

**The Role of Platelet-Activating Factor in the Adherence of Circulating Cells to the Endothelium**

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Running Title: PAF in cell-cell interaction

## Abstract

Platelet-activating factor (PAF) is a biologically active phospholipid metabolite that is produced by numerous cell types and tissues. PAF serves as both an intercellular and intracellular messenger and is involved in the development and progression of several physiological and pathological processes. In this review, we will focus primarily on the effects that PAF produces in the endothelium, in particular, the role that PAF plays in the adherence of circulating cells to the endothelium. Previous studies in our laboratory have demonstrated that PAF production in human coronary artery endothelial cells is produced via the remodeling pathway, involving the initial production of lysoplasmenylethanolamine that is the direct result of the activation of calcium-independent phospholipase A<sub>2</sub> (iPLA<sub>2</sub>). Lysoplasmenylethanolamine then participates in a transacylation reaction with alkyl acyl-glycerol-3-phosphocholine (Pathway 2, Figure 1) to produce lyso-PAF that is then acetylated by lyso-PAF acetyltransferase to form PAF. Once produced by the endothelial cell, PAF remains cell associated, and in concert with increased cell surface expression of P-selectin, results in the tethering and migration of circulating cells through the endothelium. In addition to the well-described role this process plays in inflammation, we will explore our hypothesis that endothelial PAF production also may play a role in homing of stem cells to specific organs, an area of research that is presently commanding intense interest.

## Introduction

Platelet-activating factor (PAF) is a potent lipid autotoxin rapidly synthesized and presented on the surface of endothelial cells in response to a variety of agonists and conditions. Endothelial cell PAF production significantly contributes to the recruitment of leukocytes and monocytes to inflamed tissue by promoting the initial adherence to the endothelium [12] and thus can play a major role in the progression of inflammatory diseases [81]. Inflammation is a central feature of atherosclerosis and thrombosis, both processes in which PAF is intimately involved. PAF has been implicated in the development of myocardial ischemia-reperfusion injury and plays a role in hypotension and the cardiac dysfunction occurring in cardiovascular stress situations, such as cardiac anaphylaxis, and hemorrhagic, traumatic, and septic shock syndromes.

This review will focus on the pathways of PAF production in endothelial cells and discuss the role of PAF in mediating the adherence of circulating cells to the endothelium. Due to the recent interest in the therapeutic potential of stem cells, we will also present current knowledge regarding the homing and adherence of stem cells to the endothelium and propose a role for PAF in this process.

## Pathways of PAF Synthesis

The synthesis of platelet-activating factor (PAF) occurs through two pathways, the remodeling pathway and the *de novo* pathway. Evidence indicates that the remodeling pathway for PAF synthesis is activated during inflammation and hypersensitivity

responses, whereas the *de novo* synthetic pathway is thought to be the source of PAF required for physiologic functions [3]. The remodeling pathway for PAF production is a tightly coupled reaction involving the sequential action of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) to produce lyso-PAF, followed by acetylation of lyso-PAF with acetyl-CoA:1-alkyl-*sn*-glycero-3-phosphorylcholine 2-*O*-acetyltransferase [1]. Lyso-PAF may be produced by the direct action of PLA<sub>2</sub> on alkylacyl-glycerol-3-phosphocholine (Pathway 2, Figure 1). Alternatively, since PLA<sub>2</sub> is not selective for alkylacyl phospholipids, lyso-PAF may be generated via a CoA-independent transacylation between a lysophospholipid acceptor and alkyl acyl-glycerol-3-phosphocholine (Pathway 1, Figure 1) [18-21].

In recent studies, we have demonstrated that thrombin stimulation of human endothelial cells results in a time dependent increase in membrane-associated, calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) activity. This results in accelerated PLA<sub>2</sub>-catalyzed hydrolysis of membrane plasmalogen phospholipids and the increase in production of plasmalogen lysophospholipids, PAF, and free fatty acids. The accelerated hydrolysis of plasmenylethanolamine and resultant increase in lysoplasmenylethanolamine appears to be a requirement for PAF synthesis in thrombin stimulated endothelial cells, demonstrating that the remodeling pathway of PAF significantly contributes to PAF synthesis in thrombin-stimulated endothelial cells [1, 12].

The production of PAF can also occur through the *de novo* pathway. This pathway is mainly operative in the kidney and central nervous system and proceeds via the synthesis of 1-*O*-alkyl-2-acetyl-glycerol which is then converted to PAF by a specific CDP-choline:1-alkyl-2-acetyl-*sn*-glycerol choline phosphotransferase. The direct precursors of PAF are 1-alkyl-2-acetyl-*sn*-glycerols that are formed by an acetylation-dephosphorylation sequence catalyzed by CoA:1-alkyl-2-lyso-*sn*-glycero-3-phosphate acetyltransferase and 1-alkyl-2-acetyl-*sn*-glycero-3-phosphate phosphohydrolase [22-24].

### **PAF Catabolism by PAF-acetylhydrolase**

The concentration of PAF in plasma and tissues is tightly regulated by the balance of its synthesis and degradation [82]. Since PAF is a potent inflammatory phospholipid metabolite, it is normally maintained at low concentrations by being rapidly hydrolyzed by PAF acetylhydrolase (PAF-AH). PAF-AH is classified as a cytosolic calcium-independent PLA<sub>2</sub> that preferentially hydrolyzes phospholipids with short chain or oxidized fatty acids at the *sn*-2 position [82,83]. PAF-AH hydrolyzes the acetyl group at the *sn*-2 position of PAF to form the biologically inactive lyso-PAF [3]. PAF-AH causes PAF to lose its ability to activate cell polarization and cell spreading in vitro and abolishes the inflammatory effects of PAF that involve adherence of leukocytes to the vasculature [2]. It exists as both an intracellular molecule and as a secreted enzyme. The plasma form of PAF-AH, a scavenger of both PAF and oxidized phospholipids, is generally found in a complex with low- and high-density lipoproteins [3,112]. PAF-AH is thought to play a role in atherosclerosis by degrading PAF-like oxidized phospholipids that bind PAF receptors [115] and are implicated in the pathogenesis of atherosclerosis. [116]. This enzyme is inactive against long chain fatty acids at the *sn*-2 position. Interestingly, we have recently demonstrated that pretreatment of endothelial cells with methyl arachidonyl fluorophosphonate (MAFP, an inhibitor of cytosolic Ca-dependent and -independent PLA<sub>2</sub> isoforms) [90,91] increases basal and thrombin-stimulated PAF production as a direct result of the inhibition of PAF-AH activity [12]. The increase in PAF production in

MAFP-pretreated, thrombin-stimulated endothelial cells is accompanied by increased expression of P-selectin (CD62P) on the endothelial cell surface (Figure 2) and by increased neutrophil adherence to the endothelial cell monolayer (Figure 3). These data illustrate an example of how a PLA<sub>2</sub> inhibitor, initially designed as a potential anti-inflammatory agent, could in fact act as a pro-inflammatory agent, prolonging the inflammatory response and increasing the recruitment of inflammatory cells to areas of injury [12].

