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**Platelet-derived SDF-1 regulates recruitment, proliferation
and differentiation of human CD34⁺ progenitor cells:
implications in patients with acute myocardial infarction**

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To my parents

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1. Introduction

Although most of the current research being conducted is focused on atherosclerosis, its major consequences (acute myocardial infarction and ischemic stroke) top the list of the leading causes of death in the western world. On the other hand, angiogenesis is important in patients recovering from an acute thromboembolic event since the development of new vessels is of vital importance to the vulnerable hypoxic tissues. Recent studies revealed the pathophysiological and the physiological role of circulating haematopoietic stem cells (HSCs) in atherosclerosis and angiogenesis, respectively, shedding more light into the onset and evolution of both atherosclerosis and angiogenesis. The chemokine that mainly regulates the trafficking of progenitor cells is the stromal cell-derived factor-1 (SDF-1).

1.1 SDF-1 chemokine and its receptor CXCR4

Stromal cell-derived factor-1 (SDF-1, designated also as CXCL12), is a CXC chemokine highly conserved among different species (99% homology between mouse and man) which is known to be an effective chemotactic factor for a variety of cell types that contain the G-protein-linked receptor CXCR4 including progenitor (CD34⁺) and mature blood cells (lymphocytes and monocytes)^{3,8,54}. Bone marrow stromal cells constitutively produce SDF-1 and create cellular niches facilitating retention, growth and differentiation of HSCs^{14,15}. Chemotactic responsiveness of HSCs is restricted to SDF-1 and its sole counter receptor CXCR4⁶¹. In contrast to pro-inflammatory chemokines, SDF-1 is constitutively produced in many organs, including bone marrow, spleen, heart, liver and kidney, playing a vital role in organ homeostasis, in retention of HSCs and progenitors within the bone marrow and in general in development.

SDF-1 / CXCR4 axis regulates primitive hematopoiesis by suppressing apoptosis and enhancing proliferation and differentiation of immobilized peripheral blood CD34⁺ in synergy with cytokines, acting as a survival factor for CD34⁺ cells^{30,31}. Studies have reported a crucial role for this chemokine in stem

cell engraftment and myelopoiesis ⁴⁸. SDF-1 gene mice knockout experiments have demonstrated multiple fatal defects including impaired myelopoiesis that lead to embryonic lethality ^{65,43}.

SDF-1 and its counter receptor have been involved in cellular processes associated to malignant transformation such as proliferation, migration and angiogenesis. CXCR4 and SDF1 sustain proliferation and migration of glioma cells to promote malignant progression ⁷. SDF-1 also plays a significant role in HIV infection because it is the natural ligand for a well-known CD4 co-receptor, CXCR4, which is used by HIV T-tropic strains to enter into the cells in advanced stages of the disease ⁵⁵. HIV-infected patients have higher rates of subclinical atherosclerosis ¹². The SDF1-3'A allele is associated with a lower presence of subclinical atherosclerosis as determined with carotid ultrasonography in an HIV-infected population implying that SDF-1 is directly involved in atherosclerosis ¹². Taken together, SDF-1/CXCR4 axis is instrumental in many pathophysiological pathways (Figure 1).

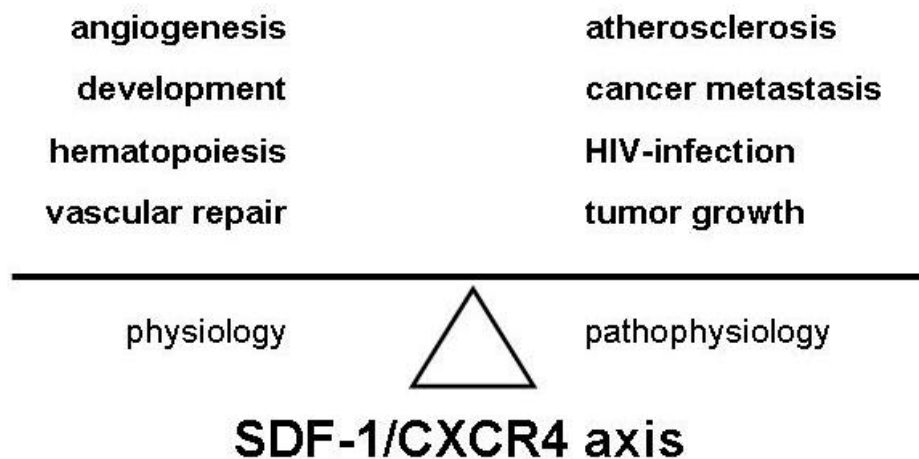


Figure 1. Multiple functions of SDF-1/CXCR4 axis affect both physiological and pathological pathways.

1.2 Progenitor cells in atherosclerosis and angiogenesis

Disruption of endothelial integrity in the means of vascular injury induces neointimal formation and hyperplasia leading to the development of the atherosclerotic plaque and at the last stage the occlusion of the diseased vessel⁵⁰. Endothelial cells and smooth muscle cells being the main pathological components of an occluded vessel were thought to originate from adjacent cells within the vessel which migrate to the site of vascular injury and start to proliferate, while foam cells, found in the fatty streak of atherosclerotic plaque, were believed to solely come from circulating monocytes which differentiate first to macrophages and then to foam cells⁹. Recently bone marrow-derived progenitor cells came up to the scene and shed light to the pathogenesis and progression of atherosclerosis^{44,23}. Endothelial turnover and/or dysfunction are critical events in the initiation of atherosclerotic plaque development. Bone marrow-derived endothelial progenitor cells are mobilized to re-establish an intact endothelial layer following denudation of endothelium. Progenitor cells are able to differentiate to endothelial cells, smooth muscle cells, macrophages and foam cells, being therefore a source of endothelial repair, smooth muscle cell accumulation and foam cell formation in atherosclerotic lesions^{60,51}.

Bone marrow-derived endothelial progenitor cells (EPCs) participate in angiogenesis in response to certain cytokines and/or tissue ischemia^{56,46}. Ex vivo expanded EPCs from peripheral blood, transplanted into animal models of ischemic hindlimbs and acute myocardial infarction, successfully augmented neovascularization resulting in physiological recovery documented as limb salvage and improvement in myocardial function^{27,26}. Correspondingly, initial pilot trials indicate that bone marrow-derived or circulating blood-derived progenitor cells are therapeutically useful for improving blood supply of ischemic tissue^{57,5}.

1.3 SDF-1 chemokine and progenitor cells

It is well reported that CD34⁺ cells, considered as hematopoietic stem cells, express CXCR4, while SDF-1 induces CD34⁺ cell migration in vitro and in vivo

^{63,41}. Accordingly, SDF-1 is considered as one of the key regulators of hematopoietic stem cell trafficking between bone marrow and peripheral circulation. SDF-1 promotes mobilization of bone marrow-derived hematopoietic cells to the circulation ^{49,22,21}. SDF-1 activates the integrins LFA-1, VLA-4 and VLA-5 on human CD34⁺ cells and was found to be essential for homing and repopulation of immune-deficient NOD/SCID mice by human stem cells ⁴⁷. Lately, SDF-1 has been implicated in attracting progenitor cells to the vascular intima playing a role in neointimal formation after vascular injury in Apo-E mice ⁵³.

Another highly important interaction between the SDF-1 / CXCR4 axis and HSCs is related to tissue repair. Vascular repair process involves the selective recruitment of HSCs. Hypoxia-inducible factor-1 (HIF-1), a decisive mediator of tissue hypoxia, induces SDF-1 expression in ischemic endothelial cells in direct association to reduced oxygen tension in vivo ¹⁰. During tissue regeneration the expression of SDF-1 normalizes after regular oxygen tension has been restored ¹⁰. Low oxygen concentration induces high expression of CXCR4 in different cell types (monocytes, monocyte-derived macrophages, tumor-associated macrophages, endothelial cells and cancer cells) which is parallel to increased chemotactic responsiveness to SDF-1 ⁵².

SDF-1 α /CXCR4 axis is also instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells ⁶⁴. SDF-1 protein is highly expressed in platelets, smooth muscle cells, endothelial cells and macrophages in human atherosclerotic plaques but not in normal vessels ^{2,37}.

1.4 Platelet-derived SDF-1 and progenitor cells in atherosclerosis and vascular repair

The first response to vascular injury is platelet adhesion either to the exposed subendothelium or to inflamed endothelium ^{35,11}. Platelet adhesion not only triggers vascular atherothrombosis but also represents the essential step for the targeting of progenitor cells to sites of endothelial disruption ³⁷. Using real-time in vivo double fluorescence microscopy of the mouse carotid artery, CD34⁺ and

c-Kit⁺ Sca-1⁺ Lin-1⁻ (KSL) bone marrow-derived progenitor cells directly adhered to platelets after vascular injury in a process that involves platelet P-selectin and GPIIb integrin³⁷. Platelet-progenitor cell adhesion was proven to be an essential step for the recruitment of EPCs to vascular injury areas because EPCs do not directly adhere to subendothelial matrix proteins under high arterial shear³⁷. Flow cytometric experiments showed that EPCs do not express on their surface the respective adhesion receptors to collagen, fibronectin, fibrinogen and vitronectin, the main components of extracellular matrix (such as GPIb-V-IX and GPVI)³⁷. Moreover in vivo experiments in mice proved that the absence of adherent platelets in vascular injured areas through the usage of blocking monoclonal antibodies to GPIb and GPVI virtually completely blocked the recruitment of CD34⁺ cells³⁷.

Platelets are also believed to have pro-inflammatory and chemotactic properties that contribute to the progression of atherosclerosis. Platelet activation releases a plethora of growth factors, inflammatory mediators and chemokines into the microenvironment¹⁸. Platelets secrete also the chemokine SDF-1, which supports primary adhesion of PCs on the surface of arterial thrombi in vivo³⁷. It was recently proven that arterial thrombi isolated from ruptured atherosclerotic plaques of human carotid arteries demonstrated substantial SDF-1 expression^{2,37}. Consistent with previous studies^{2,64}, smooth muscle cells (SMCs) were a major source of SDF-1 at 4 and 24 h after endothelial disruption indicating that medial SMCs express SDF-1 very early after vascular injury. However, 30 min after vascular injury in murine carotid arteries, SDF-1 expression in SMCs was negligible, indicating that SMC-derived SDF-1 is unlikely to contribute to PCs accumulation on arterial thrombi during the first minutes after endothelial denudation³⁷. So, thrombi that develop at sites of endothelial denudation are the initial source of SDF-1, whereas SMCs account for SDF-1 at later stages. Conducting ELISA, confocal microscopy and RT-PCR experiments in isolated platelets, it was reported that platelet α -granules contain the SDF-1 protein being released in the surface upon activation³⁷. When mice were pre-treated with a function-blocking monoclonal antibody to SDF-1, a significant attenuation of EPCs accumulation within the

growing platelet-rich thrombus occurred ³⁷. On the other hand, it was reported that plasma elevation of SDF-1 induced mobilization of mature and immature hematopoietic progenitor and stem cells ²¹. EPCs recruited to platelet aggregates give rise to neointimal cells, indicating that accumulation of EPCs in arterial thrombi may contribute to vascular repair and pathological remodelling ³⁷ (Figure 2).

