UltraRapid Communication

Adherent Platelets Recruit and Induce Differentiation of Murine Embryonic Endothelial Progenitor Cells to Mature Endothelial Cells In Vitro

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Abstract—The homing and differentiation mechanisms of endothelial progenitor cells (EPCs) at sites of vascular lesions are unclear. To investigate whether platelets play a role in the recruitment and differentiation of EPCs, we made use of a robust mouse embryonic EPC (eEPC) line that reliably differentiates to a mature endothelial phenotype. We found that platelets stimulate chemotaxis and migration of these murine eEPCs. Further, the substantial adhesion of murine eEPCs on immobilized platelets that occurs under dynamic flow conditions is inhibited by neutralizing anti–P-selectin glycoprotein ligand-1 and anti–VLA-4 (β1-integrin) monoclonal antibodies but not by anti-CD11b (αM-integrin; macrophage antigen-1). Coincubation of murine eEPCs with platelets for 5 days induced differentiation of EPCs to mature endothelial cells as verified by positive von Willebrand factor immunofluorescence and detection of Weibel Palade bodies through electron microscopy. We conclude that platelets may play a critical part in the capture and subsequent differentiation of murine eEPCs at sites of vascular lesions, revealing a possible new role of platelets in neoendothelization after vascular injury. (Circ Res. 2006;98:e2-e10.)

Key Words: endothelial progenitor cells ■ platelets ■ endothelization ■ vascular lesion ■ adhesion

Vascular endothelium represents a barrier between circulating blood cells and the subendothelial matrix. Discontinuity of this barrier (eg, at sites of vascular injury or rupture of an atherosclerotic plaque) exposes various components of the subendothelial matrix, including collagen, fibronectin, or von Willebrand factor (vWF).1 Platelets rapidly adhere to vascular lesions, resulting in thrombus formation.1,2 If this process is controlled, platelets passivate vascular injury and initiate the healing process.1 However, uncontrolled platelet-mediated thrombus formation leads to acute thrombotic occlusion or plaque progression.3 However, the underlying mechanisms of platelet-mediated vascular remodeling and healing of lesions is poorly understood so far.

Endothelial progenitor cells (EPCs) are a circulating, bone marrow–derived cell population of large nonleukocyte cells that appear to participate in vascular repair and homeostasis. In response to cytokine stimulation and ischemia, these cells are mobilized from the bone marrow, home to the ischemic tissue, and contribute to neovascularization and angiogenesis.3–6 Furthermore, EPCs are regarded to have a key role in the maintenance of vascular integrity and to act as “repair” cells in response to endothelial injury.7,8 Several groups have raised colonies in vitro from CD34+ cells, which resemble mature endothelial cells, implying that EPCs are capable of promoting endothelization.9 Recruitment of EPCs toward vascular lesions has been regarded as a critical initial step in atherosclerosis and a result of the actions of various cardiovascular risk factors.10 Current data suggest that decrease in circulating EPCs contributes not only to impaired angiogenesis but also to the progression of atherosclerosis,11 and patients at risk for coronary artery disease have a decreased number of circulating EPCs with impaired activity.9,12–14

However, the mechanisms that regulate recruitment of EPCs toward vascular lesions and initiate neoendothelization are not understood. In general, recruitment of circulating cells is a well-controlled process that involves chemotaxis, tethering, adhesion, and migration of cells into the subendothelial tissues. Several adhesion molecules have been described to regulate adhesion and migration of EPCs, including...
P-selectin glycoprotein ligand (PSGL-1) and β1- and β2-integrins.15–17

Adherent platelets secrete potent chemokines at sites of vascular injury and display several ligands for adhesion receptors on their surface, and therefore, they represent potential mediators of progenitor cell homing.18 In this study, we investigated the capacity of platelets to induce chemotaxis, adhesion, and migration of EPCs and provide evidence that platelets may also contribute to the differentiation of EPCs into endothelial cells.