### PAF Receptors

Once expressed on the endothelial cell surface, PAF activates PAF receptors on tethered cells, inducing a program of adhesion and  $\beta$ -integrin-mediated migration [2]. The PAF receptor (PAFR) is a 342 amino acid, seven transmembrane spanning, G-protein coupled receptor [92-94]. To date, no subtypes of PAFR have been identified [3]. PAFR mRNA has been detected in several tissues and isolated cells, such as polymorphonuclear cells [100,101], monocytes-macrophages [11,107] eosinophils [108], endothelial cells [109], smooth muscle cells [102], spleen [95], kidney [95], Kupffer cells [95,104-106], cardiomyocytes [95,103], skeletal muscle [95], and brain [95]. Additionally, *in situ* hybridization detected PAFR mRNA in human blood vessels, smooth muscles and the alveolar wall of the lungs [95]. PAFR regulates initial G-protein coupled receptor second messengers. It augments inositol 1,4,5-triphosphate (IP<sub>3</sub>) synthesis and calcium mobilization and suppresses forskolin-stimulated cAMP synthesis in Chinese Hamster ovary (CHO) cells [96]. PAFR-mediated IP<sub>3</sub> synthesis, thought to be responsible for the regulation of cell polarization/motility and cell survival and growth, utilizes G $\beta\gamma$ -activatable PI-3 kinase in a macrophage cell line [96]. PAFR has been reported to regulate other downstream signaling molecules, including phospholipase D (PLD), phospholipase C $\gamma$  (PLC $\gamma$ ), MAP kinases and G proteins [96,98,99]. The PAF receptor present on the surface of circulating cells recognizes certain phospholipid structures on the endothelial cell surface, specifically the *sn*-1 ether bond- of PAF, its short *sn*-2 acetyl residue and the choline headgroup [2]. An alteration in any of these structures dramatically decreases signaling through the PAF receptor.

### PAF production in Endothelial Cells

Thrombin, vasoactive mediators and pro-inflammatory cytokines are all capable of eliciting PAF synthesis in the endothelium. This synthesis may transduce, or amplify, the signals delivered by these various mediators. Several important endothelial cell functions are regulated by PAF synthesis, including impairment of the barrier function of the endothelium, and the adhesion of leukocytes to the endothelial monolayer prior to the transmigration of the leukocytes across the endothelial cell layer. *In vitro*, PAF has been shown to enhance the permeability of the endothelial cell monolayer, and to induce changes in the cell cytoskeleton, which lead to cell retraction and the formation of intercellular gaps [25]. Changes in the shape of the endothelial cells are associated with the activation of calcium-dependent potassium channels and the hyper-polarization of the cell membranes [27-30]. PAF stimulation of endothelial cells has also been shown to induce a dose-dependent synthesis of both prostacyclin and thromboxane A<sub>2</sub>, or alternatively the release of plasminogen activator, cleaving plasminogen to plasmin to break up blood clots [26]. Activation of PLA<sub>2</sub> may lead to the synthesis and release of

leukotrienes and thromboxane  $A_2$  which are, respectively, involved in PAF induced permeability changes and arteriolar constriction [33]. The ability of PAF receptor antagonists to inhibit the permeability of the endothelial cell monolayer and to inhibit changes in the cytoskeleton leading to cell retraction and the formation of intracellular gaps has been demonstrated *in vitro* [25].

### **The role of PAF in leukocyte adherence to the endothelium**

A major component of the inflammatory response is the multi-step molecular interaction between “signaling cells,” such as endothelial cells and platelets, and “responding cells,” such as neutrophils and monocytes [2]. The most widely described role for PAF in cell-cell interactions has been that of the adherence of circulating leukocytes to the endothelium. The adhesion molecules involved in leukocyte adherence to the endothelial cell monolayer are presented in Table 1. The adhesion molecules expressed on the endothelial cell surface include E-selectin (CD62E), P-selectin (CD62P), vascular cell adhesion molecule-1 (CD106), and intracellular adhesion molecule-1 (CD54). Adhesion molecules located on the leukocyte which are involved in its adhesion to the endothelium include E-selectin ligand 1 (ESL-1), L-selectin (CD62L),  $\beta$ -1 integrin very late activating antigen-4 (CD49d),  $\beta$ -2 integrin lymphocyte function-associated antigen-1 (CD11a/CD18), and P-selectin ligand glycoprotein-1 (CD162). (Table 1).

The process of migration of neutrophils from the bloodstream to inflamed tissues involves three major steps [56]. The first is the transient interaction of leukocytes with the endothelial cell monolayer mediated by cell surface adhesion molecules such as selectins. The initial adhesion of the leukocyte to the endothelial cell is mediated by P-selectin, which is preformed and stored in endothelial cells within Weibel Palade bodies and, upon stimulation of the endothelial cells, is rapidly translocated to the cell surface [5]. P-selectin binds to counter-receptors such as P-selectin ligand glycoprotein on leukocytes, thus tethering them to the activated endothelial cells. This interaction induces the rolling of leukocytes along the vessel wall, but fails to completely immobilize the cells. The binding of the leukocyte to the endothelial cells, via its P-selectin receptor, brings the leukocyte into close proximity with endothelial cells. This interaction allows the polymorphonuclear leukocyte (PMN) to scan the endothelial cell monolayer for the presence of an activating signal, such as members of the integrin family. If the presence of such a signal is detected, the leukocyte is activated and expresses integrins to augment the adhesive response [2]. Alternatively, the interaction between leukocytes and the endothelial cell monolayer may also be mediated through a delayed adhesion response. In this response, activated endothelial cells use E-selectin as the tether to temporarily bind the leukocyte. A chemokine, such as interleukin-8, is used as the secondary activating molecule to strengthen the leukocyte’s adhesion to the endothelial cell. Unlike P-selectin, E-selectin is not preformed, therefore, the presence of agonists, such as tumor necrosis factor (TNF)- $\alpha$ , is necessary in order to transcriptionally activate the E-selectin gene, resulting in the accumulation of E-selectin within a few hours. Maximal surface expression of E-selectin occurs within 12 hours. Secondary signaling molecules are synthesized in response to inflammatory stimuli. Among these secondary signaling molecules is interleukin-8 (IL-8), which is released as a soluble factor [2,31].