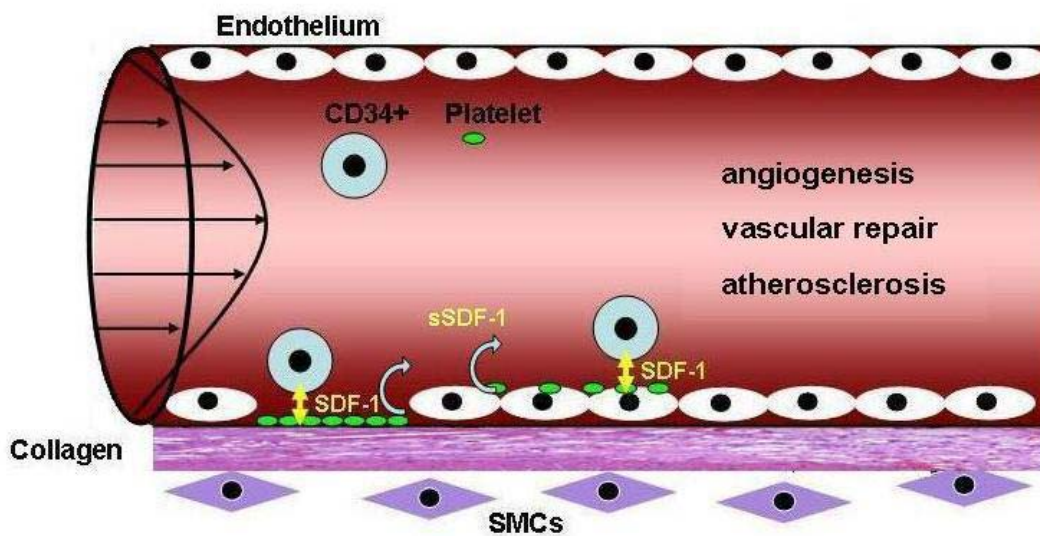


Figure 2. Platelet-derived SDF-1 is involved in angiogenesis, vascular repair and atherosclerosis.

1.5 Platelet-derived SDF-1 and progenitor cells in angiogenesis

Combination of SDF-1 local administration and HSCs transplantation has potential as a strategy for therapeutic neovascularization, since SDF-1 augments local accumulation of transplanted HSCs in ischemic tissues in vivo ⁶³. Recently, it was shown in mouse genetic models that cytokine-mediated release of SDF-1 from platelets and the subsequent recruitment of CXCR4⁺ VEGFR1⁺ hematopoietic progenitors, hemangiocytes, constitute the major determinants of revascularization ²⁵. Ischemia increased plasma levels of the cytokines sKitL and TPO. Soluble Kit-ligand (sKitL) and thrombopoietin (TPO, encoded by *Thpo*) induced the release of SDF-1 from platelets, enhancing

neovascularization through mobilization of CXCR4⁺ VEGFR1⁺ hemangiocytes²⁵. Profound impairment in neovascularization was detected in sKitL-deficient mice Mmp9^{-/-} as well as in thrombocytopenic and TPO receptor deficient mice²⁵. Elevation of SDF-1 supports mobilization of hemangiocytes, while inhibition of CXCR4 signalling by a neutralizing monoclonal antibody effectively blocked mobilization of CXCR4⁺ VEGFR1⁺ cells mediated sKitL and TPO²⁵. Cytokine-induced mobilization of hemangiocytes is partially mediated through elevation of plasma SDF-1 levels and activation of the CXCR4 signalling pathway. Elevation of SDF-1 and mobilization of hemangiocytes are impaired in thrombocytopenic Thpo^{-/-} after hindlimb ischemia in comparison to wild type mice indicating that TPO, by increasing platelet levels and enhancing release of SDF-1, contributes to the mobilization of CXCR4⁺ VEGFR1⁺ cells and thereby accelerates revascularization²⁵. Elevation of SDF-1 plasma levels restored revascularization in Thpo^{-/-} and Mmp9^{-/-} mice in a dose-dependent manner. SDF-1/CXCR4 axis activation retains hemangiocytes within the ischemic niche, since local delivery of CXCR4⁻ cells was less effective in restoring revascularization of ischemic hindlimbs in Mmp9^{-/-} mice²⁵. Therefore it is implied that delivery of SDF-1 may be effective in restoring angiogenesis in individuals with vasculopathies (Figure 2). Delivery of hematopoietic cytokines, SDF-1 or hemangiocytes can be considered as a plausible therapeutic strategy for accelerating ischemic revascularization.

Consistent with these data, another study showed that human cord blood cells containing CD34⁺ cells injected in NOD/SCID mice migrated to infarcted, but not to normal, myocardium, where they engrafted, participated in angiogenesis, and beneficially influenced remodelling processes³³. It was also reported that in infarcted myocardium, expression of SDF-1 mRNA was approximately 7-fold higher indicating the crucial role of SDF-1 in myocardium after infarction in vivo³³. Accordingly, it was reported that recruitment of CXCR4⁺ cells into infarcted myocardial tissues in rabbits via stimulation of the CXCR4/SDF-1 axis plays a critical role in the G-CSF induced myocardial repair³⁹. Furthermore, it was recently shown that impaired CXCR4 signalling

contributed to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease ⁵⁹.

Expression of CXCR4 on marrow-derived cells has been shown to mediate oncogene addiction in breast tumors. Elevation of plasma SDF-1 levels by tumor fibroblasts supported recruitment of marrow-derived Sca1⁺CD31⁺ cells, thereby accelerating tumor angiogenesis and growth ⁴⁵. Blockade of the SDF-1/CXCR4 axis attenuated in vivo tumor growth by inhibiting angiogenesis in a vascular endothelial growth factor-independent manner ²⁰. Thus, blocking of CXCR4 could reduce the metastatic potential of cancer cells, whereas administration of SDF-1 could support revascularization of ischemic tissues. Therefore it is tempting to assume that SDF-1 levels in plasma and platelets may serve as reliable biomarkers to assess neoangiogenic activity during revascularization and tumor angiogenesis.

1.6 Needs analysis and aim

The chemokine stromal cell-derived factor-1 (SDF-1) plays a central role in homing of circulating CD34⁺ progenitor cells in peripheral tissue such as ischemic myocardium, but the mechanisms of its action remain obscure ¹. SDF-1 and its receptor CXCR4 regulates homing of bone marrow-derived cells to the bone marrow ⁴⁸. SDF-1 is also involved in recruitment of stem cells to the liver and to site of vascular injury ^{28, 53}. SDF-1 is expressed in atherosclerotic plaques and protein expression is up-regulated in the heart early after myocardial infarction ⁴. SDF-1 mRNA and protein expression is enhanced primarily in the infarct and border zone of myocardium ⁴. However, the exact role of platelet-derived SDF-1 in atherosclerosis and acute myocardial infarction has not been revealed yet.

Platelets are important mediators of recruiting circulating leukocytes towards the inflamed endothelial monolayer ¹⁶. Activated platelets adhere to endothelial cells, secrete a variety of potent proinflammatory and mitogenic mediators and thereby activate endothelial cells and change their chemotactic and adhesive properties ¹⁸. Enhanced platelet/endothelium adhesion occurs in

the microcirculation of inflamed tissue and during reperfusion of ischemic organs¹⁶.

To extravasate to target tissue, circulating progenitor cells must be recruited and arrest firmly on vascular endothelium. Recently, it was shown that murine platelets mediate adhesion of murine progenitor cells (T17b) via adhesion receptors PSGL-1/P-selectin and β_1 -integrins²⁹. However, the exact adhesion molecules that are involved in the adhesion of human CD34⁺ progenitors and human platelets have not been defined yet. In addition, it has been reported that activated platelets secrete SDF-1, but the role of surface bound SDF-1 has not been clarified so far. Moreover, platelets regulate differentiation of human CD34⁺ cells into foam cells and mature endothelial cells¹³. However, the molecular requirements are poorly understood so far.

The aim of the present dissertation was to analyse the role of platelet-derived SDF-1 for recruitment and differentiation of human progenitor cells and to study the surface expression of SDF-1 on platelets obtained from patients with myocardial infarction.

2. Subjects and Methods

2.1 Patients and platelet flow cytometric analysis

Platelets obtained from 450 consecutive patients, who were referred to our hospital for symptomatic coronary artery disease, were studied for surface expression of SDF-1. The demographic and clinical data of the patients are summarized in the Table (on page 25). Three-hundred six patients presented with stable coronary artery disease and 144 patients suffered from acute myocardial infarction. Surface expression of the platelet SDF-1 (R&D Systems, Minneapolis, USA; clone 79014), P-selectin (Immunotec, Marseille, France; clone CLB-Thromb/6) and GPIb (Immunotec, Marseille, France; clone SZ2) in patients whole blood was determined by a two-colour flow cytometry as previously described ¹⁹. In brief, 10µl CPDA-blood having been resuspended 50:1 with phosphate buffer saline (PBS; Invitrogen Corporation, Paisley, Scotland, UK) was incubated for 30 minutes with the relevant conjugated antibodies in dark at room temperature. After staining, the cells were fixed with 0.5 % paraformaldehyde and were stored at 4 °C till the FACS measurement was taken place with a FACS-Calibur (Becton-Dickinson, Heidelberg, Germany). CD42b-PE was used as a control antibody to identify the region of platelets in whole blood. Specific monoclonal antibody binding was expressed as mean immunofluorescence intensity (MFI) and was used as a quantitative measurement of glycoprotein surface expression.

2.2 Isolation of platelets

Human platelets were isolated as previously described ²⁹. Briefly, venous blood was drawn from the antecubital vein of healthy volunteers and collected in acid citrate dextrose (ACD)-buffer. After centrifugation at 430g for 20 min, platelet-rich plasma (PRP) was removed, added to Tyrodes-HEPES buffer (HEPES, 2.5 mM/L (Carl Roth GmbH, Karlsruhe, Germany), NaCl, 150 mM/L, KCl, 1 mM/L,

NaHCO₃, 2.5 mM/l, NaH₂PO₄, 0.36 mM/l, glucose 5.5 mM/L (Sigma, Steinheim, Germany), BSA 1 mg/ml, pH 6.5, and centrifugated at 900 x g for 10 min. After removal of the supernatant, the resulting platelet pellet was resuspended in Tyrodes-HEPES buffer (pH 7.4 supplemented with CaCl₂, 1 mM/L; MgCl₂, 1 mM/L).

2.3 Isolation and culture of human arterial endothelial cells

Human arterial endothelial cells (haECs) were isolated and passaged according to techniques described previously⁶. HaECs were identified by immunocytochemical staining against the von Willebrand factor (Boehringer, Mannheim, Germany) and their characteristic “cobblestone” growth pattern with contact inhibition between cells. Routine stainings with the DNA dye DAPI (4', 6-diamino-2-phenylindole-dihydrochloride; Boehringer, Mannheim, Germany) were used to exclude mycoplasma contaminations. Cultivation was carried out with a special medium composed of endothelial cell growth medium MV2 (PromoCell, Heidelberg, Germany) plus 10% FCS and 1% penicillin-streptomycin (Invitrogen, Karlsruhe, Germany).

2.4 Isolated cells FACS

To evaluate SDF-1 expression on resting and activated isolated platelets with ADP (20µM; Chrono-Par, Havertown, USA) or TRAP (25µM; Sigma, Steinheim, Germany) one-colour flow cytometry was used as described above. To determine SDF-1 expression on adherent platelets, isolated platelets (2x10⁸/ml) were allowed to adhere to 96-well plates pre-coated with collagen type I (10 µg/ml; BD Biosciences, Bedford, USA) for 15-30 minutes. Unspecific adhesion was prevented by blocking with 2% bovine albumin serum (BSA; Sigma, Steinheim, Germany). After two gentle washing steps with Tyrodes, in situ staining took place with an incubation time of 30 minutes. Subsequently, platelets were removed via pipeting into a FACS tube and preceded to FACS measurement. Mouse IgG1-FITC (BD Biosciences, Bedford, USA) was used as

a monoclonal immunoglobulin isotype control, while CD62P was used as a positive control.

To determine SDF-1 expression on human arterial endothelial cells, endothelial cells were cultivated in a 24-well plate till confluence. Twelve hours before the immunostaining, endothelial cells were activated with TNF- α (50 ng/ml) and INF- γ (20ng/ml) or IL-1 β (20ng/ml), or co-incubated with isolated platelets (2×10^8 /ml) where indicated (the referred cytokines were purchased from Peprotech Inc., New Jersey, USA). In situ immunostaining was performed after removal of the supernatant and a gentle wash with PBS. Anti-SDF-1 monoclonal antibody together with a positive control CD54 (ICAM-1; Immunotec, Marseille, France) and a negative isotype control mouse IgG1 FITC-conjugated antibodies were incubated one hour with the cells in a dark environment. Cells were removed with Trypsin-EDTA (Invitrogen Corporation, Paisley, Scotland, UK) and were immediately processed to FACS measurement.

