

Elisabeth Dernbach  
Voahanginirina Randriamboavonjy  
Ingrid Fleming  
Andreas M. Zeiher  
Stefanie Dimmeler  
Carmen Urbich

## Impaired interaction of platelets with endothelial progenitor cells in patients with cardiovascular risk factors

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E. Dernbach · A. M. Zeiher ·  
S. Dimmeler, PhD (✉) · C. Urbich  
Molecular Cardiology,  
Dept. of Internal Medicine III  
University of Frankfurt  
Theodor-Stern-Kai 7  
60590 Frankfurt, Germany  
Tel.: +49-69/6301-5158 or -7440  
Fax: +49-69/6301-7113  
E-Mail: dimmeler@em.uni-frankfurt.de

V. Randriamboavonjy · I. Fleming  
Vascular Signalling Group  
Dept. of Cardiovascular Physiology  
University of Frankfurt  
Frankfurt, Germany

■ **Abstract** *Objective* Recent studies indicate that platelets influence endothelial progenitor cell (EPC) recruitment to sites of vascular injury and promote their differentiation to an endothelial phenotype. Patients with cardiovascular risk factors (CVRF) demonstrate a reduced number and impaired function of EPC, as well as platelet hyper-reactivity. Therefore, we investigated the interaction of platelets and EPC from patients with CVRF. *Methods and results* Co-incubation of platelets and peripheral blood mononuclear cells, both from healthy volunteers, dose-dependently increased the number of adherent EPC. In contrast, patient-derived platelets failed to augment the number of adherent and migrating healthy and patient-derived EPC. However, co-incubation of platelets from healthy donors with mononuclear cells from patients with CVRF significantly enhanced the number of EPC, indicating that platelets from healthy volunteers are able to partially rescue the impairment of patient-derived EPC formation. Likewise, healthy donor-derived platelets augmented the impaired migration and clonal capacity of patient-derived EPC. Analysis of individual CVRF of platelet donors revealed that only diabetes mellitus inversely correlated with EPC number, colony formation and migration. The platelet supernatants from healthy volunteers that significantly increased EPC number contained IL-6, SDF-1, sCD40L and PDGF. While sCD40L and PDGF levels were comparable in platelet supernatants from healthy volunteers and patients with CVRF, the release of IL-6 and SDF-1 by patient-derived platelets was rather increased, thus, indicating that these soluble factors are not mediating the effect of platelet supernatants. *Conclusion* Healthy volunteer-derived platelets provide a source of soluble factors to improve the number and function of EPC from patients with cardiovascular risk factors, particularly diabetes mellitus.

■ **Key words** platelets – diabetes – endothelial progenitor cells – cardiovascular risk factors

### Introduction

The disruption of the endothelial function and integrity is fundamental for the initiation and pro-

gression of multiple cardiovascular diseases [26]. However, disease progression is modulated by a number of additional cell types and soluble factors including platelets and platelet-derived products [10, 13, 18]. Indeed, the initial response to endothelial cell

injury involved the adherence of activated platelets to the vascular surface, resulting in thrombus formation [12, 27]. At such sites of injury, platelets not only secrete potent chemokines and growth factors but they also expose ligands for adhesion receptors on their surface and may therefore represent potential mediators of progenitor cell homing [22].

Endothelial progenitor cells (EPC) are mobilized from the bone marrow in response to vascular injury or ischemia and contribute to accelerated re-endothelialization and neovascularization [1, 14, 19, 38]. Recent studies demonstrate that platelets stimulate the chemotaxis and migration of EPC via the P-selectin glycoprotein ligand-1 (PSGL-1) and  $\beta_1$ -integrin [21, 22] and recruit other progenitor cell populations such as CD34<sup>+</sup> and c-Kit<sup>+</sup>Sca-1<sup>+</sup>Lin<sup>-</sup> bone marrow-derived progenitor cells to the sites of vascular injury. It appears that the recruitment of CD34<sup>+</sup> progenitor cells is also mediated by PSGL-1 as well as by  $\beta_1$ - and  $\beta_2$ -integrins [4, 24]. Homing is not the only progenitor cell process that can be affected by platelets as the latter can also stimulate the differentiation of CD34<sup>+</sup> progenitor cells into mature foam cells and endothelial cells [7].