Prescott et al. have demonstrated that exposure of endothelial cells to oxidants results in the generation of oxidatively fragmented phospholipids that have biological activity similar to PAF [2]. Leukocytes adhere to these oxidant treated endothelial cells by an adhesion which can be blocked by antibody treatment to the P-selectin. Upon *in vitro* treatment of endothelial cells with oxidants, vesicles from the endothelial cells are shed into the supernatant that contain small amounts of P-selectin and oxidized phospholipids, but no PAF. Leukocytes treated with this supernatant adhere to gelatin-coated plates by a mechanism that requires integrin activation [2]. Thus, in this setting, oxidatively modified phospholipids mediate PAF-like biological activity.

During the second step of the adhesion process, the tethered leukocytes are activated by additional chemical signals released from the activated endothelial cell monolayer. Leukocytes are activated as they are brought into contact with the endothelium via selectin tethers. Their activation leads to a more stable adhesion, dependent on the interaction of integrins expressed on surface of leukocytes with endothelial counter receptors belonging to the Ig superfamily. Integrins, such as the  $\beta_1$ -integrins, are upregulated by PAF and are critically involved in PAF induced leukocyte locomotion in extravascular tissue [31]. Finally, chemoattractants stimulate the transmigration of leukocytes across the vessel wall [31].

PAF is a known mediator of leukocyte-endothelium interaction, and may be involved in the cell activation phase [35,36]. During the process of leukocyte adherence to endothelial cells, PAF serves as a spatially-regulated juxtacrine signal between the cells, facilitating them to adhere to one another [87,88], specifically acting at the interface between the endothelium and circulating leukocytes [43,85]. Stimulation of endothelial cells with agents such as thrombin leads to the increased production of PAF, which is co-expressed with P-selectin, on the endothelial cell surface. Newly synthesized PAF remains cell-associated with the endothelial cell monolayer and can directly activate circulating leukocytes [40,84]. PAF is also able to induce the action of  $\beta_2$  integrins, such as the CD11a/CD18 complex, on activated neutrophils [36,110] and acts in concordance with P-selectin on the endothelial cell surface [86]. PAF activates neutrophils tethered by P-selectin, leading to the activation of the  $\beta_2$  integrins. The P-selectin on the endothelial cells in conjunction with the  $\beta_2$  integrins on activated neutrophils act to mediate tight adhesion of the leukocytes to the endothelium [111]. Additionally, activation of leukocytes by PAF has been shown to modify the distribution and function of P-selectin ligand glycoprotein-1 on the leukocyte surface, possibly leading to the termination of the receptor-ligand bond between the P-selectin ligand glycoprotein-1 and P-selectin, allowing movement and transmigration to occur [89].

Exogenously added PAF causes an increase in the endothelial cell adhesive properties for leukocytes [40-43] and promotes the transendothelial migration of the leukocytes [44]. Studies have demonstrated the ability of PAF receptor antagonists to block the migration of neutrophils across monolayers of cytokine pretreated endothelial cells [32]. Leukocytes express a PAF receptor that further enhances their adhesion and induces their  $\beta$ -integrin-mediated migration. PAF is ultimately responsible for priming leukocytes against any agonists they might encounter following their migration out of the vasculature [2].

The synthesis of PAF induces an increase in the cytosolic free calcium through the production of inositol triphosphate (IP<sub>3</sub>) in endothelial cells [27-30]. It is hypothesized

that the opening of receptor operated calcium channels is a signal to increase the transport and activation of PLA<sub>2</sub>. PAF also promotes an influx of calcium into adherent neutrophils. This influx enhances their response to IL-8, a chemokine that is a potent neutrophil chemoattractant produced by endothelial cells [3].

### **PAF and Adherence of Monocytes and Platelets**

In addition to mediating adherence of leukocytes to the endothelium, PAF is involved in the adherence of monocytes and platelets to the endothelium and to each other [2]. The interaction between monocytes and platelets occurs following the activation of platelets by thrombin, PAF, or oxidized phospholipids. In addition to monocyte adhesion, other processes, including intercellular signaling, altered gene expression, and the synthesis of mediators, are results of platelet activation [2]. Monocytes engage adhesion molecules, resulting in the tethering of the monocyte, and the induction of intracellular signals delivered through surface receptors. Once activated, as with endothelial cells, platelets translocate P-selectin to their surfaces from intracellular storage granules [2]. Monocytes bind to endothelial cells and platelets via receptors corresponding to the P-selectin molecule [97,117,118]. Platelets activated by thrombin cause significant release of the chemokine monocyte chemoattractant protein (MCP)-1 from monocytes [58]. Chemokine secretion, however, depends on the presence of P-selectin ligand glycoprotein-1, a sialomucin constitutively present on monocytes and other myeloid leukocytes to which P-selectin preferentially binds [59,60]. Monocytes bound to P-selectin by corresponding receptors secrete cytokines, such as MCP-1 and TNF- $\alpha$ , in response to PAF stimulation. The presence of P-selectin is necessary for the expression of these chemokines [2]. Antibodies neutralizing P-selectin attenuated MCP-1 secretion and therefore the ability of platelets to adhere to monocytes [2]. Adhesion of monocytes to P-selectin causes the translocation of NF- $\kappa$ B to the nucleus. This nuclear translocation is enhanced following PAF stimulation. NF- $\kappa$ B is a key regulator of immediate early gene in monocytes and is required for the expression of MCP-1 and TNF- $\alpha$  [2].