Materials and Methods

Reagents

RB40.34 (function-blocking anti-mouse P-selectin) and 4RA10 (function-blocking anti-mouse PSGL-1) were raised as described previously.19,20 A goat anti-human polyclonal antibody (monoclonal antibody [mAb]) directed against vWF (C-20), which cross-reacts with mouse vWF, and N.2′-O-dibutyryladenosine3′,5′-cyclic monophosphate (cAMP) were purchased from Santa Cruz Biotechnology. Phycoerythrin or fluorescein isothiocyanate (FITC)–conjugated mAb rat anti-mouse CD162 (PSGL-1; clone 2PH1); clone 9C10), CD49d (VLA-4; α-chain; clone 9C10), CD11b (clone M1/70), and blocking mAb anti-mouse CD29 (β1-integrin; clone 18) were purchased from Pharmingen. mAb anti-CD41 (GPIIb; clone P2) was from Beckmann Coulter. For immunofluorescence microscopy, a goat polyclonal antibody to eEPCs has been described previously.15,21 The cells are cultured in medium alone as control. Migrated cells were counted after 24 hours. Subsequently, T17b cells were added and incubated for 60 minutes. In a second adhesion assay, T17b (1×10^5/mL) were incubated for 30 minutes with isolated platelets (2×10^4/mL) or buffer as control under agitation (1000 rpm) using a conventional platelet aggregometer. Thereafter, cell suspension was washed 3 times with Tyrodes buffer, and eEPCs were incubated with FITC–conjugated anti-CD41 or control anti-CD11b mAb. The percentage of CD41-positive T17b cells was analyzed by flow cytometry and was used as index of platelet/eEPC adhesion.

Dynamic Adhesion

Adhesion experiments under flow conditions were basically performed as described previously.22 In brief, glass coverslips were coated with collagen type I (5 μg/mL) as described previously23 and used in a flow chamber (Oliogene). Thereafter, isolated platelets (2×10^4/mL) were allowed to adhere to collagen for 30 minutes. In some experiments, adherent platelets were pretreated with anti-CD62P mAb (5 μg/mL) before eEPCs were perfused over immobilized platelets. In other experiments, eEPCs (T17b) were preincubated with anti-CD29, anti-CD162, or anti-CD11b (5 μg/mL). Perfusion was performed with T17b cells resuspended in Tyrodes–HEPES buffer (pH 7.4 supplemented with 1 mmol/L CaCl_2 and 1 mmol/L MgCl_2) at shear rates of 2000 s^{-1} (high shear). All experiments were recorded in real time on videotape and evaluated offline using Capimage software (Zeintl).

Scratch Assay
eEPCs were grown to confluence in a 24-well plate. Subsequently, the cell monolayer was wounded with a plastic pipette tip generating a gap of ~1-mm width, and cells were incubated with isolated platelets resuspended in medium (0.1 to 1×10^5/mL) or growth medium alone as control. Migrated cells were counted after 24 hours. Thereafter, wells were incubated with an anti-vWF antibody (5 μg/mL) and a secondary Alexa Fluor 488–conjugated rabbit anti-goat antibody, as described above, and analyzed by immunofluorescence microscopy, as described below. To detect specific mediators of this platelet-mediated migration, blocking mAbs to platelet-derived growth factor-AB (Promega), CD40L (Biozol), CD11b (clone M1/70; Pharmingen), and CD54 (Beckmann Coulter), using concentrations of 5 μg/mL for each substance, were used. Activation protein tumor necrosis factor-α (Tebu-bior; 20 ng/mL) served as positive control.

Scanning and Transmission Electron Microscopy

For scanning electron microscopy (SEM), T17b cells were cultivated on coverslips in the absence or presence of platelets (2×10^5
cells/mL) for 24 hours. Thereafter, the cells were washed 2 times with PBS, and the coverslips were fixed and examined using a field emission scanning electron microscope (JSM-6300F; Jeol Ltd.). For transmission electron microscopy, EPCs were grown to 70% to 80% confluence and coincubated with isolated platelets (2×10⁵/mL) for 5 days in culture medium. Subsequently, cells were washed and fixed using a buffer consisting of glutaraldehyde (2.5%) and tannin (0.02%) in a sodium cacodylate buffer, pH 7.4, before electron microscopy was performed.²⁷