Diabetes mellitus is a major risk factor for vascular diseases and is associated with accelerated atherosclerosis and a high rate of arterial thrombotic complications. A number of studies support the concept that platelets contribute to the pathogenesis and progression of the vascular complications of diabetes [3]. Indeed, platelets obtained from patients with type I or type II diabetes are hyper-reactive and exhibit increased adhesiveness as well as exaggerated aggregation and thrombus generation [39]. Patients with cardiovascular risk factors (CVRF), particularly diabetes mellitus, have a reduced number of circulating EPC and those that are detectable demonstrate an impaired function [16, 37]. In addition, endothelial function correlates with the number of circulating EPC in patients with coronary artery disease [40]. However, how an alteration in platelet function can affect EPC number and function remains unclear. We therefore investigated the interactions between platelets and EPC derived from healthy volunteers and compared the effects with those observed using platelets and cells isolated from patients with diabetes and other cardiovascular risk factors.

## Methods

### ■ Study population and patient characteristics

Peripheral blood mononuclear cells (PBMC) and platelets were isolated from healthy human volunteers

and patients with several cardiovascular risk factors. Exclusion criteria were the presence evidence of malignant diseases. The ethics review board of the Hospital of the Johann Wolfgang Goethe University of Frankfurt, Germany, approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. An informed consent was obtained from each patient.

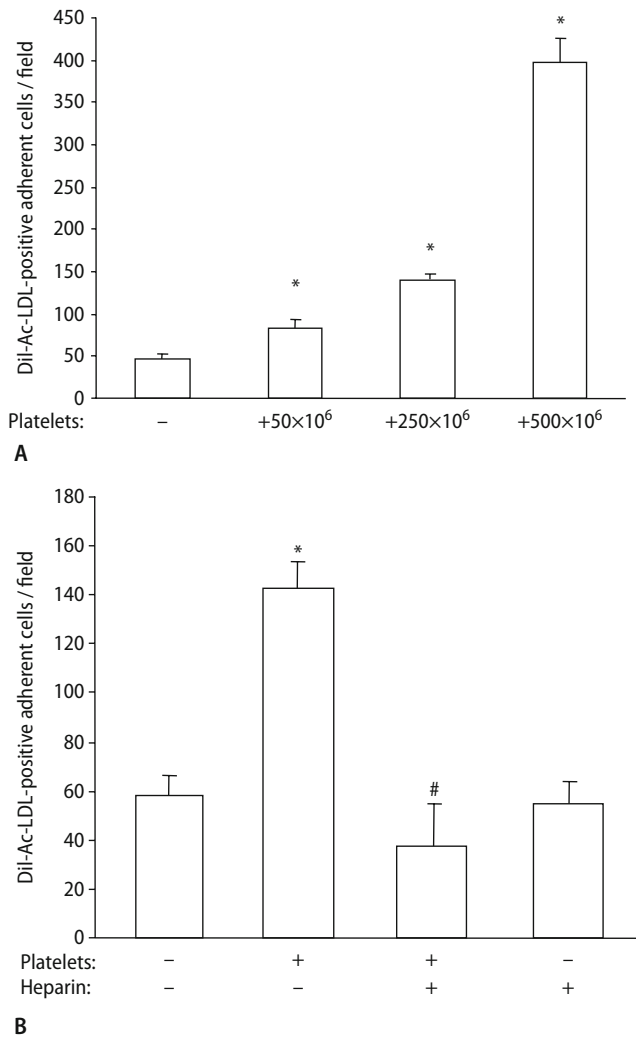
### ■ Human EPC culture

Mononuclear cells (MNC) were isolated from the peripheral blood of healthy volunteers and patients as described previously [9], and  $8 \times 10^6$  cells/ml were plated on 6 cm culture dishes coated with human fibronectin (Sigma) and maintained in endothelial basal medium (EBM; Cambrex) supplemented with EGM SingleQuots, and 20% FCS [9]. Cells were cultured for 3 days alone or together with platelets ( $250 \times 10^6$ /ml if not stated otherwise) freshly isolated from patients, healthy volunteers or platelet-concentrates from healthy donors (Blood Transfusion Center, Frankfurt, Germany).