PAF induces the adhesion of platelets to the endothelium in the presence of activated polymorphonuclear lymphocytes [37-39]. As described previously, PAF activates neutrophils tethered by P-selectin, leading to the activation of the  $\beta_2$  integrin complex of CD11a/CD18. The P-selectin on the endothelial cells in conjunction with the  $\beta_2$  integrins on activated neutrophils act to mediate tight adhesion of the leukocytes to the endothelium [111]. Platelet/endothelial cell adhesion molecule-1 (CD31) is involved in PAF induced cell activation, suggesting that platelet/endothelial cell adhesion molecule-1 may be a costimulatory agonist receptor capable of modulating integrin function in human platelets during adhesion and aggregation [34].

### **Adhesion Molecules involved in Transendothelial Migration Across Bone Marrow Endothelial Cells**

Transmigration across the bone marrow endothelial cells occurs in both directions. In one direction, cells formed in the bone marrow must cross the bone marrow endothelium in order to reach the circulation. Conversely, in order for bone marrow transplant procedures to be successful, a transplant of a sufficient population of cells possessing the ability to reproduce within the bone marrow is necessary. These cells are injected intravenously into the recipient and consequently have to cross the bone marrow

endothelium in the opposite direction to bone marrow-derived cells. Currently, there are three major sources available for bone marrow transplant: donor bone marrow, donor peripheral circulating stem cells and umbilical cord blood-derived stem cells. A variety of adhesion molecules have been demonstrated to be involved in the transmigration of this population of cells across the bone marrow endothelium. The most significant of these molecules are listed in Table 1. Importantly, if PAF is involved in homing these cells to the bone marrow, it would be necessary for the cells to possess PAFR. It has been established that cells capable of forming colonies in culture are positive for CD34, VLA-4, and ICAM-1.

CD34 has been shown to be a ligand for L-selectin (Table 1) [4]. It is a 110-kD glycosylated protein, expressed on pluripotent hematopoietic progenitor cells and other vascular endothelial cells such as HUVEC, capillaries of different tissues, and neoplastic tissues. CD34 is thought to function as an adhesion molecule that mediates the transit of peripheral progenitor cells to the bone marrow. Adhesion studies show that CD34 positive progenitor cells display an affinity for resting bone marrow endothelial cell (BMEC) monolayers that is divalent cation dependent and partially inhibited by anti-CD34 antibodies [5]. Rafii et al [5] were able to detect significant expression of CD34 on first passage BMEC in culture, but were only able to barely detect levels of CD34 after the second passage. Additionally, levels of CD34 expression are downregulated in BMEC monolayers in the first 4 to 5 days of tissue culture [11]. In contrast, endothelium from large veins, arteries, placenta and lymphatics fail to express CD34. This differential expression of CD34 on endothelial cells from different regions of the circulation when combined with the expression of other adhesion molecules may play a role in the preferential homing of stem cells to the bone marrow following transplant. Stem cells expressing CD34 demonstrate a higher efficiency of rolling than CD34 negative human progenitor cells (HPC) [79] and have been shown to bind to E-selectin expressed on human BMEC [9,64,80].

Populations of CD34 positive cells also expressing E-selectin ligand-1 demonstrate a greater tendency than cells lacking these markers to possess the capabilities of either progenitor or long-term culture-initiating cell (LTC-IC) [9]. In contrast, L-selectin is present on the majority of non-migrating CD34 positive cells and is expressed at significantly lower levels on transmigrating progenitor cells [4]. Möhle et al [4] were able to demonstrate the loss of L-selectin on transmigrated CD34 positive cells suggesting that shedding occurs during migration across the endothelium. Signaling through L-selectin has been shown to increase the affinity state and expression level of integrins, and enhances the proliferation of hematopoietic progenitor cells [4].

Aiuti et al [119] found that stromal cell derived factor-1 (SDF-1) is chemotactic for CD34 positive hematopoietic progenitor cells from both the bone marrow and peripheral blood. CD34 positive bone marrow cells express increased amounts of the chemokine receptor CXCR-4, the receptor for SDF-1, as compared to levels expressed by mobilized progenitors. This suggests a downregulation of the SDF-1 ligand in progenitor mobilization. The presence of increased amounts of CXCR-4 on the surface of bone marrow-derived CD34 positive cells causes them to be more efficiently mobilized than peripheral blood CD34 positive cells in their response to SDF-1. The presence of IL-1 $\beta$  induces the production of cytokines, such as IL-6 and IL-8, by endothelial cells [8] and IL-6 has been shown by Peled et al [17] to upregulate the expression of CXCR-4 receptor on

CD34 positive cells. Möhle et al [120] demonstrated that circulating CD34 positive cells efficiently transmigrated the bone marrow endothelium *in vitro* when SDF-1 containing conditioned medium was added to the lower chamber of their transmigration system.

$\beta$ -1 integrin (very late activating antigen) and  $\beta$ -2 integrin (lymphocyte function associated antigen) [45,75], have also been shown to be downregulated on circulating progenitors, thus implying the function of these integrins in both the mobilization of progenitors from the bone marrow and the homing of stem cells to the bone marrow [45,46,50-52]. Möhle et al [4] published conflicting results, finding that similar amounts of  $\beta$ -1 integrin can be detected on both migrating and non-migrating cells and low levels of  $\beta$ -2 integrin can be detected on both migrating and non-migrating cells. Papayannopoulou et al [13,14] and Möhle et al [4] have shown the *in vivo* administration of antibodies to  $\beta$ -1 integrin mobilizes progenitors, whereas antibodies to  $\beta$ -1 integrin and vascular cell adhesion molecule-1 (VCAM-1) interfere with the homing of the stem cells to the bone marrow. Stem cell factor (SCF), also known as c-kit ligand or CD117, may also play a role in bone marrow transendothelial migration since it has been shown to be expressed on bone marrow stromal and endothelial cells [47,48]. Möhle et al [4] identified the corresponding cytokine receptor, c-kit, in hematopoietic progenitor cells. Reduced levels of c-kit are detected on circulating progenitor cells in comparison to the levels expressed on bone marrow-derived CD34 positive cells [49].