Reverse Transcription–Polymerase Chain Reaction

After incubation of T17b cells with buffer alone, cAMP (0.5 mmol/L), or isolated platelets (4×10⁶/mL) for 5 days, the 6-well plates were carefully washed with PBS and analyzed by RT-PCR as described.²⁸ Briefly, total RNA was extracted using the RNeasy Mini Kit (Qiagen). Contaminating DNA was removed by DNase using the Qiagen RNase-free DNase Set. RT-PCR was performed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and Taq Polymerase (GIBCO BRL). Annealing temperature was 94°C for 30 seconds, extension at 72°C for 30 seconds, with a final 7-minute extension at 72°C. The primer sequences were as follows: c-kit forward 5′-ATA AAG GGT TCA GTG TGT TAC GTG G-3′, reverse 5′-TTG GAA AGG TGC AAG AGT GTA GC-3′; tié-2 5′-CGG TCA GCA TTG TAC TGA GAG-3′; CD31 (platelet-endothelial cell adhesion molecule-1 [PECAM-1]) forward 5′-ATT CAG GCA TTG TAC TGA GAG-3′, reverse 5′-CGT GCT TCC GTA GGA TCT TCA G-3′; flk-1 5′-GCA GCG CTT CAAG-3′, 5′-CAC ATA CTG-3′; aldolase, 5′-GAA AAG GCT GGG TTG CTT GAT C-3′; and CD31, 5′-GAA AAG GCT GGG TTG CTT GAT C-3′; cyclase, 5′-AGC TGT CTG ACA TCG CTC ACC G-3′; tie-2 5′-CAA CAG CGT CTA TCG GAC TCC-3′, 5′-GAA AAG GCT GGG TTG CTT GAT C-3′; aldolase, 5′-AGC TGT CTG ACA TCG CTC ACC G-3′; and CD31, 5′-GAA AAG GCT GGG TTG CTT GAT C-3′. Lowercase letters indicate nucleotides added for cloning purposes.

Immunofluorescence Microscopy

EPCs were coincubated with medium or platelets for 5 days on chamber slides and processed for immunofluorescence microscopy. The mouse endothelial cell line MHEC5-T was used as positive control. Between each incubation step, cells were gently washed with PBS. T17b cells were fixed using a buffer consisting of glutaraldehyde (2.5%) and tannin (0.02%) in a sodium cacodylate buffer, pH 7.4, before electron microscopy was performed.²⁷

Data Presentation and Statistics

Comparisons between group means were performed using Student t test or ANOVA analysis. Data are presented as mean±SD. P<0.05 was considered statistically significant.

Results

Platelets Induce Chemotaxis and Migration of EPCs

Adherent platelets secrete various chemotactic compounds on activation.¹⁸ Thus, we evaluated the effect of platelets on eEPCs chemotaxis in a trans-well chamber culture system. Chemotaxis of eEPCs (T17b cells) toward adherent platelets was substantially increased compared with controls (P<0.01; Figure 1). Next, we studied the effect of platelets on eEPC migration. T17b cells were grown to confluence in 24-well plates, and the cell monolayers were wounded in a linear fashion with a pipette tip. Thereafter, the wounded eEPC monolayer was incubated for an additional 12 hours in the absence or presence of platelets (0.1 to 1×10⁶ cells/mL medium), and the number of eEPCs that migrated into the denuded area was evaluated (Figure 2). We found that eEPC migration was significantly increased, depending on platelet number (Figure 2). At a platelet concentration of 1×10⁶/mL, eEPC migration was enhanced by ∼2.5-fold (P<0.01) compared with control, indicating that platelets stimulate migration of eEPCs. To detect the specific mediators of platelet-induced migration, blocking antibodies were used, as indicated in figure legends. By addition of blocking anti–platelet-derived growth factor-AB, a substantial reduction of migration was found (Figure 2D and 2E).

eEPCs Adhere to Platelets Under Static and Dynamic Flow Conditions

Platelets play a critical role in the recruitment and adhesion of circulating leukocytes to the vascular wall.¹⁸ To test whether EPCs bind to platelets, isolated platelets (2×10⁶/mL) were allowed to adhere to 96-well plates for 2 hours. Subsequently, EPCs were added to the wells, and adhesion of EPCs on platelets was evaluated as described in Materials and Methods. Under these static conditions, EPCs showed an ∼4-fold
enhanced adhesion to platelets compared with the plastic surface control ($P<0.01$; Figure 3A and 3B). In similar fashion, a flow cytometric adhesion assay showed enhanced coadhesion of platelets with EPCs under stirring conditions (Figure 3C). Furthermore, adhesion of isolated platelets to EPCs could be visualized by scanning electron microscopy (data not shown).