### ■ Isolation of platelets and platelet supernatant

Peripheral venous blood (20–45 ml) was drawn from patients and healthy volunteers in heparinized tubes (NH<sub>4</sub>-Heparin, Sarstedt Monovette<sup>®</sup>, Nümbrecht-Rommelsdorf). After centrifugation for 20 min at 130g, platelet-rich plasma was removed, added to 400 U heparin/ml in a new tube and centrifuged for 7 min at 902g. After removal of supernatant, the resulting pellet was washed twice in phosphate buffered saline (PBS) containing 400 U heparin/ml and was finally resuspended in EBM without supplements. Platelets were activated by centrifugation (10 min at 10.000g). The activation of platelets by centrifugation was comparable to that achieved by thrombin (2 U/ml) after 10 min as measured by the release of sCD40L ( $804 \pm 25\%$  Vs.  $723 \pm 44\%$ ; non-activated platelets were set as 100%,  $n = 3$ ), platelet factor-4 ( $154 \pm 24\%$  Vs.  $148 \pm 21\%$ ,  $n = 3$ ), and  $\beta$ -thromboglobulin ( $125 \pm 1\%$  Vs.  $127 \pm 3\%$ ,  $n = 3$ ). Heparin was added to the wash buffer (PBS) in each platelet preparation; however, heparin was additionally added directly to the medium of the co-culture only in the experiments shown in Fig. 1b. Unless stated otherwise,  $250 \times 10^6$  platelets/ml were co-incubated with MNC at day 0 of culture.

For preparation of the platelet supernatant,  $250 \times 10^6$  platelets/ml were resuspended in PBS and activated by centrifugation (10 min at 10.000g). The supernatant thus obtained was used either for co-



**Fig. 1** Platelets increase the number of EPC. **a** MNC from healthy controls were co-cultivated with different doses of platelets derived from platelet concentrates of healthy controls for 3 days. Adherent EPC were stained with Dil-Ac-LDL and were counted (cells/field).  $n = 4$ ,  $*P < 0.05$  Vs. control. **b** MNC from healthy controls were co-cultivated with platelets derived from platelet concentrates of healthy controls for 3 days. Heparin (400 U/ml) was directly added to the co-culture medium. Adherent EPC were stained with Dil-Ac-LDL and were counted (cells/field).  $n = 4$ ,  $*P < 0.05$  Vs. control and  $#P < 0.05$  Vs. platelets

incubation with MNC at day 0 of culture or to measure secreted factors by ELISA.

### Assay of colony-forming units

After co-cultivation of MNC with platelets for 3 days, the adherent cells which we have previously identified as EPC, were washed twice with PBS and detached with trypsin. The EPC ( $5 \times 10^4$  cells) were seeded in methylcellulose (Methocult GF H4434; CellSystems)

with 100 ng/ml human recombinant VEGF. Colonies were monitored under phase-contrast microscopy, and were counted after 14 days of incubation. Colonies that contained a minimum of 50 cells were defined as endothelial cell colony-forming units (EC-CFU) as described previously [2, 33].