Several studies have demonstrated the release of significant amounts of vascular endothelial growth factor (VEGF) by hematopoietic cells [7,9]. VEGF is a chemokine which acts on endothelial cells to support endothelial proliferation and vascular remodeling, and increase endothelial fenestration [50-52]. Release of VEGF in the bone marrow (BM) supports angiogenesis, inducing the proliferation of a specific endothelial phenotype which expresses E-selectin [8,53]. *In vivo*, BMEC, like other endothelial cells [8,9], constitutively express E-selectin [64]. E-selectin is detectable in BMEC monolayers in the first four to five days of tissue culture. The expression of E-selectin is upregulated upon IL-1 $\beta$  activation [9,11]. E-selectin is thought to play a role in the tethering of CD34 positive cells (Table 1) in response to SDF-1 stimulation since Naiyer et al [9] found the addition of monoclonal antibodies to E-selectin inhibited the SDF-1 induced migration of CD34 positive cells through a BMEC monolayer activated by VEGF. However, Voermans et al [8] showed that the addition of monoclonal antibodies to E-selectin did not inhibit SDF-1 induced transendothelial migration. Therefore, it is not entirely clear whether E-selectin mediates SDF-1 induced transendothelial migration of CD34 positive cells or not. As discussed previously, E-selectin interacts with leukocyte counter receptors E-selectin ligand-1 and P-selectin ligand glycoprotein [65-67].

Experiments aimed at determining the presence or absence of various adhesion molecules and their receptors on BMEC have obtained variable results. Rafii et al [5] demonstrated the presence of Weibel-Palade bodies that store von Willebrand Factor, interleukin-8 (IL-8), P-selectin and endothelin within the BMEC monolayer. Unstimulated BMEC fail to express P-selectin, but are capable of expressing P-selectin following stimulation with cytokines [4], but not thrombin [61]. The importance of the selectins in stem cell homing is further illustrated by a study by Frenette et al showing that recruitment of transplanted hematopoietic progenitor cells to the BM of recipient P-selectin and E-selectin knock out mice P/E(-/-) is significantly reduced [113].

Platelet/endothelial cell adhesion molecule-1 is thought to function as a transigratory bridge permitting the exit of mature cells such as neutrophils, lymphocytes and monocytes out of the BM [54,55]. Rafii et al [5] demonstrated that BMEC stain positively for this adhesion molecule, showing increased amounts in areas of cell-cell contact. However, BMEC failed to stain with antibodies to L-selectin, intracellular adhesion molecule-1 or vascular cell adhesion molecule-1 [5]. Möhle et al [4] obtained contradicting results, demonstrating the basal expression of intracellular adhesion molecule-1 on BMEC and the positive staining of BMEC for vascular cell adhesion molecule-1 following cytokine stimulation. In separate studies, Voermans and coworkers [8] along with Jacobsen and coworkers [74] also obtained results contrary to those of Rafii, demonstrating the constitutive expression of vascular cell adhesion molecule-1 on BMEC. Additionally, Naiyer et al [9] was able to demonstrate the ability of interleukin-1 $\beta$  to induce the expression of vascular cell adhesion molecule-1 and intracellular adhesion molecule-1 on BMEC. Levels of vascular cell adhesion molecule-1, a molecule involved with mediating the firm adhesion of CD34 positive cells to BMEC [9], were shown to be downregulated in the first four to five days of tissue culture and are upregulated upon interleukin-1 $\beta$  activation [11]. Intracellular adhesion molecule-1, the ligand for lymphocyte function associated antigen-1, is involved with mediating firm adhesion of CD34 positive cells to BMEC [9] and may be constitutively expressed on BMEC *in vivo* [4]. Identical to expression of vascular cell adhesion molecule-1, intracellular adhesion molecule-1 is also downregulated in the first four to five days of tissue culture [11] and is upregulated upon IL-1 $\beta$  activation [9,11]. Blocking monoclonal antibodies to either vascular cell adhesion molecule-1 or intracellular adhesion molecule-1 only slightly reduced the level of stromal derived factor-1 induced transendothelial migration of CD34 positive cells [9]. Antibodies to very late activating antigen-4, located on CD34 positive cells, induce significant mobilization of progenitors [7]. However, monoclonal antibodies to VLA-4 and its ligand, vascular cell adhesion molecule-1, induce the mobilization of CD34 positive cells [14] and interfere with stem cell homing to the bone marrow [7]. This suggests the role of this ligand pair in the control of homing as well as the mobilization of CD34 positive cells [62,63].

Phagocytic glycoprotein-1 (CD44) is an E-selectin ligand expressed on primitive CD34 positive human HPCs but not on more mature hematopoietic cells [10]. Phagocytic glycoprotein-1 is responsible for mediating E-selectin dependent rolling interactions over a wide shear range and promotes HPC rolling interactions with E-selectin expressed on BMEC [10]. E-selectin binding determinants on Phagocytic glycoprotein-1 are displayed on sialofucosylated N-linked carbohydrates and expression is restricted to primitive CD34 positive human BM cells [10]. A HECA-452 reactive glycoform of phagocytic glycoprotein-1 functions as the major E-selectin glycoprotein ligand on human HPCs [10]. Expression of this glycoform on HPC promotes functional E-selectin-mediated rolling interactions with BMEC over a wider shear range than P-selectin ligand glycoprotein alone [10]. Phagocytic glycoprotein-1 engagement with E-selectin predominates over P-selectin ligand glycoprotein at higher physiologic shear stress and possesses overlapping contributions to E-selectin binding at low shear stress [10]. BMEC expresses high levels of phagocytic glycoprotein-1, however it fails to possess any E-selectin activity [10].

Studies by Tavassoli et al [15] and Aizawa et al [16] have demonstrated that the homing of murine progenitor cells to BM is regulated by a calcium dependent C-peptide.

The binding of CD34 positive cells to BMEC can be prevented upon addition of EDTA, supporting the idea that a calcium-dependent adhesion molecule may play a critical role in the homing of CD34 positive cells to the BM [5,9].