Next, we evaluated the determinants that mediate EPC adhesion to platelets under arterial flow conditions. In a parallel plate flow chamber, EPCs were perfused over platelets immobilized on collagen at a wall shear rate of 2000 $s^{-1}$ as described.27 EPCs substantially adhered to immobilized platelets under flow conditions but not to immobilized collagen type I ($P<0.01$; Figure 4A). Interestingly, rolling of EPCs on immobilized platelets was almost completely inhibited in the presence of blocking anti-CD62P (P-selectin) or anti-CD162 (PSGL-1) antibodies but not by anti-CD29 ($\beta_1$-integrin; Figure 4). In contrast, firm adhesion of EPCs to platelets was attenuated by both anti-CD62P/anti-CD162 and anti-CD29 mAbs (Figure 4A). Virtually no inhibition of adhesion of EPCs on immobilized platelets was found in the presence of a blocking anti-CD11b (anti-integrin $\alpha_M$, macrophage antigen-1 $\alpha$) mAb (Figure 4A). Flow cytometric analysis of EPCs showed substantial cell surface expression of CD162 (PSGL-1) and of CD49d (VLA-4) on T17b cells (data not shown), confirming previously published results.15

Together, these data indicate that adhesion of EPCs onto immobilized platelets occurs under dynamic flow conditions. The adhesion of EPCs to immobilized platelets is specifically regulated by selectins (PSGL-1/P-selectin; rolling) and integrins ($\beta_1$-integrin; firm adhesion). To test whether these findings can be transferred from the murine EPC model to adult progenitor cells, dynamic adhesion of adult human CD34$^+$ cells isolated from cord blood was evaluated. Com-
were cultured in the presence of platelets for 5 days and analyzed by light and fluorescence microscopy. We found that a noticeable amount of eEPCs acquired an endothelium-like shape with a 3- to 5-fold larger, spindle-shaped appearance with cellular protrusions (Figure 5B). In contrast, no change in the phenotype of eEPCs was noted in the absence of platelets.

Immunofluorescence verified that eEPCs transform into mature endothelial cells in the presence of platelets. We found that eEPCs coincubated in the presence of platelets for 7 to 10 days showed enhanced vWF expression compared with untreated cells (Figure 6A). Moreover, eEPCs cocultured with platelets revealed a typical endothelial cell-like cytoskeleton rearrangement, as verified by staining with phalloidin. As a representative control of the differentiated endothelial phenotype, we used the mouse endothelial cell line MHEC5-T (Figure 6A). Transmission electron microscopy showed the formation and presence of Weibel Palade bodies in eEPCs cocultured in the presence of platelets similar to mature endothelial cells (Figure 6B). Additionally, RT-PCR analysis of native eEPCs showed virtually no expression of the mature endothelial cell marker CD31 (PECAM-1) and a distinct signal for the stem cell marker c-kit (Figure 6). However, after incubation of EPCs with isolated platelets for 5 days, CD31 mRNA expression increased, whereas c-kit decreased substantially (cAMP-induced differentiation served as a positive control). In a second RT-PCR analysis, the endothelial cell markers flk-1 (vascular endothelial growth factor receptor), vascular endothelial-cadherin, and thrombomodulin were increased after incubation with isolated platelets or cAMP. Consistent with previous results, expression of Tie-2 was not enhanced after incubation with cAMP, nor with platelets. To test, whether these findings can be transferred from the murine eEPC model to adult human progenitor cells, differentiation of human CD34+ cells by platelets was evaluated. After 10 days of coincubation, a change in morphology of CD34+ cells could be observed with regard to increased size and spindle-shaped morphology (Figure 6D). Furthermore, these cells displayed substantial amounts of cytoskeleton, characteristic for endothelial cells, but not progenitor cells, as verified by phalloidin staining.

**Discussion**

In this study, we have shown that platelets regulate chemotaxis, adhesion, and differentiation of murine EPCs (eEPCs).