### Migration assay

After co-cultivation of MNC with platelets for 3 days or after cultivation of MNC alone for 3 days, adherent EPC were detached and  $2 \times 10^4$  EPC were resuspended in 250  $\mu$ l EBM containing 20% FCS, and seeded in the upper chamber of a modified Boyden chamber (6.5 mm, 8- $\mu$ m pore size; BD Falcon). The chamber was placed in a 24-well culture dish containing 500  $\mu$ l of EBM 20% FCS supplemented with either 100 ng/ml SDF-1 $\alpha$  or  $125 \times 10^6$  platelets. After 24 h at 37°C, migrated cells were stained with DAPI, fixed and counted in 4 fields per measurement.

### Cytokine assay

Platelet supernatants were isolated as described above. CD40L, IL-6, PDGF and SDF-1 concentrations were measured in platelet supernatants by enzyme-linked immunosorbent assays (ELISA, R&D Systems, Wiesbaden) according to the manufacturer's instructions.

### Statistical analysis

Results from at least three independent experiments are expressed as mean  $\pm$  SEM. For comparisons of multiple groups the statistical analysis was performed by ANOVA followed by post-hoc analysis with LSD adjustment. For comparison of two groups the Mann-Whitney U test was used. Linear regression analysis and bivariate correlation (Pearson correlation coefficient [ $r$ ]) was used to compare HbA1c with functional parameters of EPC (cell number, colony forming capacity and migration).  $P$  values  $< 0.05$  were considered statistically significant. All analyses were performed with SPSS 12.0 (SPSS Inc.).

## Results

### Platelets dose-dependently increased the number of adherent EPC

EPC were obtained from peripheral blood mononuclear cells by incubating MNC on fibronectin-coated

dishes with medium favoring endothelial differentiation for 3 days as previously described [9, 36]. After this period the adherent cells take up Dil-Ac-LDL, bind lectin and express a variety of endothelial marker proteins [9, 36]. To investigate the effect of platelets on EPC generation, platelets isolated from healthy volunteers were added to MNC also from healthy subjects. After 3 days platelets had dose-dependently increased the number of cells that stained positive for Dil-Ac-LDL (Fig. 1a). Although a significant increase of EPC number was observed using  $50 \times 10^6$  platelets/ml, the physiological concentration of  $250 \times 10^6$  platelets/ml was used in all further experiments. Moreover, platelets ( $250 \times 10^6$ /ml) increased the number of CD34<sup>+</sup>/VEGFR-2<sup>+</sup> cells (data not shown). To elucidate a potential mechanism by which platelets from healthy volunteers may increase EPC numbers, we added heparin to the co-culture medium. Heparin abolished the platelet-induced augmentation of EPC number (Fig. 1b).

#### ■ Platelets from healthy subjects improve impaired EPC formation and clonal capacity in MNC from patients with CVRF

Risk factors for coronary artery disease, like hypertension and diabetes mellitus, decrease the number and impair the function of EPC [37]. Having demonstrated that the addition of platelets increases the number of EPC, we investigated whether the addition of platelets from healthy volunteers rescues the impaired EPC formation from MNC from patients with CVRF. Indeed, platelets from healthy individuals significantly increased the number of Dil-Ac-LDL-positive adherent EPC derived from patients with CVRF after 3 days of culture (Fig. 2a). In contrast, platelets from patients failed to increase the number of healthy individual- or patient-derived EPC (Fig. 2a). The progenitor cell characteristic of EPC is evidenced by their capacity to form endothelial cell colonies [2, 33]. Therefore, we measured the number of EC-CFU generated from EPC with or without addition of platelets. Consistent with the results described above, the addition of platelets from healthy volunteers significantly increased the number of EC-CFU that could be cultivated from the MNC of patients, whereas the addition of patient-derived platelets did not increase the number of EC-CFU (Fig. 2b).