2.5 Isolation and culture of human CD34⁺ cells

Human CD34⁺ cells were isolated from human cord blood and cultured as previously described¹³. The isolated cells were $\geq 95\%$ positive for CD34⁺ cells as determined by flow cytometry after every isolation procedure. Human mononuclear cells were obtained from human umbilical cord blood by density gradient centrifugation on Biocoll separation solution (Biochrom, Berlin, Germany) at 600g for 15min. CD34⁺ cells were enriched by immunoaffinity selection (CD34 Progenitor Cell Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. For cell culture, IMDM with Glutamax was used, supplemented with 5% heat-inactivated fetal calf serum, 100 U/ml penicillin-streptomycin, 1% MEM-vitamines and 1% non-essential amino acids (all purchased from Invitrogen, Karlsruhe, Germany).

2.6 Adhesion assays under static and dynamic conditions

Evaluation of CD34⁺ cell adhesion to immobilized platelets under static conditions was performed as previously described ²⁹. In brief, isolated platelets (2×10^8 /ml) were allowed to adhere to 96-well plates coated with collagen type I (10 µg/ml) for 2 hours. Subsequently, CD34⁺ cells were added and incubated for 30 minutes. Unspecific adhesion was prevented by blocking with BSA (2%). After two gentle washing steps with PBS, residual adherent CD34⁺ cells were counted by direct phase contrast microscopy. As negative control, similar experiments were performed with CD34⁺ cells adherent to collagen (10 µg/ml). Where indicated, adherent platelets were pre-incubated for 30 minutes with a blocking monoclonal antibody (mAb) to SDF-1 (20 µg/ml) or with an irrelevant IgG control mAb (2D1; 20 µg/ml) ³⁶. In another series of experiments, where indicated, EPCs or immobilized platelets were pre-incubated for 30 minutes with mAbs against known EPCs surface adhesion receptors (anti-P-selectin glycoprotein ligand-1 (CD162), anti- α_4 (CD49d), and anti- β_2 integrins (CD18)) and against platelet P-selectin (anti-CD62P), respectively.

To evaluate cell adhesion to immobilized platelets or haECs under flow conditions, cells were allowed to adhere to collagen-coated glass coverslips or cultivated till confluence, respectively, and then used in a flow chamber (Oligene, Berlin, Germany). Where indicated, adherent platelets or monolayers of haECs were pre-incubated for 30 min with an anti-SDF-1 mAb or with a control mAb (2D1) (20 µg/ml each) before perfusion was started. Where indicated, haECs were activated with TNF- α (50 ng/ml) and INF- γ (20 ng/ml) or IL-1 β (100 pg/ml), or co-incubated with isolated platelets (2×10^8 /ml) for 12 hours. Perfusion experiments were performed at shear rates of 2000 s⁻¹ (high shear). All experiments were recorded in real time on video-CD and evaluated off-line ²⁹.

2.7 Platelet-induced formation of foam cells

To analyze the effect of anti-SDF-1 on platelet-induced foam cells formation, isolated platelets were co-incubated with CD34⁺-progenitor cells as previously

described ¹³. In brief, CD34⁺ progenitor cells (50,000 cells) were co-cultured with platelets (2x10⁸/ml) in 96-well plates pre-coated with 0.2% gelatin at 37°C and 5% CO₂ for 10 days. Developing foam cells were counted per well at the phase contrast microscope. Furthermore, to evaluate the effect of atorvastatin and PPAR agonist on platelet-dependent foam cell generation, CD34⁺ progenitor cells were cultured in presence of platelets and of atorvastatin or PPAR agonist.

2.8 Colony Forming Unit Assay

To analyze the effect of anti-SDF-1 on platelet-induced endothelial cells maturation, isolated platelets were co-incubated with CD34⁺ progenitor cells as previously described ¹³. In brief, CD34⁺ progenitor cells were seeded on human fibronectin (Becton Dickinson, Heidelberg, Germany) or on immobilized platelets (2x10⁸ cells/ml) and cultivated for several days in endothelial cell growth medium MV 2 containing 5% heat-inactivated fetal calf serum, 5.0 ng/ml epidermal growth factor, 0.2 µg/ml hydrocortisone, 0.5 µg/ml vascular endothelial growth factor, 10 ng/ml basic fibroblast factor, 20 ng/ml R3 insulin-like growth factor-1, 1µg/ml ascorbic acid (PromoCell, Heidelberg, Germany). After 48 hours, non adherent cells were removed. Endothelial colony-forming units were counted between days 5 and 10 (number of colonies). Cells were washed and resuspended in PBS, incubated with polyglobin (Bayer Vital; Leverkusen, Germany) for 15 min, washed, and incubated with FITC- labeled antibodies to CD146 (clone 128018; R&D Systems) for 30 min at room temperature. After washing, cells were analyzed on a FACS Canto flow cytometer (Becton Dickinson).

2.9 Gelatin Zymography

MMP-9 activity was determined in the supernatants by gelatin zymography as previously described ⁴². SDS gels containing 10% gelatine were from Invitrogen. Equal amounts of supernatants were loaded on the gels and after

electrophoresis, renaturation and further incubation of the gels for 12 hours at 37°C, gelatinolytic activity of MMP-2 and MMP-9 was detected as transparent bands on the Comassie brilliant blue stained gels.

2.10 Sudan red staining

Cells were washed with phosphate-buffered saline (PBS) before each incubation step, fixed with 2% formaldehyde solution (20 min) and incubated with 0.5% sudan red (Sigma; 20 min). Nuclei were counterstained with hematoxyline solution (Sigma) for 5 min and analyzed by standard microscopy.

2.11 Immunofluorescence microscopy and cell staining procedures

CD34⁺ cells were co-incubated with medium or platelets for 10 days on chamber slides and processed for immunofluorescence microscopy. Between each incubation step, cells were gently washed with PBS. CD34⁺ cells were fixed with 2% formaldehyde solution for 20 minutes. Afterwards, cells were washed with 2% glycine, permeabilized with 0.2% Triton-X100 and incubated with PBS containing a monoclonal mouse anti-human CD68 antibody (4.7µg/ml) for 1h. As secondary antibody an Alexa Fluor anti-mouse IgG₁ antibody (10µg/ml) was added for another hour and finally DAPI staining (3.3µg/ml) was performed to visualize cell nuclei. Unspecific binding was prevented by bovine serum albumin (3%, 1h). Furthermore, rhodamine phalloidin (5 units/ml, detection of cytoskeletal actin) was applied for 30 min. For labelling, a rabbit anti-human vWF Ab (Dako Cytomation) and a secondary sheep anti-rabbit Ab (Sigma) were used. Samples were analyzed by immunofluorescence microscopy.

2.12 Data presentation and Statistical Analysis

Data are presented as mean \pm SEM, unless otherwise indicated. For the patients data distribution of continuous variables was tested for normality with Kolmogorov-Smirnov test or P-P test and the following tests were recruited as follows: a two-tailed unpaired t-test was used for comparisons between two groups and the Pearson correlation coefficient test was performed to estimate the correlation between SDF-1 and CD62P after logarithmic transformation of the data. For the *in vitro* experiments means were compared with a two-tailed unpaired t-test (between two categories) or with a one-way ANOVA using the Scheffé post hoc analysis (between three or more categories). All reported probability values are two-sided, and a value of $P < 0.05$ was considered as statistically significant. All statistical analyses were performed using SPSS version 13 for windows.

3. Results

3.1 Platelet surface expression of SDF-1 is increased in myocardial infarction

Recently, it was shown that adherent platelets recruit and induce differentiation of endothelial progenitor cells and that platelets secrete SDF-1 upon activation indicating a critical role of SDF-1 for progenitor cell homing in the peripheral vasculature of the myocardium during ischemia/reperfusion. Resting platelets do not substantially surface express SDF-1 (Figure 3).

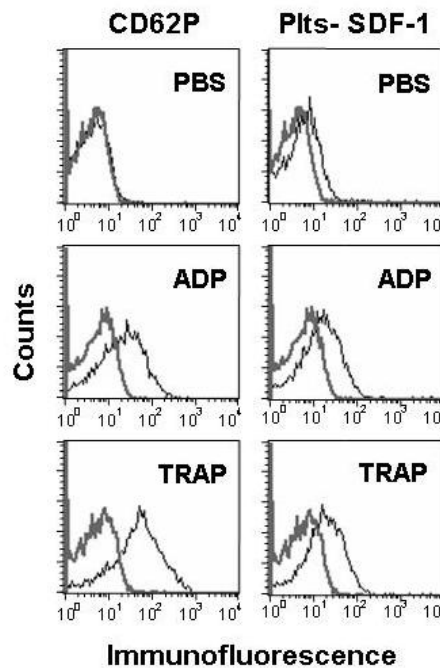


Figure 3. Representative FACS histograms showing P-selectin (CD62P; left panel) and SDF-1 (right panel) expression on unstimulated or ADP (20 μ m) or TRAP (thrombin receptor activating peptide; 25 μ M) -stimulated washed platelets and isotype IgG1 control (overlay).

However, upon activation with ADP (20 μ mol/l) or TRAP (10 μ mol/l) platelets degranulate and surface express significant amounts of SDF-1 (Figure 3 and 4). Similarly, adhesion of platelets to immobilized collagen results in enhanced surface expression of SDF-1 and CD62P (Figure 4).

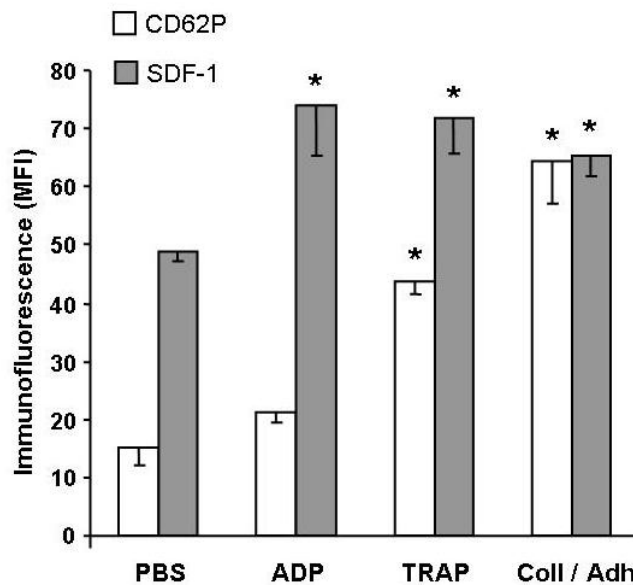


Figure 4. Quantitative analysis of the expression of P-selectin and SDF-1 on the surface of washed or platelets adhering to collagen evaluated by flow cytometry. The results (mean \pm SEM) of 6 independent experiments are shown. * $P < 0.05$ vs. unstimulated platelets (PBS).

To analyze whether surface expression of SDF-1 is enhanced in myocardial infarction we evaluated a cohort of 450 patients with symptomatic coronary artery disease (Table; page 25). Surface expression of SDF-1 on platelets was significantly enhanced in acute myocardial infarction (n=144) compared to stable angina pectoris (n=306) (Figure 5). SDF-1 expression correlated with platelet degranulation of P-selectin ($r=0.448$, $P < 0.001$) (Figure 6).

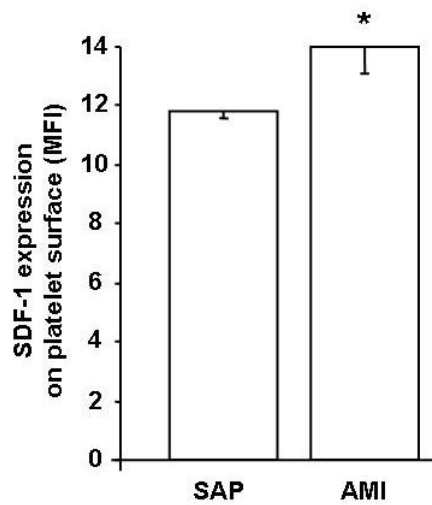


Figure 5. Whole-blood flow cytometry from 450 consecutive patients with coronary artery disease demonstrated an increased platelet-derived SDF-1 expression in patients with acute myocardial infarction (AMI; n=144) in comparison to those with stable angina pectoris (SAP, n=306). Results (mean±SEM) expressed as mean fluorescence intensity (MFI) were AMI vs. SAP: 13.97±0.87 vs. 11.82±0.27 (P= 0.021).