In brief, the major findings of the present study are: (1) platelets induce chemotaxis and migration of a mouse eEPC line (T17b); (2) murine eEPCs specifically adhere to immobilized platelets via PSGL-1 and VLA-4 under high-shear dynamic flow conditions; and (3) platelets induce differentiation of murine eEPCs and maturation into endothelial cells as evidenced by specific immunological (vWF) and morphological (Weibel Palade bodies) endothelial markers. The findings imply that platelets are critical for recruitment of circulating murine eEPCs to areas of vascular or ischemic lesions. Interaction of these eEPCs with activated platelets may play a prominent physiological role for vascular or tissue
remodeling. A disturbed platelet interaction with EPCs may in turn be associated with defective vascular and tissue repair mechanisms involved in pathologies such as atherosclerosis and myocardial infarction.

The maintenance of endothelial integrity is of crucial importance for preventing atherosclerotic plaque erosion and rupture, causing myocardial infarction and sudden cardiovascular death. Recently, circulating EPCs have been shown to incorporate into sites of neovascularization and to home to sites of endothelial denudation. EPCs recruited at site of vascular lesions accelerate re-endothelization and lesion repair. However, the mechanisms that regulate EPC homing to vascular or tissue lesions are incompletely understood.

Platelets accumulate within seconds to sites of vascular injury and release a variety of potent chemotactic factors that induce recruitment of circulating blood cells toward sites of vessel injury. Adherent platelets express various receptors (e.g., P-selectin) on their surface that regulate adhesion of neutrophils and mononuclear cells. Previous studies indicated that platelet-derived microparticles could influence

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Figure 4. Adhesion of eEPCs to immobilized platelets under arterial shear conditions. Coverslips were precoated with collagen I (5 μg/mL) and additionally preincubated with or without freshly isolated platelets (2×10^9/mL) to achieve adherent platelet layers as described in Materials and Methods. Resuspended T17b cells (5×10^4/mL) were perfused over these coverslips in the presence or absence of blocking mAbs (5 μg/mL) as indicated. After 5 minutes, adherent eEPC were quantified by offline counting. The mean and SD of 4 independent experiments is shown. *P<0.05 compared with control (collagen without platelets).

Figure 5. Effect of platelets on ePC migration and morphology. A, A confluent monolayer of eEPCs was wounded with a plastic pipette and coincubated with isolated platelets (2×10^9/mL). After 24 hours, at the edge of the wound, migrating EPCs showed enhanced expression of vWF as verified by immunofluorescence microscopy. B, T17b cells were left untreated (right panel, magnification ×80) or coincubated with platelets (left panel, magnification ×40) as described in Materials and Methods. After 5 days, photomicrographs were taken to assess cell morphology. ! indicates original morphology of the cells. ≈ indicates modified cell morphology after coincubation with platelets.

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minutes, adherent CD34^+ cells were quantified by offline counting. The mean and SD of 4 independent experiments is shown. *P<0.05 compared with control (collagen without platelets).
Number and biological functions of stem cells. Thus, we hypothesized that platelets mediate chemotaxis and adhesion of murine eEPCs. As reported previously, β3-integrin CD49d (VLA-4) and PSGL-1 are the major adhesion receptors present on the cell surface of EPCs and mediate progenitor cell homing to the endothelial monolayer of bone marrow microvessels via binding to their counterpartner P-selectin and vascular cell adhesion molecule-1. A subset of cord blood–derived CD42+ cells, which are enriched in primitive CD38+ cells, demonstrate reduced rolling attributable to partial expression of a nonfunctional form of PSGL-1. Neutralizing the β3-integrin VLA-4 blocks homing of human CD34+ cells to the bone marrow of fetal sheep. In the present study, we show that murine eEPCs adhere to immobilized platelets under flow conditions similar to arterial shear stress rates. We demonstrate that rolling of these eEPCs onto immobilized platelets is mediated by PSGL-1 and that VLA-4 is required for firm adhesion.

Recruitment and incorporation of EPCs require a coordinated sequence of multistep adhesive and signaling events, including chemotraction, adhesion, and migration, and finally the differentiation to endothelial cells. Preliminary data suggest that EPCs have the capacity to regenerate the injured endothelial monolayer. However, the mechanisms that induce differentiation of EPCs into mature endothelial cells remain unexplained. Platelets store a variety of potent growth factors within their granules, including vascular endothelial growth factor-A, that are released into the microenvironment of activated platelets. We found that in the microenvironment of platelets, murine eEPCs migrate and adhere to immobilized platelets and start to differentiate into mature endothelial cells, as evidenced by expression of vWF and formation of Weibel-Palade bodies, organelles that are specific for mature endothelial cells.