#### ■ Platelets from healthy individuals improve the impaired migration of EPC derived from patients with CVRF

To assess the influence of platelets on the migratory capacity of EPC, the latter were co-cultured with

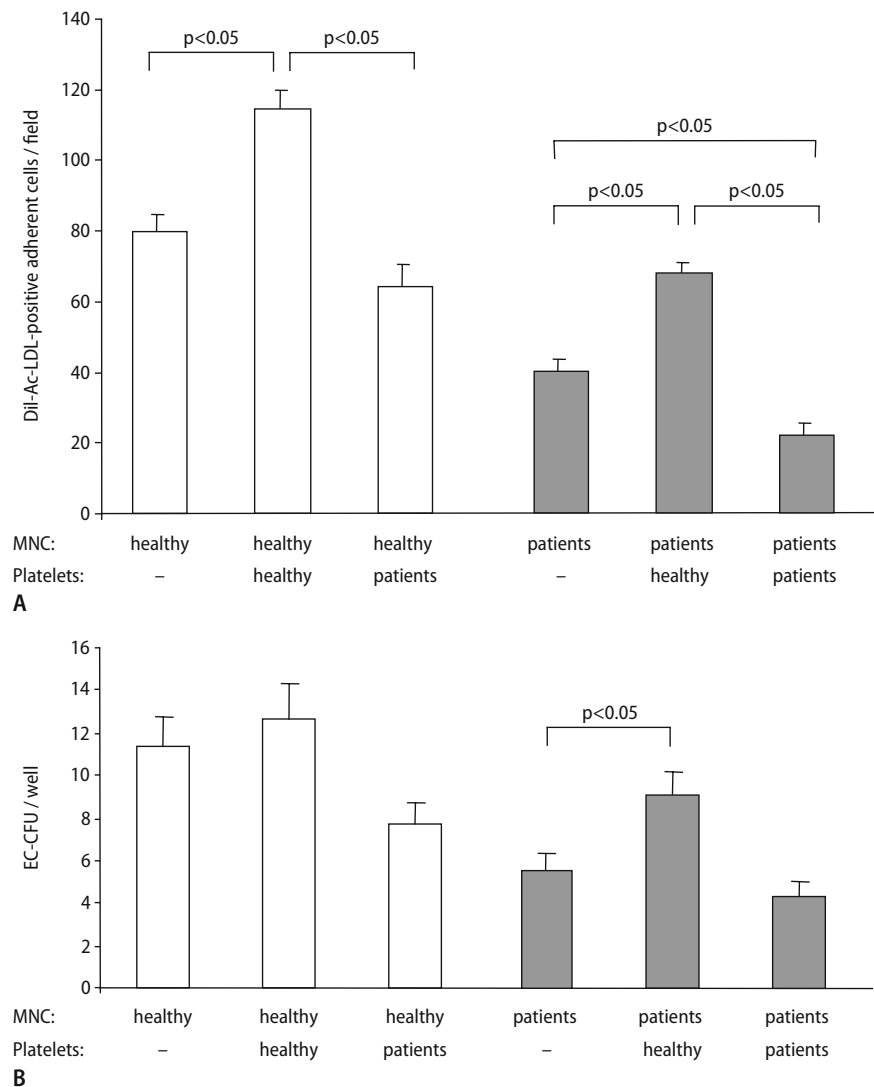
platelets from healthy individuals or from patients with CVRF. After 3 days, the adherent cells were harvested and seeded in the upper chamber of a modified Boyden chamber. After a further 24 h, the number of cells that had migrated to the lower chamber was counted. As shown in Fig. 3a, patient-derived EPC exhibited a markedly reduced migratory capacity compared to cells from the control group. The addition of platelets from healthy donors improved the migration of control and patient-derived EPC, whereas the co-incubation of patient-derived MNC with patient-derived platelets led to a further impairment of EPC migration (Fig. 3a).

