAL MATERIAL

Supplemental Methods

Culture and characterization of EPCs

Human PBMCs were isolated from peripheral blood by centrifugation on a Ficoll density gradient according to the manufacturer’s instructions (StemCell Technologies, Vancouver, BC). The procedures of PBMC-derived EPCs in culture were adapted with modifications from previously published methods.1-4 Briefly, unFractioned mononuclear cells were seeded onto fibronectin coated 35-mm diameter 6-well tissue culture plates (BD Biosciences, Mississauga, ON) at a density of 5 x 10^6 cells/well, and supplemented with EndoCult™ Liquid Medium (StemCell Technologies). After 48 hours, non adherent cells were discarded by gentle washing with phosphate buffer saline (PBS), and fresh media was applied. The adherent cells were continually cultured in a complete media that was changed every 48 hours to allow formation of a well-circumscribed monolayer of spindle-shaped cells. Freshly isolated PBMCs and the differentiated cells after 5 and 10 days of culture were examined by optical and confocal microscopy. At day 5 of culture, the number of colonies, per well for each sample, was counted. Colonies were defined as a central core or round cells with elongated sprouting cells at the periphery. Direct fluorescent staining was used to detect the binding of FITC-Conjugated Ulex-lectin (Sigma-Aldrich, Oakville, ON) and the uptake of Dil-Acetylated Low Density Lipoprotein (Ac-LDL, Molecular probes, Eugene, OR) by laser confocal microscopy. The cells were plated with fresh media onto Microwell Dishes (MatTek corporation, Ashland, MA) coated with fibronectin (5 μg/cm^2, Roche, Laval, Qc) for 1 hour at 37°C. Cells were then incubated with Ac-LDL (2.5 μg/mL) and Ulex-lectin (10 μg/mL) for 1 hour at 37°C, and fixed with 2% paraformaldehyde for 10 minutes. Cells
positive for both Ulex-lectin and Ac-LDL were classified as EPCs. Fluorescent confocal images (Z stacks) were acquired with a LSM 510 confocal microscope (Zeiss, Oberkochen, Germany). A 100x/1.3 Plan-Neofluar objective (Zeiss) was used for magnification. Z stacks-images were deconvolved with the Huygens Pro 3.0 software (Scientific Volume Imaging, Hilversum, The Netherlands), and the final images were reconstructed with the LSM510 software.

The growth capacity of the ex vivo expanded EPCs was examined by passing the cells after 10 days of culture onto fibronectine coated 35-mm diameter 6-well tissue culture plates, and grown in complete endothelial media. Two, 5, and 11 days after plating, the grown cells were passed with Trypsin 0.25% -EDTA 1 mM mixture (US. Biological) and counted with a hematological counter under phase-contrast microscopy (Leicha), with non viable cells discarded through utilization of 4% trypan blue staining (Invitrogen).

**Surface marker expression**

The expression of specific pan-monocyte/leukocyte and progenitor/endothelial surface markers on freshly isolated PBMCs and EPCs were assessed by flow cytometry using saturating concentrations of mouse anti-human PE-conjugated monoclonal antibodies against CD14 (AbD Serotec, Raleigh, NC), CD34 (BD Biosciences), CD162 or PSGL-1 (Santa Cruz, Santa Cruz, CA), CD62P or P-selectin (BD Biosciences), CD62L or L-selectin (AbD Serotec), VEGF Receptor 2 (VEGFR2, Abcam, Cambridge, MA), vWF (AbD Serotec), VE-Cadherin (BD Biosciences) or CD41 (Dako Mississauga, ON). Briefly, freshly isolated PBMCs or cultured cells at days 5 and 10 were detached from the culture plates by gentle scraping in 1 mM EDTA-PBS (pH 7.2) under inverted microscopy. Cells were then re-suspended in PBS containing 0.5% bovine serum albumin, plated in 96-microwell plates at 200 x 10^3 cells/well, blocked with normal
mouse serum for 15 minutes at 4°C, washed, and labeled with saturating concentrations of the above monoclonal antibodies for 20 minutes. The samples were then washed, fixed with paraformaldehyde, and analyzed with an Altra flow cytometer (Beckman Coulter, Mississauga, ON) as described previously. Results were expressed as the percentage of positive cells obtained from 5000 events. Fluorescent isotype-matched antibodies were used as negative controls.