In this study, we made use of a mouse eEPC line. A hindering factor for a systematic analysis of EPC homing and differentiation mechanisms is the fact that adult EPCs are difficult to isolate and maintain and vary substantially between individuals with respect to functional properties. The major advantages of eEPCs are their robust growth properties in culture and practical genetic manipulation. The eEPCs express early endothelial markers, differentiate to mature endothelial cells, form vascular tubes in vitro, and build blood vessels after transplantation during embryogenesis. In this study, we made use of a mouse eEPC line. A hindering factor for a systematic analysis of EPC homing and differentiation mechanisms is the fact that adult EPCs are difficult to isolate and maintain and vary substantially between individuals with respect to functional properties. The major advantages of eEPCs are their robust growth properties in culture and practical genetic manipulation. The eEPCs express early endothelial markers, differentiate to mature endothelial cells, form vascular tubes in vitro, and build blood vessels after transplantation during embryogenesis.

Figure 6. Differentiation of eEPCs by platelets. A, T17b cells were left untreated or incubated in 6-well plates with platelets. After 5 days, platelets were removed and eEPCs were incubated with FITC-conjugated goat anti-mouse vWF antibody (5 μg/mL) for 30 minutes. After washing, an Alexa Fluor rabbit anti-goat IgG was added for an additional 30 minutes. For detection of cell cytoskeleton, eEPCs were incubated with rhodamine phalloidin (5 μg/mL) and 505 to 530 nm, respectively. The mouse endothelial cell line MHEC5-T served as a positive control. B, Electron microscopic analysis of T17b cells after incubation with medium served as a control. Analysis revealed Weibel Palade bodies (indicated with ←, 311×61 nm; magnification ×80 000) in eEPCs treated with platelets, which show comparable appearance to those in the mouse endothelial cells MHEC5-T (320×89 nm; magnification ×80 000). T17b cells incubated with medium alone showed no Weibel-Palade-bodies. C, RT-PCR analysis of the stem cell marker c-kit and of the marker for mature endothelial cells CD31 (PECAM-1) in T17b cells treated with medium, cAMP (0.5 mmol/L), or isolated platelets (4×10⁹/mL), as indicated for 5 days. The image shown is representative for 2 independent experiments. D, RT-PCR analysis of the markers for mature endothelial cells thrombomodulin, flk-1 (vascular endothelial growth factor receptor), vascular endothelial-cadherin, and Tie-2 in T17b cells treated with medium, cAMP (0.5 mmol/L), or isolated platelets (4×10⁹/mL) as indicated for 5 days. The image shown is representative for 2 independent experiments. Aldolase mRNA was analyzed as internal control. E, CD34+ cells (5×10⁶) were coincubated with isolated platelets (2×10⁹/mL) in 96-well plates for 10 days. Compared with untreated CD34+ cells (arrows, top panel), cells coincubated with platelets developed an endothelial spindle-shaped phenotype (arrows, top panel). vWF immuno-staining and DAPI staining of CD34+ cells (bottom panel) and progenitor cells after 10 days coculture with platelets (bottom panel).
dition, the homing of eEPCs in hypoxic tumors, their participation in tumor vessel formation, as well as their stimulation of angiogenesis in chronic and acute ischemia render eEPCs a relevant model system to study the biology of EPCs. To evaluate, if the central findings of this study derived from the murine progenitor cell model can be transferred to human progenitor cells, key experiments were performed using adult human CD34+ cells. We show that adult CD34+ cells adhere to platelets under high shear rates and that platelets induce differentiation of CD34+ cells into endothelial cells. Nevertheless, further studies using human bone marrow–derived stem cells have to be performed to confirm the exact mechanisms of this interaction within the human system.

In conclusion, we provide first evidence that platelets mediate recruitment of murine eEPCs and stimulate differentiation of these eEPCs into endothelial cells. If these findings can be transferred to the human system, the mechanism described in the present study may play a central physiological role for maintenance of vessel integrity and neoendothelialization of vascular lesions. Further, an altered interaction of platelets and EPCs may play a critical role in atheroprotection.

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References


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