We next compared the chemotactic effect of healthy or patient-derived platelets on EPC migration with that of the chemokine SDF-1 $\alpha$ . To this end, healthy volunteer- and patient-derived EPC were cultured for 3 days, seeded in a modified Boyden chamber and migration towards SDF-1 $\alpha$  or a suspension of healthy or patient-derived platelets was assessed. Platelets from healthy individuals significantly increased the migration of patient-derived EPC (Fig. 3b). The chemotactic effect of the normal platelets on the migration of patient-derived EPC was greater than that of the well established pro-migratory cytokine, SDF-1 $\alpha$  (Fig. 3b). In contrast, patient-derived platelets only increased the migration of healthy volunteer-derived EPC and failed to stimulate the migration of patient-derived cells (Fig. 3b). To elucidate which cardiovascular risk factor had an influence on platelet function, we made a sub-analysis of age, diabetes mellitus (Type I, II and cortisone-induced), hypertension, gender and hyperlipidemia. Interestingly, only HbA1c levels showed a strong correlation with cell number, colony forming capacity and migration indicating a link with diabetes (Fig. 3C-E). Other risk factors such as hypertension, hyperlipidemia and age had no significant effect (data not shown). The characteristics of platelet donors included in the sub-analysis are summarized in Table 1.

#### ■ Supernatants of platelets from patients with CVRF failed to augment EPC number

The activation of platelets results in the release of various cytokines, which might be able to affect EPC numbers in a paracrine manner. Therefore, we incubated MNC with the supernatant of platelets activated by centrifugation. Platelet supernatants from healthy volunteers significantly increased the number of the EPC, whereas supernatants from patients with CVRF were without effect (Fig. 4a). In order to identify a factor mediating the effect, we measured the concentrations of several cytokines known to be released

**Fig. 2** Platelets from healthy donors improve impaired EPC formation and clonal capacity. **a** MNC were co-cultured with freshly isolated platelets from healthy volunteers or patients with CVRF for 3 days. Adherent EPC were stained with Dil-Ac-LDL and were counted (cells/field),  $n = 12$ . **b** MNC were co-cultured with platelets for 3 days. Adherent EPC were then seeded in methylcellulose. After 14 days, EC-CFU were counted (colonies/well),  $n = 6$