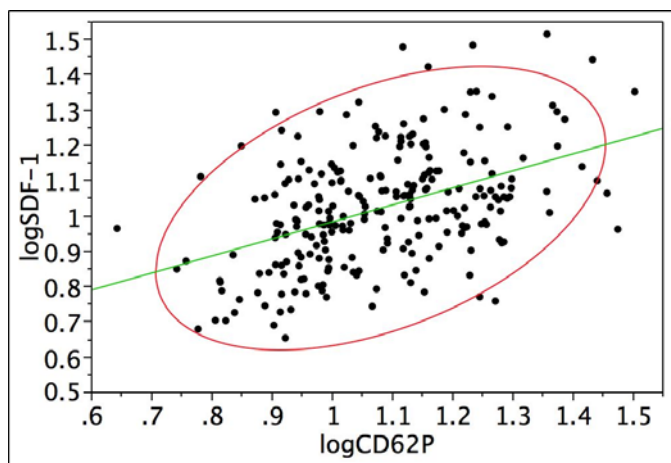


Figure 6. In a subpopulation of 243 consecutive patients, parallel assessment of platelet surface expression of SDF-1 and P-selectin (CD62P) demonstrated a significant correlation between these two platelet glycoproteins (r=0.448; P<0.001).

Table: Baseline patients' characteristics

Characteristics	Total (n=450)	SAP (n=306)	AMI (n=144)	P value for trend
Age – yrs*	66.9±10.8	67.5±9.6	65.4±12.8	0.053
Female, n (%)	115 (25.6)	70 (22.9)	45 (31.3)	0.057
Cardiovascular Risk				
Factors – no. (%)				
Arterial Hypertension	338 (75.3)	239 (78.4)	99 (68.8)	0.028
Hyperlipidemia	284 (63.3)	216 (70.8)	68 (47.2)	<0.001
Diabetes	141 (31.4)	93 (30.5)	48 (33.3)	0.545
Family History of CAD	89 (19.8)	69 (22.6)	20 (13.9)	0.03
Smoking	180 (40.1)	113 (37)	67 (46.5)	0.056
CAD – n (%)				<0.001
1 Vessel	88 (19.8)	41 (13.4)	47 (33.6)	
2 Vessels	155 (34.8)	110 (36.1)	45 (32.1)	
3 Vessels	200 (44.9)	153 (50.2)	47 (33.6)	
Left Ventricular				
Ejection Fraction – n (%)				<0.001
normal	220 (50)	174 (58)	46 (32.9)	
slightly reduced	84 (19.1)	50 (16.7)	34 (24.3)	
moderate	87 (19.8)	47 (15.7)	40 (28.6)	
low	49 (11.1)	29 (9.7)	20 (14.3)	

*mean ± standard deviation. CAD denotes coronary artery disease

3.2 Adherent platelets recruit human CD34⁺ progenitor cells

To determine the molecular requirements of adhesion of human adult CD34⁺ cells to platelets, we performed experiments with human CD34⁺ cells isolated from cord blood. Isolated platelets (2×10^8 /ml) were allowed to adhere to collagen-coated 96-well plates, and adhesion of CD34⁺ cells was evaluated under static conditions as described in Methods.

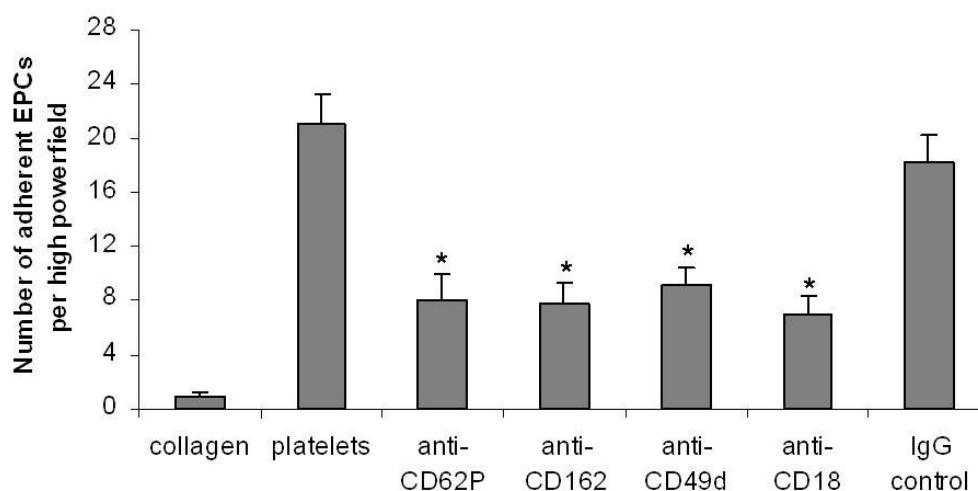


Figure 7. Adhesion of CD34⁺ cells (EPCs) to immobilized platelets. Under static conditions, EPCs adhere to immobilized platelets via P-selectin/PSGL-1 interaction and β_1 - and β_2 - integrins. 96-well plates pre-coated with collagen type I ($10 \mu\text{g}/\text{ml}$) were incubated with or without freshly isolated platelets in order to achieve an adherent platelet layer as described in Materials and Methods. Human EPCs ($20,000 \text{ CD34}^+$ cells per well) were allowed to adhere to these platelets for one hour. Where indicated, CD34⁺ progenitor cells or platelets were pre-incubated for 30 minutes with blocking mAbs. Then, the plates were gently washed twice. The adherent EPCs were quantified by direct phase contrast microscopy. The mean and SD of 4 independent experiments are shown. Pre-incubation with blocking mAbs against CD62P, CD162, CD49d and CD18 reduces the adhesion of CD34⁺ to immobilized platelets. * $P < 0.05$ compared to positive control (IgG control).

Human CD34⁺ cells adhere to immobilized platelets but not to immobilized collagen type I alone, which represents the major extracellular matrix component of the injured arterial wall (Figure 7). Adhesion of CD34⁺ cells to immobilized platelets that were activated by adherence to collagen was significantly attenuated in the presence of blocking mAbs anti-CD162 or anti-CD62P indicating that the platelet P-selectin interacts with the EPCs P-selectin glycoprotein ligand-1. Moreover, pre-incubation of CD34⁺ with the blocking monoclonal antibodies to α_4 -integrin (CD49d) or to β_2 -integrin (CD18) resulted in a significant decrease of adherent CD34⁺ to immobilized platelets suggesting that both β_1 - and β_2 -integrins located on EPCs surface are involved in the adhesion process between these two types of human cells (Figure 7).

3.3 Platelet-derived SDF-1 mediates adhesion of CD34⁺ progenitor cells under static and flow conditions

Next it was examined whether platelet-derived SDF-1 modulates adhesion of progenitor cells to immobilized platelets. It was found that human CD34⁺ cells adhere to immobilized platelets but not to immobilized collagen type I alone, which represents the major extracellular matrix component of the injured arterial wall (Figure 8). Adhesion of CD34⁺ cells to immobilized platelets, which were activated by adherence to collagen, was significantly attenuated in the presence of a blocking monoclonal antibody (mAb) to SDF-1 but not control IgG, indicating that the platelet-derived SDF-1 is critical for progenitor cell/platelet interaction (Figure 8).

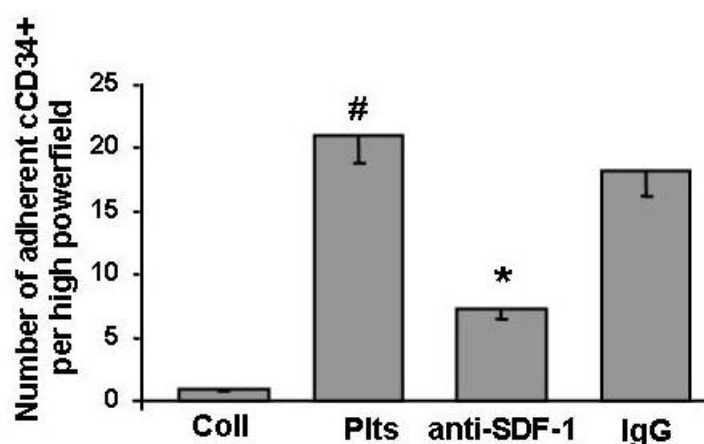


Figure 8. CD34⁺ progenitor cells adhere to immobilized platelets but not to collagen type I under static conditions. A blocking monoclonal antibody (mAb) to platelet SDF-1, but not an isotype control IgG1, significantly attenuated the number of adherent CD34⁺ cells to immobilized platelets. The mean and SEM of 4 independent experiments are shown. #P<0.001 vs. collagen, *P=0.004 vs. IgG control.

In perfusion experiments it was observed that both rolling and firm adhesion of CD34⁺ cells to immobilized platelets were significantly reduced in the presence of neutralizing anti-SDF-1 mAb compared to IgG control (Figure 9 and 10). Thus, release and presentation of SDF-1 on activated platelets is critical for interaction with CD34⁺ cells.

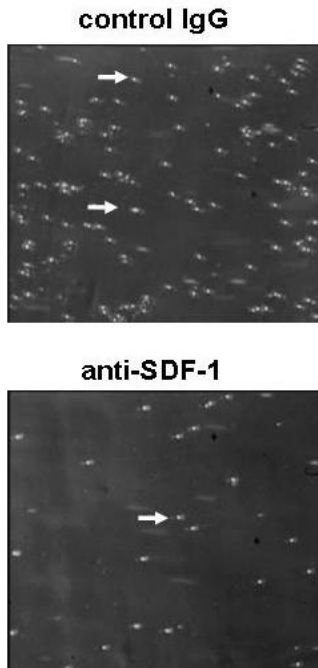


Figure 9. Representative phase contrast images of adherent CD34⁺ cells under dynamic conditions (flow chamber). Arrows show adherent CD34⁺ cells.

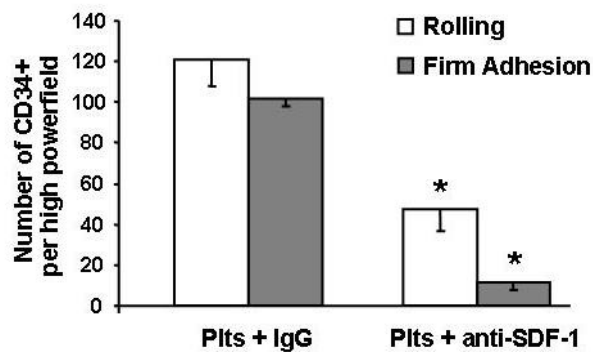


Figure 10. In a flow chamber, both rolling and adhesion of CD34⁺ cells on platelets were significantly blocked by a mAb to SDF-1 at high shear conditions of 2000^s. *P<0.01 vs. control IgG.

3.4 Platelets activate endothelial cells and support recruitment of CD34⁺ cells towards endothelium via platelet-derived SDF-1

Interaction of circulating progenitor cells with the endothelial monolayer of the vasculature of peripheral organs is critical for homing. Previously it was shown that platelets activate endothelial cells and support adhesion of leukocytes to the endothelium. Thus, whether platelets support recruitment of CD34⁺ cells to cultured human arterial endothelium (haEC) was examined.