Voermans et al [8] demonstrated that CD34 positive cells require  $\beta$ 1-integrins,  $\beta$ 2-integrins, PECAM-1, and O-glycosylated proteins for efficient migration across the BM endothelium. Monoclonal antibodies to  $\beta$ 1-integrins,  $\beta$ 2-integrins, or PECAM-1 partially inhibited SDF-1 induced migration of CD34 positive across BMEC [8]. Any combination of these antibodies causes a stronger inhibition of CD34 positive cell migration. Surprisingly, antibodies to E-selectin alone or in combination with antibodies to  $\beta$ 1-integrins,  $\beta$ 2-integrins, or platelet/endothelial cell adhesion molecule-1 failed to significantly inhibit SDF-1 induced CD34 positive cell migration [8]. Treatment of CD34 positive cells with glycoproteases inhibits their migration across IL-1 $\beta$  prestimulated BMEC. This suggests the involvement of O-glycosylated adhesion molecules such as CD34, leukocyte sialoglycoprotein (CD43), phagocytic glycoprotein-1 or leukocyte-common antigen (L-CA, CD45) in the transmigration process [8]. These findings demonstrate the complex nature of the system involved in the adhesion and migration of CD34 positive cells across the BM endothelium.

### **Factors Inducing Stem Cell Migration**

SDF-1 expression on BMEC is crucial for the arrest of human stem cells under shear flow, an essential step for transendothelial migration [4]. SDF-1 induces the activation of the major adhesion molecules involved in transendothelial cell migration, including phagocytic glycoprotein-1, integrin lymphocyte function associated antigen-1, integrin very late activating antigen-4 and -5 on migrating stem cells [6]. The addition of stromal derived factor to the BMEC enhanced the rate of transendothelial migration of CD34 positive cells over the rate at which the cells migrated across either unstimulated or IL-1 $\beta$  prestimulated BMEC [8]. Optimal levels of transendothelial migration of CD34 positive cells occurred when either 30 or 100ng/ml stromal derived factor-1 was used to treat the BMEC. Surprisingly, a reduction in the rate of transendothelial migration was seen at very high concentrations of SDF-1, demonstrating the optimal response of CD34 positive cells to lower concentrations of SDF-1 in the presence of an endothelial layer [8]. In the absence of stromal derived factor-1, spontaneous migration of approximately 50% of progenitors occurs, with the addition of stromal derived factor-1 causing the migration of an additional approximately 40% of colony-forming cells (CFC) [9]. IL-1  $\beta$  prestimulation of endothelial cells increased the stromal derived factor -1 induced migration [8]. Using IL-1 $\beta$  activated endothelial cells, in the absence of stromal derived factor-1, spontaneous migration of LTC-IC occurs. Treatment with stromal derived factor-1 causes the migration of additional LTC-IC [9].

The migration of CD34 positive cells with LTC-IC potential across stromal derived factor-1 treated endothelium is dependent on the interaction of the CD34 positive cells with E-selectin molecules on the endothelium [9]. The engagement of E-selectin by its ligand on CD34 positive cells plays an important role in the stromal derived factor-1 induced chemokinesis of CD34 positive cells. The addition of monoclonal antibodies to E-selectin inhibit approximately 90% of stromal derived factor-1 induced LTC-IC transendothelial migration and approximately 90% of stromal derived factor-1 induced CFC transendothelial migration [9].

In separate studies, mice lacking stromal derived factor-1 fail to develop hematopoiesis in the bone marrow [7]. Targeted gene knockout of either stromal derived factor-1 [71] or its receptor CXCR-4 [72,73] resulted in a defect in BM hematopoiesis, but failed to alter fetal liver hematopoietic activity.

### **The Potential Role of PAF in Stem Cell Homing to the Endothelium**

Numerous studies have characterized the various adhesion molecules involved in the adherence of stem cells to the endothelium. However, the role that PAF plays in this process has not been studied.

As previously mentioned, PAF serves as a juxtacrine signal facilitating cell-cell adherence [87,88], acting at the interface between the endothelium and circulating leukocytes [43,85]. We hypothesize that, in a process similar to that of leukocyte adherence to the endothelium, CD34 positive cells may be able to adhere and subsequently migrate across an endothelial cell monolayer. In preliminary studies, our lab has been able to demonstrate the presence of mRNA for PAFR on CD34 positive cells isolated from umbilical cord blood. The presence of this mRNA suggests that there may be a role of PAF in the adherence of stem cells to the endothelium. PAF is known to be responsible for the upregulation of  $\beta_1$ -integrin, a process critical to PAF's role in leukocyte locomotion [31]. The presence of  $\beta_1$ -integrin mRNA in CD34 positive cells, (our unpublished findings), also suggests that this same process may be able to induce the transmigration of CD34 positive cells. As discussed above, PAF activates neutrophils that are tethered by P-selectin to the endothelial cell surface [86]. Our lab has demonstrated the presence of both P-selectin and its receptor P-selectin ligand glycoprotein-1 on CD34 positive cells. Since CD34 positive cells have been shown by our lab and others [76-78] to express P-selectin ligand glycoprotein, this supports our hypothesis that the processes of stem cell homing and leukocyte adherence may share common mechanisms. In our laboratory, we propose to determine whether BMEC synthesize PAF, resulting in upregulation of P-selectin expression on the cell surface and to determine whether PAF plays a role in BMEC transendothelial cell migration.

### **Conclusion**

As a result of synthesis primarily through the remodeling pathway, PAF is produced by endothelial cells and plays a significant role in a variety of inflammatory diseases. In addition, PAF is known to play a significant role in the adherence of leukocytes to the endothelium. Our lab has demonstrated the presence of several adhesion molecules on CD34 positive cells isolated from umbilical cord blood, among the most significant of these is the PAFR. Due to similarities between adhesion molecules expressed on the surface of leukocytes and CD34 positive cells, it is possible that PAF also plays a significant role in the adhesion and subsequent transmigration of these cells.

The expression of PAF on bone marrow endothelial cells in response to an as yet unknown signal may initiate or mediate the release of CD34 positive cells from the bone marrow into the circulation where these cells can be trafficked to sites in need of tissue repair, or may mediate the homing of CD34 positive cells to the bone marrow following transplant procedures. PAF produced by damaged vasculature in conditions of ongoing atherosclerosis could induce the transmigration of CD34 positive cells bearing the PAFR across the coronary artery to assist in the repair of the damaged tissues. Evidence of the

presence of PAFR on cells capable of producing colony forming units provides strong support for this theory.