Platelet activation and binding
Platelets were prepared as previously described, and adjusted to a final concentration of 250 x 10⁶/mL. The binding of thrombin-activated platelets to PBMCs and EPCs, in mixed cell populations of 50:1 ratio, was determined using a dual-labeling technique with monoclonal antibodies directed against platelet-CD41 (FITC) and PBMC-CD14 (PE) or EPC-CD34 (PE). The binding was assessed with and without blocking monoclonal antibodies directed against P-selectin (AK6, 10 μg/mL, AbD Serotec) and PSGL-1 (KPL-1, 10 μg/mL, Santa Cruz) for 5 minutes at 37°C, as detailed previously. Control experiments for specificity were done with a blocking anti-CD62L (Dreg-56, 10 μg/mL, BD Biosciences). The fluorescence threshold was set to analyze only dual FITC-and PE-labeled cells, corresponding to CD14⁺ or CD34⁺ that exhibited platelet-CD41 fluorescence from 5000 events. The number of platelets bound to 100 cells was calculated, as described previously.

Platelet (250 x 10⁶/mL) activation was determined by assessing P-selectin translocation (CD62P, BD Biosciences) and GPIIb/IIIa activation (PAC-1, BD Biosciences) at baseline and after stimulation with thrombin in the presence of cell-free supernatant, which was obtained by
centrifugation (600 g, 10 minutes) of 5 x 10^6/mL of freshly isolated PBMCs or EPCs culture media.

**Platelet aggregation**

Platelets, alone (250 x 10^6/mL) or in the presence of 5 x 10^6/mL PBMCs, EPCs, or their cell-free supernatants, were co-incubated in a 4-channel lumi-aggregometer (Chronolog, Havertown, PA) under shear conditions (1,000 rpm) at 37°C. Platelet aggregation was initiated by adding thrombin (0.1 U/mL) or collagen (2 μg/mL) and then monitored by Aggro-Link software (Chronolog) for 5 minutes, as described previously.6, 7

**Platelet adhesion**

The effects of PBMCs and EPCs on platelet adhesion to collagen under flow were assessed in an ex-vivo perfusion system.9 Cells (500 x 10^3/200 μL) or their supernatant were incubated with 2 mL of sodium citrate anticoagulated blood that was labeled with 10 μg/mL Rhodamine 6G (Sigma-Aldrich) for 15 minutes at 37°C. Stained samples were then perfused simultaneously in glass capillaries (0.2 x 2.0 mm I.D., Fiber Optic Center Inc., Bedford, MA), pre-coated overnight at 4°C with 250 μg/mL of fibrillar equine type 1 collagen (Chronolog), at a shear rate of 300/seconds using a KD scientific syringe pump (Fisher Scientific, Ottawa, ON). After a washing step, images of the surfaces were captured using a digital Camera (Scion Corp., Frederick, MD) connected to a Nikon Diaphot 200 epifluorescence microscope (Plan 10x 0.25, Mississauga, ON). The percentage of platelet-covered surface, which represents platelet adhesion, was then quantified by morphometric analyses using the ImageJ software (NIH, Bethesda, MD)
PGI₂ and NO measurements

To assess the release of PGI₂ and NO, cells were incubated with culture media containing either a COX inhibitor (indomethacin, 10 μmol/L, Calbiochem, Darmstadt, Germany), or a selective NO scavenger (phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide or PTIO, 100 μmol/L, Sigma-Aldrich). PGI₂ release was measured with a radioimmunoassay (RIA, Sigma-Aldrich) for the stable metabolite of prostacyclin, 6-keto-PGF₁α. NO release was measured with a nitrate/nitrite fluorometric assay kit (Cayman chemical, Ann Arbor, MI) according to the manufacturer’s instructions. The concentrations of PGI₂ and NO were normalized per unit number for each cell type. Cell culture supernatants from control, indomethacin (10 μmol/L) with and without superoxide dismutase (SOD, 250 U/mL, Sigma ), COX-2 inhibitor (NS-398, 10 μmol/L, Calbiochem), PTIO (100 μmol/L), NOS inhibitor (Nω-Nitro-L-arginine or L-NNA, 100 μmol/L, Sigma-Aldrich), or iNOS inhibitor (1400W, 10 μmol/L, Calbiochem) treated cells were collected and used for platelet function assays.