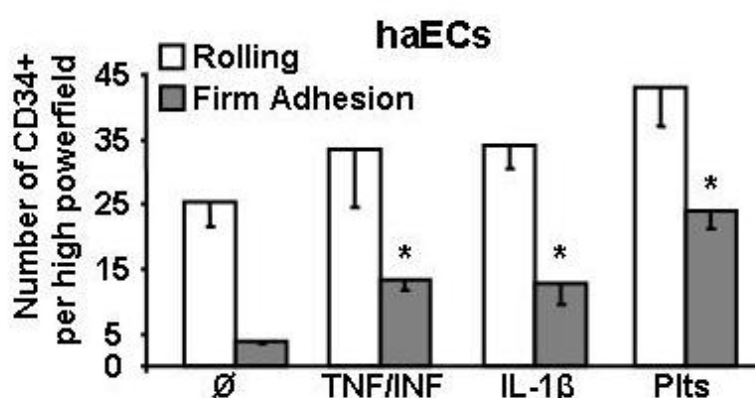


Figure 11. Perfusion of CD34⁺ cells over human arterial endothelial cells, being unstimulated, or stimulated with TNF- α /INF- γ or IL-1 β , or co-incubated with washed platelets, was conducted for 10 minutes at high shear stress of 2000^s in a flow chamber. Activation of haECs with TNF- α /INF- γ or IL-1 β induced a significant increase of CD34⁺ cell adhesion on haECs in comparison with unstimulated haECs (P= 0.016 and P= 0.04, respectively). Co-incubation of haECs with washed platelets for 12 hours caused a significant elevation of adherent CD34⁺ cells on endothelial surface compared to resting haECs (P<0.001) or compared to stimulated haECs with TNF- α /INF- γ or IL-1 β (P= 0.008 for both). Rolling of CD34⁺ cells on endothelial surface was high on resting haECs being slightly increased upon stimulation or co-incubation with platelets, although significance was not reached. The results (mean \pm SEM) are given as number of adherent CD34⁺ per high powerfield (n= 6 experiments per group). *P<0.01 vs. unstimulated haECs.

It was found that cultured non-activated endothelial cells do not support firm adhesion of CD34⁺ cells under arterial flow conditions (Figure 11). However, when monolayers of haEC were activated with TNF/INF or IL-1 β , adhesion of CD34⁺ cells was significantly enhanced (Figure 11 and 12).

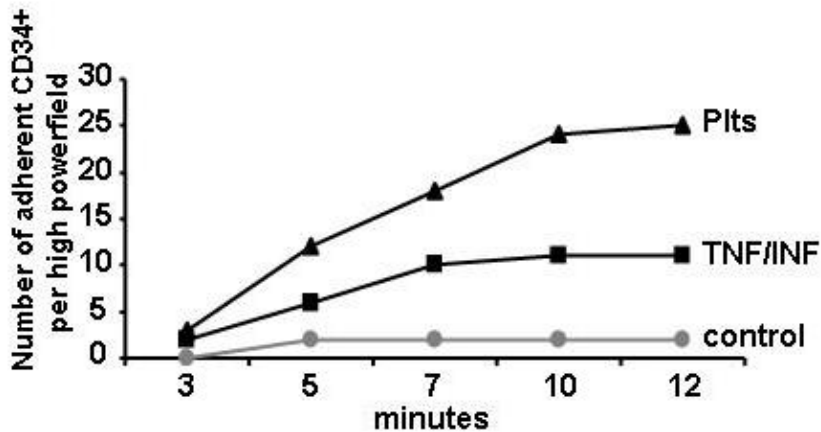


Figure 12. Representative time curve of three single flow chamber experiments.

Pre-incubation of haECs with platelets further enhanced CD34⁺ cell adhesion to immobilized endothelium (Figure 11 and 12), indicating that platelets play a critical role in homing circulating CD34⁺ cells to the endothelium. In the presence of neutralizing anti-SDF-1 mAb adhesion of CD34⁺ cells to platelet-activated but not to TNF/INF or IL-1 β -activated haECs was significantly reduced (Figures 13 and 14). This indicates that although low levels of SDF-1 are constitutively surface expressed on endothelial cells, which are not influenced upon activation with TNF/INF or IL-1 β primarily or exclusively, platelet-derived SDF-1 regulates CD34⁺ cell recruitment to endothelial monolayers (Figures 15 and 16).

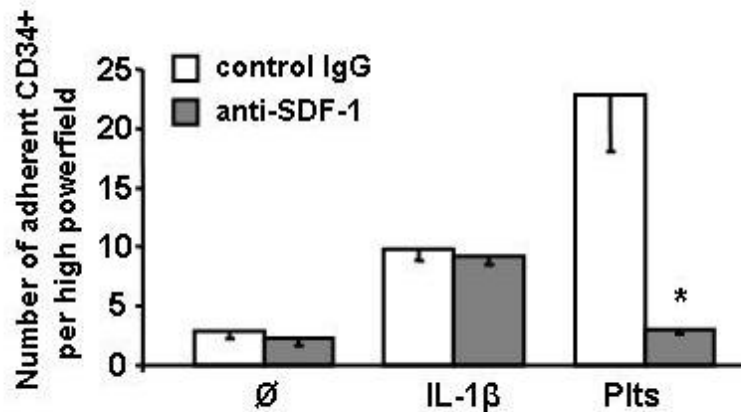


Figure 13. Dynamic adhesion assays were performed to determine the effect of endothelial- and platelet- derived SDF-1 on the recruitment of CD34⁺ cells. Pre-treatment of endothelial surface for 30 minutes with a blocking monoclonal antibody to SDF-1 did not alter the adhesion of CD34⁺ to haECs, which were unstimulated or to stimulated with IL-1β, but did decrease the number of adherent CD34⁺ cells when haECs were co-incubated with platelets compared always to isotype control IgG. *P<0.01 vs. control IgG.

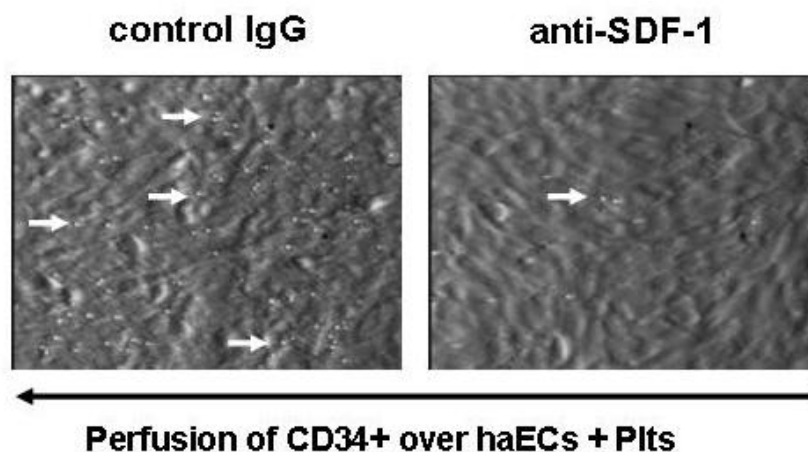


Figure 14. Representative phase contrast images of adherent CD34⁺ cells under dynamic conditions (flow chamber). Arrows show adherent CD34⁺ cells.

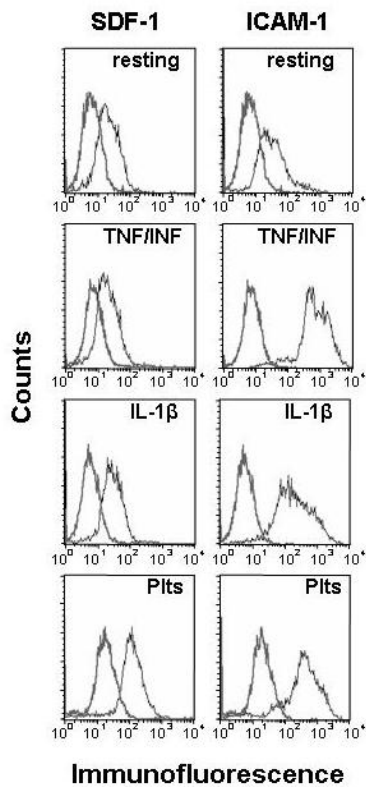


Figure 15. Representative FACS histograms showing SDF-1 (left panel) and ICAM-1 (right panel; positive control) expression on unstimulated, TNF- α /INF- γ or IL-1 β stimulated human arterial endothelial cells (haECs), and haECs co-incubated with platelets, and isotype IgG1 control (overlay).

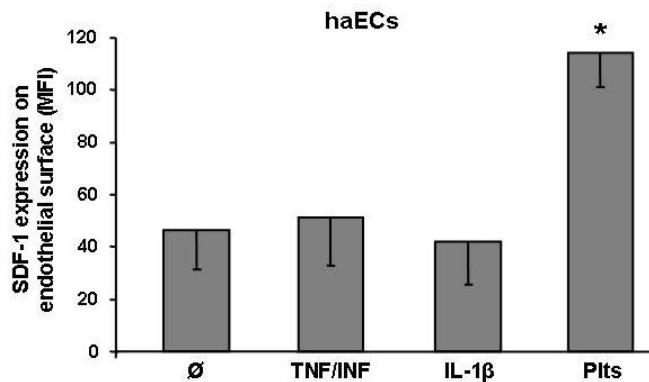


Figure 16. Quantitative analysis of the expression of SDF-1 on endothelial surface. The mean and SEM of 4 independent experiments are shown.

*P<0.001 vs. resting haECs and also vs. stimulated haECs.

3.5 Platelet-derived SDF-1 regulates differentiation of CD34⁺ cell into macrophages/foam cells

Recently it was described that platelets regulate recruitment and differentiation of CD34⁺ progenitor cells into foam cells and endothelial cells¹³. To further evaluate the molecular requirements of platelet-dependent differentiation of progenitor cells, CD34⁺ cells were co-incubated with isolated platelets as described in Methods. In the presence of neutralizing anti-SDF-1 mAb platelet-dependent foam cell formation was substantially attenuated compared to IgG control experiments (Figures 17, 18 and 19).

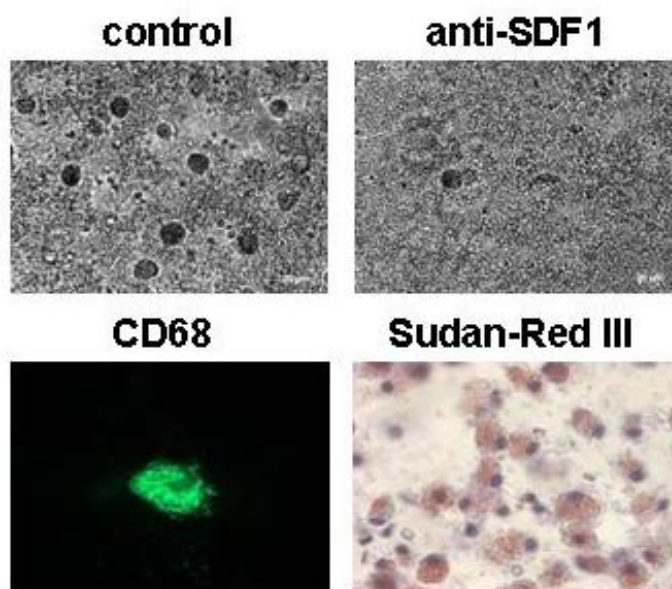


Figure 17. CD34⁺ cells were co-incubated with freshly isolated platelets (2×10^8 /ml) for 10 days in the presence of a blocking monoclonal antibody to SDF-1 or control isotype IgG1. Representative phase contrast images showing foam cell generation in the presence of control IgG1 or of anti-SDF-1 mAb (upper panel). CD68 immunostaining indicates differentiation of CD34⁺ into the macrophage/monocyte lineage, while sudan red III depicts large granular and lipid rich cells (lower panel).

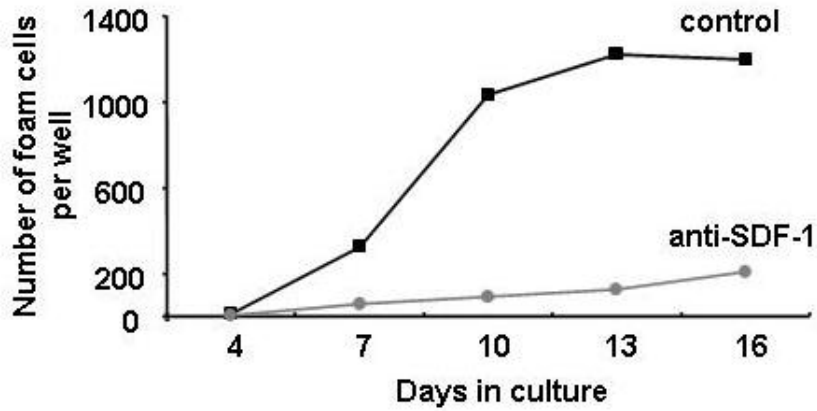


Figure 18. Representative time curve of foam cell generation in a co-culture system with CD34⁺ cells and platelets with or without a mAb to SDF-1.