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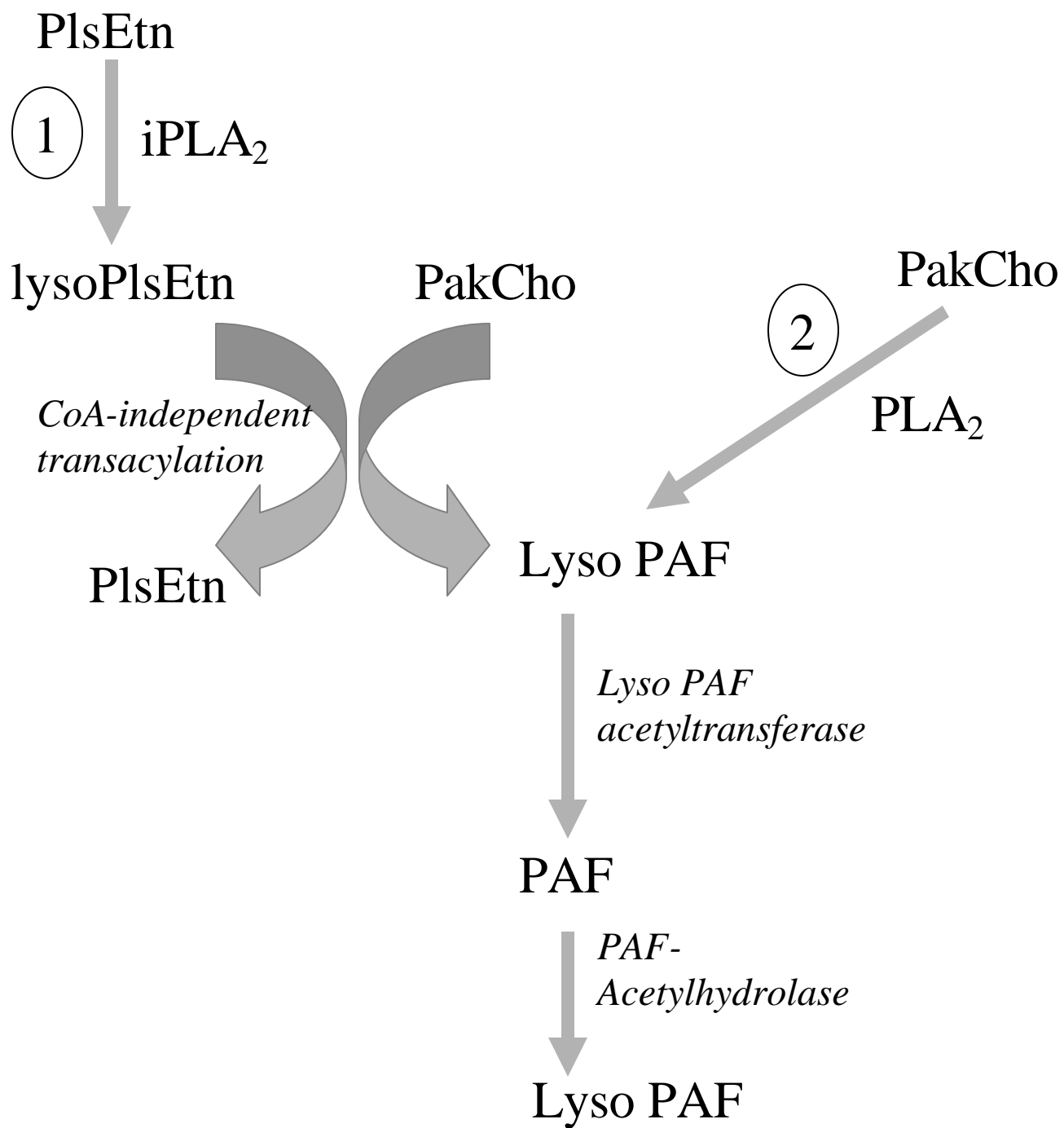
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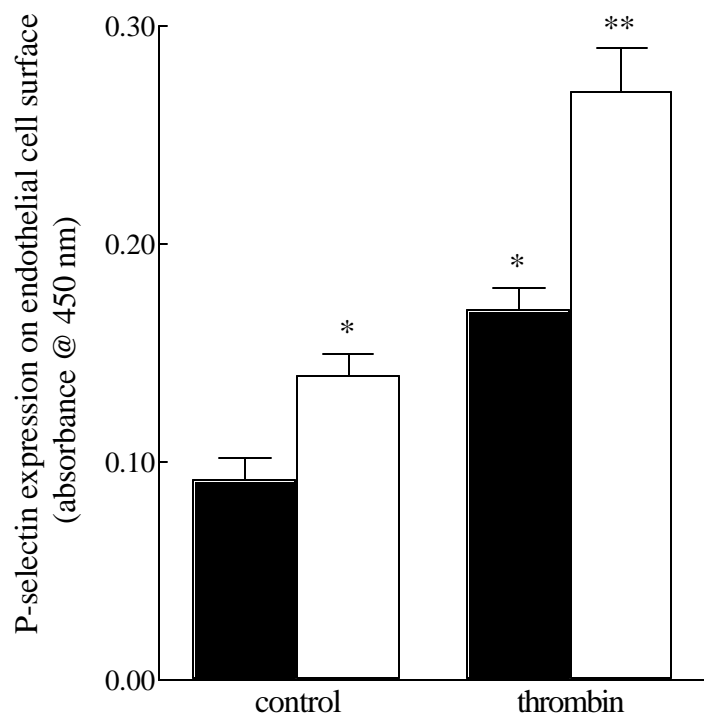
## FIGURE LEGENDS