Western Blot of COX and NOS

Twenty-five μg of protein from PBMCs, and EPCs (5 × 10⁶ cells/mL) lysates were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes. After blocking of non-specific binding, the membranes were incubated overnight at 4°C with antibodies (1:200) against COX-1, COX-2, eNOS or iNOS (Santa Cruz), and labeled with HRP-conjugated secondary antibodies (Santa-Cruz) at room temperature for 1 hour. Proteins were detected by enhanced chemiluminescence (PerkinElmer Life Sciences, Walthman, MA). β-actin was used as an internal control.
Model of mouse carotid thrombosis

The effects of freshly isolated PBMCs and culture-derived EPCs (500 x 10^3 cells)^3,10-12 or their supernatant on thrombus formation were determined in a FeCl3 mouse carotid injury model, according to a standardized protocol.13 Control experiments were done with culture media alone. Briefly, C57BL6 mice (14-16 weeks old, Jackson laboratory, Bar Harbor, ME) were anesthetized with a mixture of ketamine (Vetalar: 1.5 mg/kg I.P., Bioniche, Belleville, ON) and medetomidine (Domitor: 1 mg/kg I.P., Pfizer, Kirkland, QC) and the right carotid artery was carefully exposed. A miniature ultrasound flow probe (0.5 VB 552; Transonic Systems, Ithaca, NY), interfaced with a flow meter (T206; Transonic Systems) and a computer-based data acquisition program (Iox 2.2.17.19, Emka, Falls Church, VA), was positioned around the artery. After measuring baseline blood flow, a 0.5 x 1.0-mm strip of filter paper (Whatman no. 1) soaked in 6.5% FeCl3 was applied on the adventitial surface proximal to the flow probe for 3 minutes, after which blood flow and time to thrombotic occlusion (blood flow of 0 mL/minutes) were monitored.

Histology, immunostaining and confocal fluorescence

After completion of in vivo flow measurements in mice, the injured and the contralateral non-injured carotids were excised and fixed in 10% buffered formalin. These arterial segments were then embedded in paraffin, sectioned at 6 microns, and stained with hematoxylin and eosin, or with an anti-CD34 antibody (Santa Cruz). Samples were visualized using an Olympus BX60 microscope (Olympus imaging America Inc., Center Valley, PA) and images were captured with a Retiga 2000R camera (QImaging Corporation, Surrey, BC). The final images were acquired with Image Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD).
For confocal fluorescence, 500 x10^3 of PBMCs or EPCs were labeled with an intracellular photostable fluorescence marker (CellTracker™ CM-DiI, Molecular Probes) according to the manufacturer’s instructions. Cells were then washed with PBS, resuspended in fresh media and injected intravenously, and allowed to circulate for 15 minutes before inducing the mouse carotid injury. The carotids were excised immediately after completion of the blood flow measurements and immersed in liquid nitrogen. Labeled PBMCs and EPCs incorporated into the luminal aspect of arterial thrombi were observed on cryostat sections of 14-μm thickness using confocal microscopy. Fluorescent confocal images (Z stacks) were acquired with a LSM 510 confocal microscope (Zeiss). A 10x/1.3 Plan-Neofluar objective (Zeiss) was used for magnification. Z stacks-images were deconvolved with the Huygens Pro 3.0 software (Scientific Volume Imaging), and the final images were reconstructed with the LSM510 software.

Statistical analysis

Results are presented as mean ± SEM. The number of experiments for each test is indicated in the figure legends. Statistical comparisons were done using the InStat3 Software (GraphPad software inc. La Jolla, CA) for one-way ANOVA, followed by a Dunnett-t-test for comparison against a single group. Data with p <0.05 were considered statistically significant.

Supplemental References