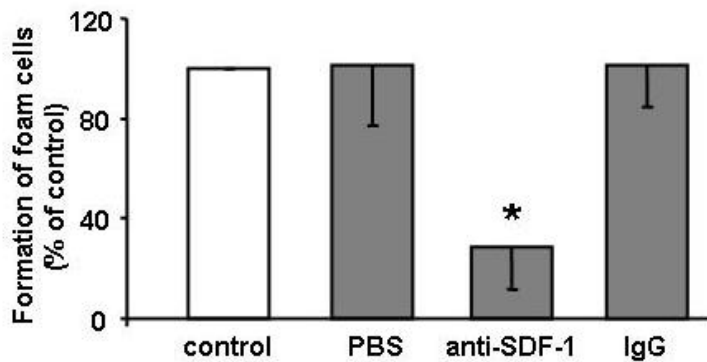


Figure 19. Quantitative analysis (mean±SEM) of 4 independent experiments is shown. Foam cells were counted per well and correlated to control (=100%). *P<0.05.

Similar results were found when statins or PPAR-agonists were added to the co-culture as recently described (Figure 20).

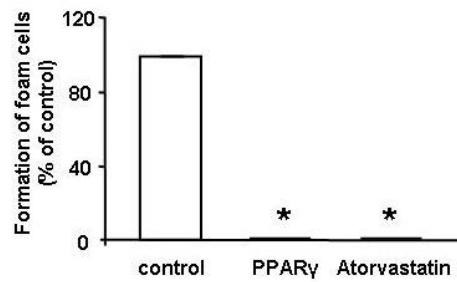


Figure 20. Co-incubation of CD34⁺ progenitors with platelets in the presence of PPAR- γ or atorvastatin caused a complete abolishment of CD34⁺ differentiation to macrophages / foam cells. Results are representative of 3 independent experiments. *P<0.001.

SDF-1 induces the expression and release of matrix metalloproteinases (MMPs) by human CD34⁺ cells²⁴. Statins inhibit secretion of MMP-9 from vascular cells, implying an antiatherosclerotic pleiotropic effect of these compounds⁵⁸. To evaluate the effect of inhibition of platelet-derived SDF-1 on secretion of MMPs in our co-cultures, CD34⁺ cells were co-cultured with platelets in the presence or absence of anti-SDF-1 mAb. We found that anti-SDF-1 mAb, but not control IgG, substantially reduced MMP-9 activity in culture supernatants (Figure 21).

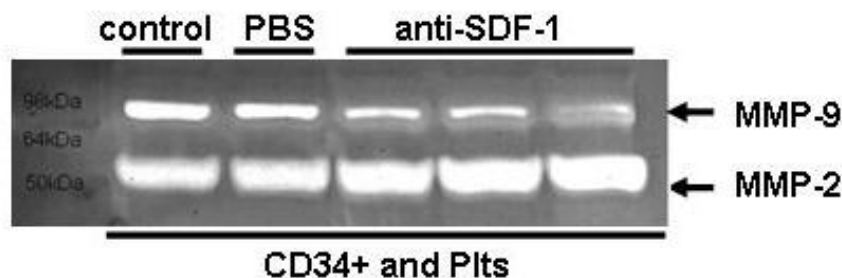


Figure 21. SDS-PAGE zymography of cell culture supernatants showed decreased secretion of MMP-9, but not MMP-2, after inhibition of platelet-derived SDF-1.

3.6 Platelet-derived SDF-1 regulates differentiation of CD34⁺ cells into endothelial cells

Recently it was reported that adherent platelets modulate CD34⁺ differentiation to mature endothelial cells^{29,13}. Whether platelet-derived SDF-1 influences also this differential pathway, colony forming unit assays were performed, in which a blocking mAb inhibited platelet SDF-1. It was observed plenty of endothelial cell colonies on immobilized fibronectin or adherent platelets as verified with vWF and phalloidin staining, and positive endothelial marker (CD146) (Figures 22-25). When a neutralizing mAb to platelet-derived SDF-1 was used, there was a significant decrease on the number of colonies in comparison with control IgG (Figure 22, 23 and 25).

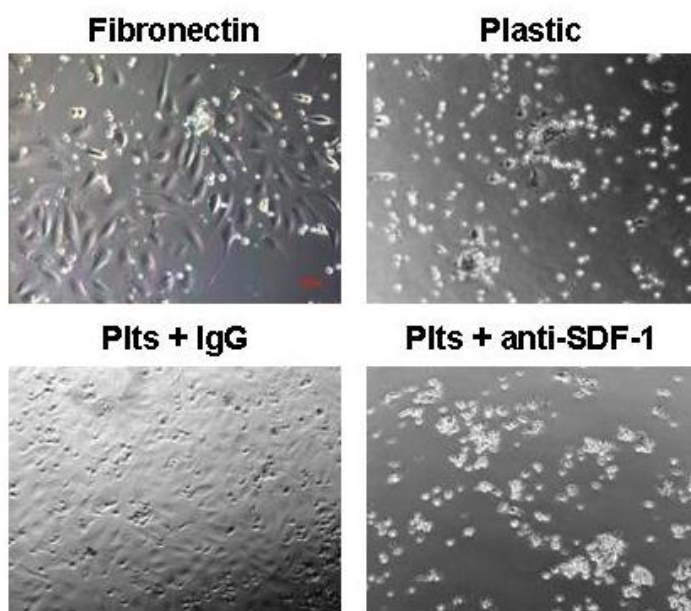


Figure 22. CD34⁺ cells formed endothelial colonies on immobilized platelets and fibronectin, but not on plastic. In the presence of anti-SDF-1 mAb, but not control IgG, almost no colonies were observed.

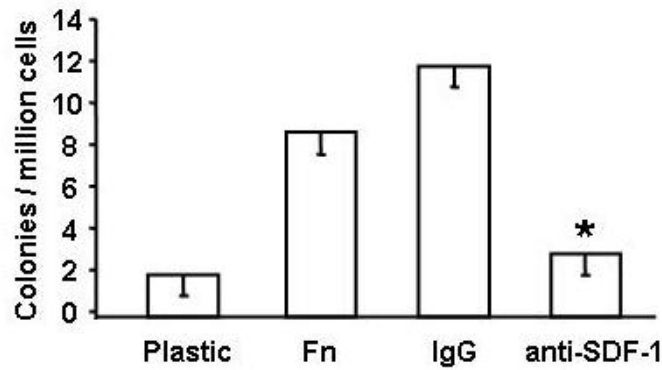


Figure 23. Endothelial cell colony forming units were counted between days 5 and 10. The mean and SEM of 3 independent experiments are shown. *P<0.05 vs. control IgG.

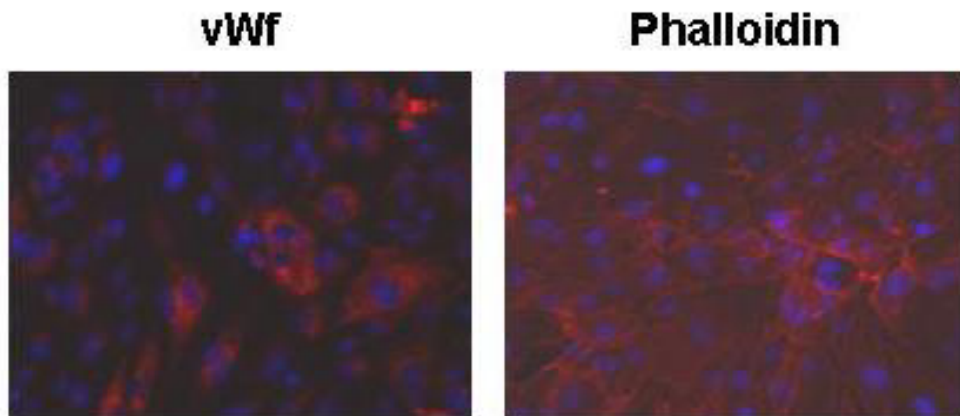


Figure 24. After 5-10 days the morphology of CD34+ cells on immobilized fibronectin or immobilized platelets turned into adherent spindle-shaped cells that were positive for vWF and rhodamine phalloidin.

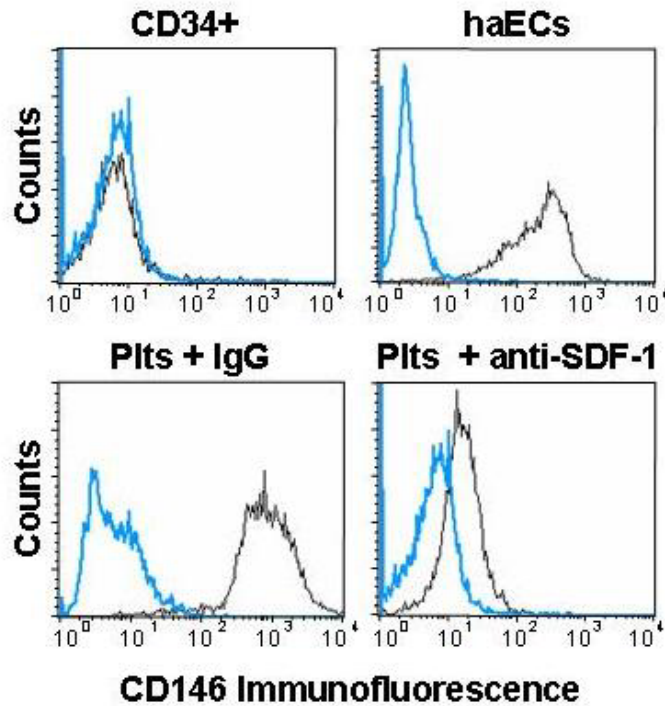


Figure 25. Cultivated CD34⁺ cells on immobilized platelets in the presence or absence of anti-SDF-1 mAb were analyzed for endothelial marker expression of CD146 by flow cytometry. Four representative histograms of CD146 expression of freshly isolated CD34⁺, human arterial endothelial cells, endothelial cells coming from CD34⁺ progenitors in the absence or presence of anti-SDF-1, are presented in comparison to control IgG (overlay).

3.7 Platelets and endothelial cells modulate proliferation of CD34⁺ progenitors via surface expression of SDF-1

In order to determine and distinguish the impact of SDF-1 coming from platelets or haECs on CD34⁺ development, co-culture experiments were performed in which CD34⁺ cells were co-cultivated on top of endothelial monolayers (haECs) in the presence or absence of platelets. Co-cultivation of CD34⁺ cells with haECs did not remarkably change the morphology of progenitor cells, while a significant increase in their number was achieved after a 2-week co-culture in comparison to CD34⁺ alone. When platelets were added to the co-culture there was a further significant increase in number of CD34⁺ cells in comparison with CD34⁺ co-cultured with haECs (Figures 26 and 27).

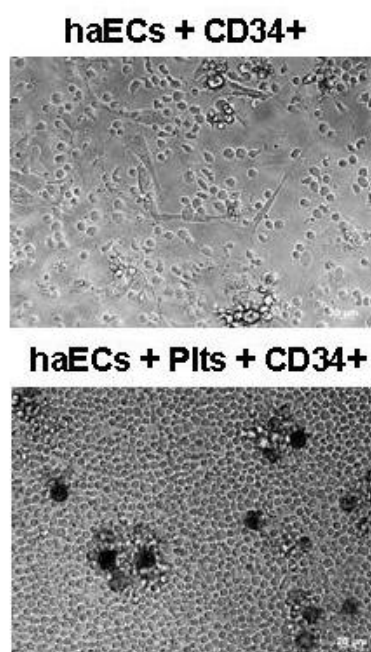


Figure 26. Representative phase contrast images showing proliferation of CD34⁺ cells in co-culture systems of haECs and CD34⁺ (upper image) or haECs and CD34⁺ in the presence of platelets (lower image) after a 2-week incubation time. Only in the presence of platelets in the co-culture system foam cells generation was observed.