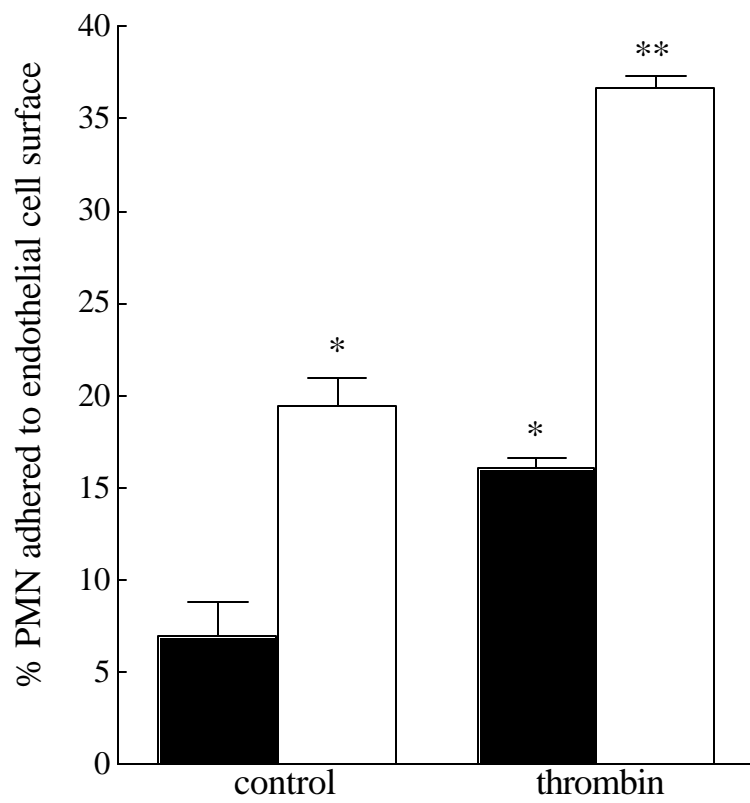
**Figure 1.** The remodeling pathway for PAF production is initiated by phospholipase A<sub>2</sub> (PLA<sub>2</sub>)-catalyzed hydrolysis of membrane phospholipids. In thrombin-stimulated endothelial cells, we have demonstrated that calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) preferentially hydrolyzes plasmenylethanolamine (PlsEtn, pathway 1) to produce lysoplasmenylethanolamine (lysoPlsEtn) that acts as a lysophospholipid acceptor. Transacylation between lysoPlsEtn and alkylacyl-glycerol-3-phosphocholine (PakCho) results in reacylation of lysoPlsEtn and the production of lyso-PAF. Lyso-PAF may also be produced by the direct action of PLA<sub>2</sub> on PakCho (pathway 2). Lyso-PAF is then acetylated by acetyl-CoA:1-alkyl-*sn*-glycero-3-phosphorylcholine 2-*O*-acetyltransferase [1] to produced PAF, which can be rapidly hydrolyzed by PAF-acetylhydrolase.

**Figure 2.** Human coronary artery endothelial cells stimulated with thrombin (0.1 IU/ml, 10 mins) demonstrate an increased cell surface expression of P-selectin (filled bars). Pretreatment with methyl arachidonyl fluorophosphonate (5μM, 10 mins, open bars) enhances P-selectin cell surface expression in both unstimulated and thrombin-stimulated cells. Cell surface expression of P-selectin was assayed as described in ref. 114 \*p<0.05, \*\* p<0.01 when compared to untreated endothelial cells, n=8

**Figure 3.** Adherence of polymorphonuclear leukocytes (PMN) to intact human coronary artery endothelial cell monolayers is significantly increased by endothelial stimulation with thrombin (0.1 IU/ml, 10 mins, filled bars). Pretreatment with methyl arachidonyl fluorophosphonate (5μM, 10 mins, open bars) enhances PMN adherence to both unstimulated and thrombin-stimulated endothelial cells. \* p<0.05, \*\* p<0.01 when compared to untreated endothelial cells, n=4







<b>Molecule Name</b>	<b>CD No.</b>	<b>Location</b>	<b>Corresponding Ligand/Receptor</b>	<b>Functions</b>
CXCR-4		CD34 positive cells		Causes CD34 positive cells to be more efficiently mobilized when stimulated by SDF-1
Stromal Cell Derived Factor SDF-1		EC	CXCR-4	Crucial for HPC arrest under shear flow, essential for transendothelial migration
E-selectin Ligand ESL-1		Leukocytes, CD34 positive cells	CD34	
B-2 Integrin Lymphocyte Function Associated Antigen LFA-1	CD11a/ CD18	CD34 positive cells		Downregulated on circulating progenitors, functions in mobilization of progenitors from BM and homing of stem cells, leukocyte adhesion
Platelet/endothelial Cell Adhesion Molecule PECAM-1	CD31			Adhesion receptor, transendothelial migration, endothelial cell-cell adhesion
	CD34	HPC, vascular EC	SELL, SELE	Cell adhesion
Leukocyte sialoglycoprotein	CD43	Leukocytes		Adhesion receptor, anti-adhesion molecule
Phagocytic Glycoprotein-1 PGP-1	CD44	CD34 positive cells	SELE	Mediates SELE dependent rolling interactions over wide shear range, promotes HPC rolling interactions
Leukocyte Common Antigen L-CA	CD45			An intracytoplasmic tyrosine phosphatase, regulator of leukocyte activation
B-1 Integrin Very Late Activating Antigen VLA-4	CD49d	Leukocytes	VCAM	Downregulated on circulating progenitors, functions in mobilization of progenitors from BM and homing of stem cells
Intracellular Adhesion Molecule ICAM-1	CD54	EC	LFA-1	Mediating firm adhesion of CD34 positive cells to EC, adhesion receptor
L-selectin SELL	CD62L	Leukocytes, CD34 positive cells	CD34	Signaling thru SELL increases affinity state and expression level of integrins, enhances proliferation of HPC, receptor for leukocyte homing
E-selectin SELE	CD62E	Constitutively expressed on BMEC	ESL-1, PSGL-1	Receptor for leukocyte rolling, possible role in tethering of CD34 positive cells
P-selectin SELP	CD62P	EC		Tethering CD34 positive cells after SDF stimulation, adhesion of platelets to monocytes and neutrophils
Vascular Cell Adhesion Molecule VCAM-1	CD106	EC		Mediates cell-cell adhesion and signaling with integrin- $\alpha$ 4-bearing cells
P-selectin Glycoprotein Ligand PSGL-1	CD162	Leukocytes, CD34 positive cells	SELL, SELE, SELP	Receptor for leukocyte rolling

Table 1. Adhesion molecules, receptors and chemotactic agents involved in the adherence of circulating cells to the endothelium. BM, Bone Marrow; EC, Endothelial Cells; HPC, Human Progenitor Cells