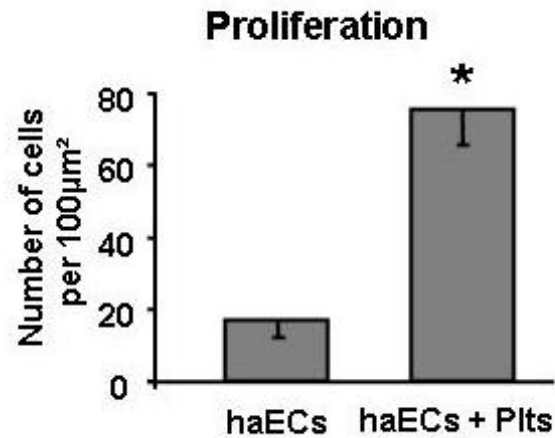


Figure 27. Quantitative analysis of proliferation (n=3).

Progenitor cell proliferation was significantly attenuated when haECs or haECs and platelets were treated with a blocking mAb to SDF-1, indicating that both haEC- and platelet-derived SDF-1 play a role in the proliferation of progenitor cells (Figures 28 and 29).

Moreover, as already described above, part of the progenitor cells differentiated into foam cells in the presence of platelets (Figures 26 and 30). Foam cell formation was significantly attenuated in the presence of a neutralizing mAb to SDF-1 but not of control IgG (Figures 31 and 32).

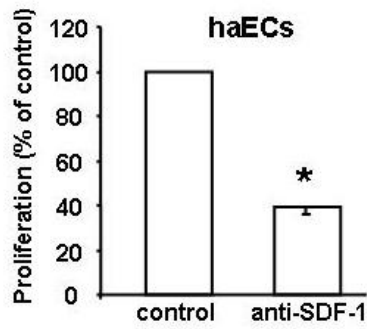


Figure 28. Co-cultures of CD34⁺ and haECs caused significant cell proliferation inhibited by a mAb to SDF-1. Cells were counted per 100 μm^2 . Results of three experiments are shown. *P<0.05.

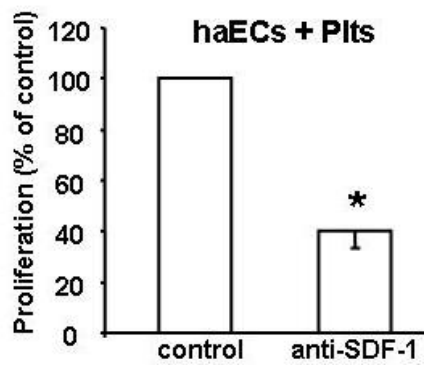


Figure 29. Co-cultures of CD34⁺ and haECs in the presence of isolated platelets caused significant cell proliferation inhibited by a mAb to SDF-1. Cells were counted per 100 μm^2 . Results of three experiments are shown. *P<0.05.

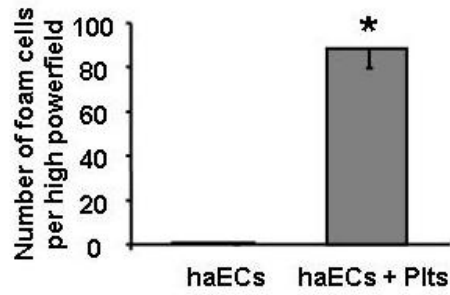


Figure 30. Foam cell formation in the absence and presence of platelets on top of endothelium (n=3). *P<0.05.

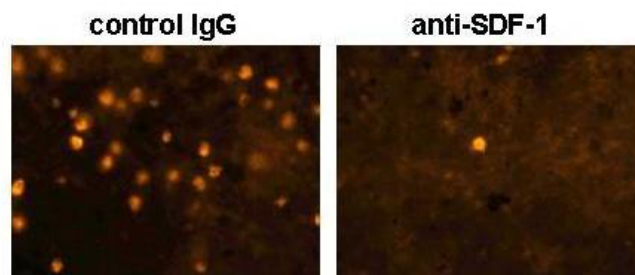


Figure 31. CD34⁺ cells were kept in culture for 6-10 days with haECs and platelets. Representative fluorescence images (rhodamine) of macrophage / foam cell formation in the presence or absence of a blocking mAb to SDF-1.

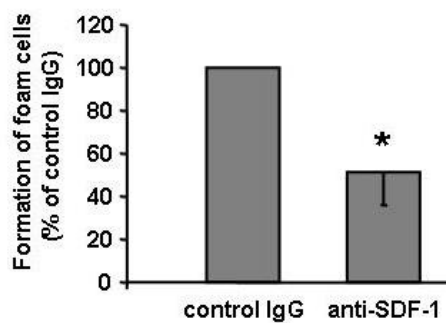


Figure 32. Foam cell formation in the absence and presence of platelets on top of endothelium (n=3). *P<0.05.

4. Discussion

The major findings of the present dissertation are: 1) Expression of SDF-1 on platelets is enhanced in patients with acute myocardial infarction and correlates with degree of platelet activation. 2) Platelet-derived SDF-1 mediates adhesion of CD34⁺ progenitor cells under static and flow conditions. 3) Platelets activate endothelial cells and support recruitment of CD34⁺ cells towards endothelium via platelet-derived SDF-1. 4) Platelet-derived SDF-1 regulates proliferation and differentiation of CD34⁺ cell into macrophages/foam cells and endothelial cells.

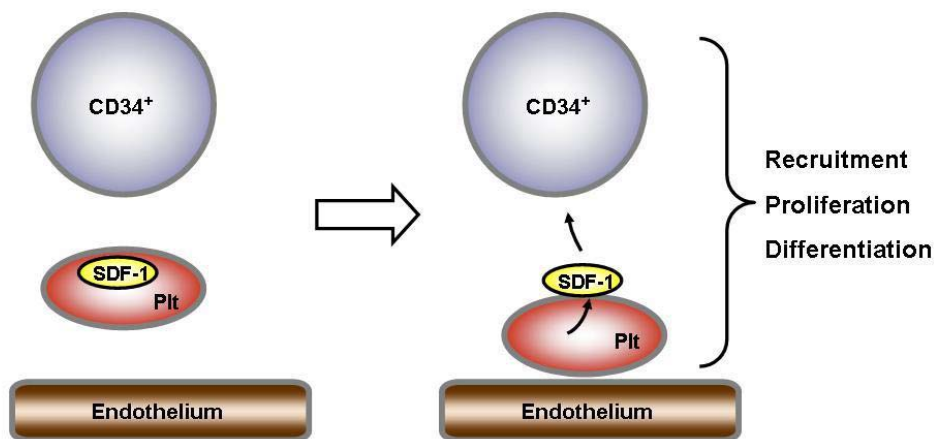


Figure 33. Proposed model of the multiple roles of platelet-derived SDF-1 on human CD34⁺ progenitors. Substantial expression of SDF-1 is observed in cases where platelet activation or adhesion occurs, as in acute myocardial infarction. Thereafter it causes recruitment of CD34⁺ cells and affects proliferation and differentiation of progenitor cells, being a key player in vascular repair mechanisms.

These findings imply that activated platelets and platelet-membrane bound SDF-1 are critically involved in the recruitment of circulating CD34⁺ progenitor

cells at site of vascular injury or in the microcirculation of ischemic myocardium where enhanced platelet adhesion and aggregate formation occurs. Furthermore, platelet-dependent proliferation and differentiation of CD34⁺ progenitor cells is regulated via platelet-derived SDF-1. Thus, platelets may play a central role for homing of circulating progenitor cells and for stem cell dependent regeneration of ischemic tissue (Figure 33).

Circulating progenitor cells have been shown to instigate new vessel formation via angiogenesis and neovascularisation but also have the potential to provide ongoing vascular and tissue repair by homing to site of vascular or tissue damage⁶². However, the mechanisms that recruit circulating progenitor cells towards vascular lesions and regulate repair mechanisms are incompletely understood. Platelets are the first circulating blood cells that interact with the injured vessel wall or that accumulate at inflamed endothelium within the microcirculation of ischemic tissue¹⁶. Activated platelets release a variety of potent proinflammatory and chemotactic factors (e.g. interleukin-1 β)^{17, 32} and growth factors (platelet-derived growth factor)³⁸ that in turn activate endothelium and support recruitment of circulating blood cells³⁴.

Recently, it was reported that platelets recruit circulating CD34⁺ cells via P-selectin/PSGL-1 and the β_1 -integrins^{29, 37}. Further, platelets regulate differentiation of progenitor cells into foam cells and mature endothelial cells¹³. Moreover, platelets secrete SDF-1 upon activation which implies a potential important mechanism of how circulating progenitor cells are recruited in ischemic tissue of peripheral organs. The present data show that platelet-derived SDF-1 supports adhesion of CD34⁺ cells to immobilized platelets and endothelium. Since platelet adhesion to endothelium is enhanced at site of atherogenesis³⁴ and in the microcirculation of ischemic tissue during reperfusion⁴⁰, it is tempting to speculate that platelet-dependent CD34⁺ cells homing in peripheral organs is critically involved in regeneration of ischemic tissue. The impact of platelets is not limited just to their role in cell recruitment. Since platelets induce differentiation of CD34⁺ cells into monocyte/macrophages/foam cells¹³ and endothelial cells²⁹, platelets also may have a major impact on tissue regeneration and angiogenesis (Figure 33).

Further studies are called to investigate the potential therapeutic value of manipulation of the SDF-1/CXCR4 axis in atherosclerosis, tissue ischemia (vasculopathy, myocardial infarction, and stroke) and angiogenesis.

5. Summary

The chemokine stromal cell derived factor-1 (SDF-1) regulates the homing of hematopoietic stem cells. The aim of this study was to examine the platelet surface expression of SDF-1 in patients with acute myocardial infarction (AMI) and to investigate the role of platelet-derived SDF-1 for homing and differentiation of CD34⁺ progenitor cells.

In a consecutive cohort of 450 patients with coronary artery disease, flow cytometric analysis demonstrated a significantly increased platelet-surface expression of SDF-1 in patients with AMI (n=144) compared to patients with stable angina (n=306) (P=0.021). Platelet surface expression was dependent on platelet activation and correlated with degranulation of P-selectin. Platelet-derived SDF-1 regulated adhesion of CD34⁺ cells to immobilized platelets and endothelium under static and dynamic flow conditions. Moreover, platelets induced formation of macrophages/foam cells and maturation to endothelial cells from CD34⁺ cells in co-culture experiments via SDF-1.

These findings imply that platelet-derived SDF-1 regulates recruitment and differentiation of progenitor cells and may play a central role for regeneration of ischemic myocardium.

6. Zusammenfassung

Das Chemokin SDF-1 reguliert unter anderem die Rekrutierung von hämatopoetischen Stammzellen. Im Rahmen dieser Arbeit wurde die Oberflächenexpression von SDF-1 auf humanen Thrombozyten von Patienten mit akutem Myokardinfarkt untersucht. Von besonderem Interesse war dabei der Einfluß von thrombozytärem SDF-1 auf das „Homing“ und die Differenzierung von CD34-positiven Progenitorzellen.

In einer Kohorte von 450 konsekutiven Patienten mit koronarer Herzerkrankung zeigte die Durchflußzytometrie, dass bei Patienten mit akutem Myokardinfarkt (n=144) die Oberflächenexpression von SDF-1 auf den Thrombozyten im Vergleich zu Patienten mit stabiler Angina pectoris signifikant erhöht ist. Die thrombozytäre SDF-1 Oberflächenexpression korreliert zum Ausmaß der Thrombozytenaktivierung und Degranulierung. Unter statischen und unter Flussbedingungen regulierte thrombozytäres SDF-1 die Adhäsion von CD34-positiven Progenitorzellen an immobilisierte Thrombozyten und an das Endothel. Dabei induzierte die Adhärenz an Thrombozyten die Differenzierung von CD34-positiven Progenitorzellen zu Makrophagen / Schaumzellen oder Endothelzellen.

Aus diesen Daten kann man schließen, dass das Chemokin SDF-1 eine zentrale Rolle in physiologischen Regenerationsprozessen des Myokards spielen kann.

7. Bibliography

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8. Publications

The results of this dissertation are subject to publication as follows:

Original Articles

Stellos K, Langer H, Daub K, Schonberger T, Gauss A, Geisler T, Bigalke B, Mueller I, Schumm M, Schaefer I, Seizer P, Kraemer B, Siegel-Axel D, May AE, Lindemann S, Gawaz M. Platelet-derived SDF-1 regulates recruitment and differentiation of human CD34⁺ progenitor cells to endothelial cells. In Revision

Stellos K, Bigalke B, Langer H, Geisler T, Mueller I, Seizer P, May AE, Lindemann S, Gawaz M. Platelet-derived SDF-1 is increased in patients with acute coronary syndromes and correlates with number of circulating CD34⁺ progenitor cells. In Revision

Stellos K, Langer H, Bigalke B, Geisler T, Mueller I, Seizer P, May AE, Lindemann S, Gawaz M. Platelet-derived SDF-1 regulates MMP-9 secretion and differentiation of CD34⁺ progenitor cells to macrophages and foam cells. In Preparation

Stellos K, Seizer P, Langer H, Bigalke B, Geisler T, Siegel-Axel D, May AE, Lindemann S, Gawaz M. Endothelial cells induce proliferation of CD34⁺ progenitor cells. In Preparation

Stellos K, Langer H, Seizer P, Bigalke B, Geisler T, Siegel-Axel D, May AE, Lindemann S, Gawaz M. Adherent platelets regulate adhesion under high shear and induce differentiation of CD34⁺ progenitor cells to endothelial cells in vitro: a comparative analysis with collagen, fibronectin and endothelial cells In Preparation

Daub K, Langer H, Seizer P, Stellos K, May AE, Goyal P, Bigalke B, Schönberger T, Geisler T, Siegel-Axel D, Oostendorp RA, Lindemann S, Gawaz M. Platelets induce differentiation of human CD34⁺ progenitor cells into foam cells and endothelial cells. *FASEB J.* 2006; 20(14):2559-61

Review Articles

Stellos K, Gawaz M. Platelets and stromal cell-derived factor-1 in progenitor cell recruitment. *Semin Thromb Hemost.* 2007; 33(2):159-64.

Stellos K, Gawaz M. Platelet interaction with progenitor cells: potential implications for regenerative medicine. In Revision

Stellos K, Gawaz M. Platelet interaction with progenitor cells: vascular regeneration or injury? In Preparation

Congress Abstracts

Stellos K, Langer H, Bigalke B, Daub K, Schumm M, Seizer P, Schönberger T, Siegel-Axel D, Lindemann S, Gawaz M. Platelet-derived SDF-1 recruits and induces differentiation of human CD34⁺ progenitor cells to endothelial cells: implications in vascular and tissue regeneration. ESC Congress 2007, Vienna, Austria, September 01-05 2007

Stellos K, Bigalke B, Geisler T, Schaefer I, Langer H, Lindemann S, May A, Gawaz M. Surface expression of SDF-1 is increased on circulating platelets and correlates with the number of progenitor cells in patients with acute coronary syndrome. ESC Congress 2007, Vienna, Austria, September 01-05 2007

Stellos K, Gauss A, Schönberger T, Langer H, Bigalke B, Daub K, Schumm M, Seizer P, Gawaz M. Recruitment and differentiation of human progenitor cells to

endothelial cells is mediated through platelet-derived SDF-1: impact on vascular regeneration. 4th European Meeting on Vascular Biology and Medicine, Bristol, UK, September 17-20 2007

Stellos K, Bigalke B, Geisler T, Schaefer I, Langer H, Lindemann S, May A, Gawaz M. Platelet-derived SDF-1 is increased in patients with acute coronary syndromes and correlates with the number of CD34⁺ progenitor cells. 4th European Meeting on Vascular Biology and Medicine, Bristol, UK, September 17-20 2007

Stellos K, Langer H, Daub K, Bigalke B, Gawaz M. Platelet-derived SDF-1 regulates recruitment and differentiation of human CD34⁺ progenitor cells to endothelial cells: potential impact on vascular regeneration. 14th Three-country Congress of the German, Austrian and Swiss Society for Angiology, Munich, Germany, September 09-12 2007

Stellos K, Bigalke B, Geisler T, Seizer P, Kraemmer B, Daub K, Mueller I, Schonberger T, Langer H, Lindemann S, May A, Gawaz M. Expression of platelet-derived SDF-1 is increased and is associated with the number of CD34⁺ progenitor cells in patients with acute coronary syndromes. 44th Congress of the southwest German Society for Internal Medicine, Esslingen, Germany, October 19-20 2007

Stellos K, Bigalke B, Gauss A, Schonberger T, Daub K, Kraemmer B, Seizer P, Mueller I, Schumm M, Geisler T, Lindemann S, May A, Gawaz M. Platelet-derived SDF-1 regulates recruitment and differentiation of human progenitor cells: potential implications for regenerative medicine. 44th Congress of the southwest German Society for Internal Medicine, Esslingen, Germany, October 19-20 2007

Stellos K, Gauss A, Schönberger T, Daub K, Kraemer B, Seizer P, Mueller I, Langer H, Schumm M, Geisler T, Bigalke B, Siegel-Axel D, Lindemann S, May

A, Gawaz M. Platelet-derived SDF-1 regulates recruitment and differentiation of human progenitor cells: impact on myocardial regeneration. 3rd International Russel-Ross-Symposium, Ulm, Germany, June 28-30 2007

Stellos K, Bigalke B, Geisler T, Seizer P, Kraemer B, Daub K, Mueller I, Schonberger T, Langer H, Siegel-Axel D, Lindemann S, May A, Gawaz M. Expression of platelet-derived SDF-1 is associated with the number of CD34⁺ progenitor cells in patients with coronary artery disease. 3rd International Russel-Ross-Symposium, Ulm, Germany, June 28-30 2007

Stellos K, Langer H, Daub K, Seizer P, Geisler T, Schönberger T, May A, Gawaz M. Role of platelet-derived SDF-1 in recruitment and proliferation of progenitor cells to inflamed endothelium. 73rd Annual meeting of the German Cardiac Society, Mannheim, Germany, April 12-14 2007

Stellos K, Langer H, Bigalke B, Daub K, Siegel-Axel D, Lindemann S, Gawaz M. Platelet-derived SDF-1 recruits and induces differentiation of human CD34⁺ progenitor cells to foam cells: a novel role in atherosclerosis. 73rd Annual meeting of the German Cardiac Society, Mannheim, Germany, April 12-14 2007

Stellos K, Bigalke B, Geisler T, Lindemann S, Gawaz M. Surface expression of SDF-1 is increased on circulating platelets in patients with acute coronary syndrome. 73rd Annual meeting of the German Cardiac Society, Mannheim, Germany, April 12-14 2007

Stellos K, Langer H, Bigalke B, Daub K, Schäfer I, Siegel-Axel D, Schumm M, May A, Lindemann S, Gawaz M. Platelet-derived SDF-1 regulates recruitment and differentiation of human CD34⁺ progenitor cells. 51st Annual meeting of the German Society of Thrombosis and Haemostasis Research, Dresden, Germany, February 21-24 2007

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Other Publications not being related to the dissertation

Original Articles

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Zissimopoulos A, Stellos K, Permenopoulou V, Petrakis G, Theodorakopoulos P, Baziotis N, Thalassinou N. [The importance of the tumor marker CYFRA 21-1 in patients with lung cancer after surgery or chemotherapy]. *Hell J Nucl Med.* 2007; 10(1):62-6

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10. Curriculum Vitae

PERSONAL INFORMATION

Name	Konstantinos Stellos
Nationality	Hellenic
Date; place of birth,	09.01.1981; Volos, Greece

EDUCATION AND TRAINING

1992	Primary School, N. Ionia, Volos, Greece
1998	Junior and Senior High School, N. Ionia, Volos, Greece
2005	Medical studies (M.D. degree), Medical School, Democritus University of Thrace, Alexandroupolis, Greece
2007	Dissertation (Dr. med. degree), Medical School, Department of Internal Medicine III, Eberhard-Karls University of Tuebingen, Tuebingen, German (dissertation advisor: Prof M. Gawaz).

ELECTIVES

August 2000	Neurology clinic, St. Luke's University Hospital, Malta
July 2001	Pathology Institute, University Hospital Essen, Germany
July 2004	Cardiology Clinic, Klinikum Rechts der Isar, Technical University of Munich, Germany

LAB WORK

1999 - 2002	Voluntary assistant; Medical Biology and Medical Genetics Laboratory of the Democritus University of Thrace (Principal Investigator: Prof. Dr. T. Lialiaris), Greece
2000 - 2002	Voluntary assistant; Histology-Embryology Laboratory of the Democritus University of Thrace (Principal Investigator: Prof. Dr. N. Papadopoulos), Greece
2004 - 2005	Voluntary assistant; Nuclear Medicine Department of the Democritus University of Thrace (Principal Investigator: Dr. A. Zissimopoulos), Greece
2005 - 2007	Graduate student; Experimental Cardiology Lab, Department of Internal Medicine III, University Hospital of Tuebingen, Eberhard-Karls University of Tuebingen (Principal Investigator: Prof. Dr. M. Gawaz), Germany
2007 -	Junior Investigator and Coordinator of the Research Team "Vascular and Regenerative Cardiology"; Department of Internal Medicine III, University Hospital of Tuebingen, Eberhard-Karls University of Tuebingen, Germany

PROFESSIONAL APPOINTMENT

September 2006 -	Resident Physician at the Department of Internal Medicine III, Medical Clinic, University Hospital of Tuebingen, Eberhard-Karls University of Tuebingen, Germany
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PERSONAL SKILLS AND COMPETENCES

LANGUAGES

Greek (mother tongue)
English (Certificate of Proficiency of the University of Cambridge, UK and the University of Michigan, USA)

German (Zeugnis Zentrale Mittelstufenprüfung, Goethe-Institut)
Spanish (Certificado Inicial de Español, La Ministra de Educación,
Cultura y Deporte del Reino de España)

NON FORMAL EDUCATION (INCL. ORGANIZATIONAL SKILLS)

- 2001 - 2003 Member of the Organising Committee of the 7th, 8th and 9th Greek Medical Students' Conferences of the Greek Scientific Association of Medical Students (Larissa 2001, Ioannina 2002, Athens 2003)
- 2001 - 2002 National Officer on Medical Education of the Hellenic Medical Students' International Committee - HelMSIC (www.helmsic.gr)
- 2002 - 2003 Regional Coordinator for Europe and Central Asia of the International Federation of Medical Students' Associations – IFMSA (www.ifmsa.org)
- Vice-President for Internal Affairs of the Executive Board of HelMSIC
- Editor-In-Chief of the national medical student's magazine "InterMEDICA"
- 2003 - 2004 Vice-President for External Affairs of the Executive Board of the IFMSA
- Member of the international preparatory team and coordinator of the report of the Study Session on "Health and Human Rights of Refugees, Immigrants and Minorities in Europe" organized by the Council of Europe and the IFMSA (8-14 December 2003; European Youth Center Budapest)
- International Coordinator of the Fundraising and Marketing Team of IFMSA
- 2003 – 2005 Peer educator of the IFMSA on project management, fundraising, human rights education, public relations and marketing
- 2003 - 2007 Adviser and peer-reviewer of "studentBMJ", published by the British Medical Journal (BMJ) Publishing Group Ltd (www.studentbmj.com)

TECHNICAL SKILLS

Statistical Programme SPSS v.15; Layout/ Design of Publications (Quark X-press, Photoshop), WindowsXP, Microsoft Office 2007

MEETINGS/CONFERENCES

- 1999 - 2007 Participation in many conferences; A list available upon request
- 2001 - 2004 6 General Assemblies and 5 Executive Board Meetings of the IFMSA
- 05/2004 57th World Health Congress of the World Health Organisation (WHO), Geneva, Switzerland
- 04/2005 Basic Life Support seminar, Alexandroupolis, Greece

AWARDS RECEIVED

- 1995-1998 Each year of High School awarded the Ministry of Education National Academic Achievement Award
- 2007 Poster prize on "Basic Research", 51st Annual meeting of the German Society of Thrombosis and Haemostasis Research, Dresden, Germany