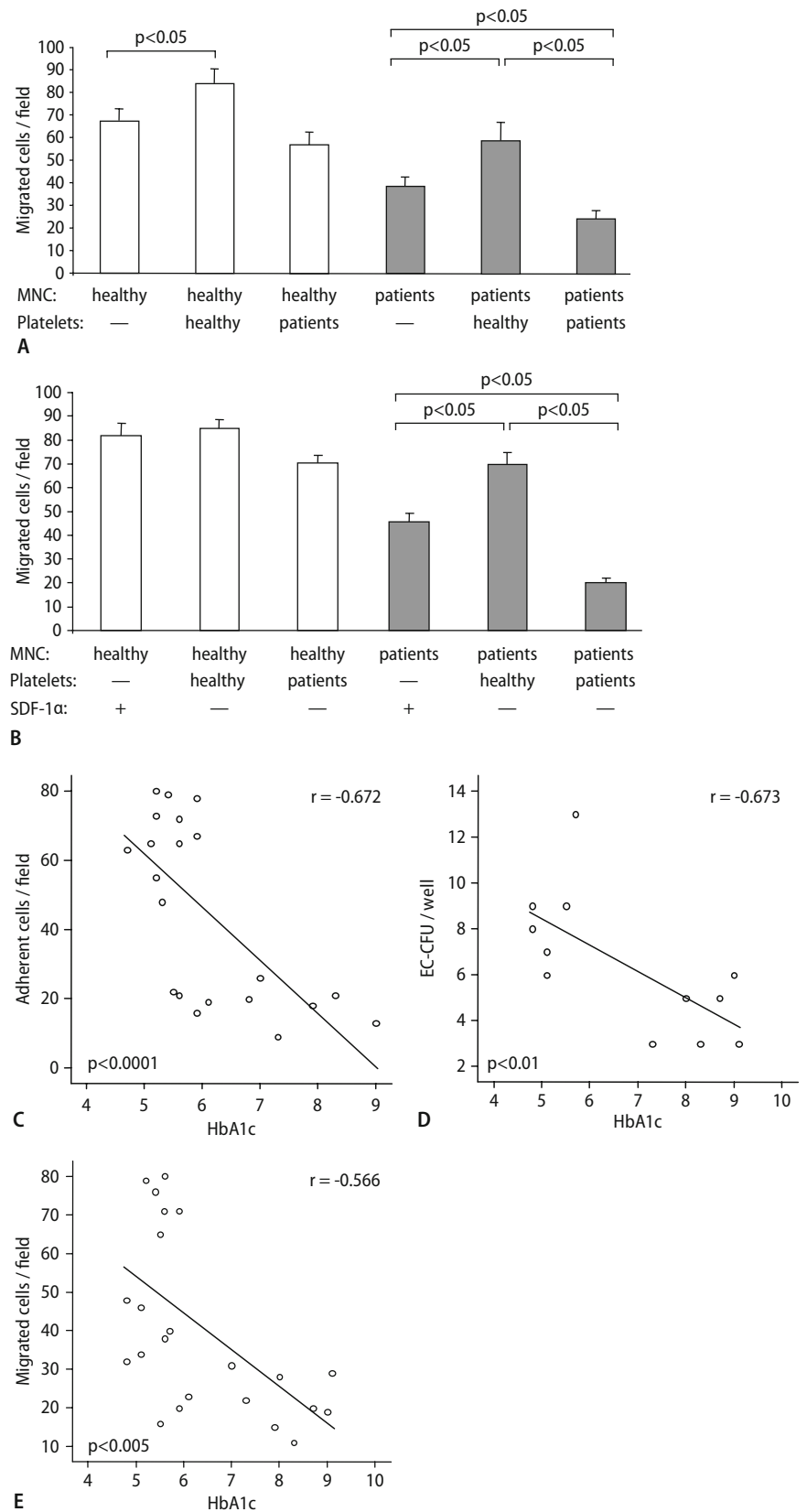


by platelets and to exert putative effects on EPC function.

Because SDF-1 is a platelet-derived factor involved in EPC recruitment, we first assayed the levels of SDF-1 in the supernatants derived from healthy volunteer- versus patient-derived platelets. Surprisingly, SDF-1 levels were even higher in platelet supernatants generated from the platelets of patients than from the healthy volunteers (Fig. 4b) indicating that the impairment of patient-derived platelets to augment EPC functions is unlikely to be mediated by SDF-1. Next, we measured the concentration of IL-6, which is known to be released from platelets and to induce the expression of the vascular endothelial growth factor (VEGF) and exert a pro-angiogenic effect on endothelial cells [5, 17]. However, IL-6 was also signifi-

cantly higher in supernatants from patients with CVRF compared to healthy donor-derived supernatants (Fig. 4c). In order to determine, whether or not IL-6 may exhibit an as yet undiscovered inhibitory effect on EPC, we added recombinant IL-6 to EPC cultures. However, consistent with the reported pro-angiogenic effects recombinant IL-6 increased eNOS expression and slightly elevated EPC numbers (data not shown) excluding its contribution to the inhibitory function of patient-derived platelets. Finally, PDGF- $\beta$  and sCD40L were detected in platelet supernatants. While sCD40L was slightly but non-significantly up-regulated, PDGF was not regulated (Fig. 4d, e) indicating that none of the cytokines measured can explain the inability of the supernatant derived from platelets of patients with CVRF to augment EPC number and function.

**Fig. 3** Platelets from healthy donors improve impaired EPC migration. **a** MNC were co-cultured with healthy or patient-derived platelets for 3 days. Adherent EPC were then seeded in a modified Boyden chamber for 24 h. Migration was determined in response to SDF-1 $\alpha$  (100 ng/ml). Migrated EPC were stained with DAPI and were counted (cells/field),  $n = 12$ . **b** MNC were cultured for 3 days. Adherent EPC were then seeded in a modified Boyden chamber. Migration was determined in response to SDF-1 $\alpha$  (100 ng/ml) or  $125 \times 10^6$  freshly isolated platelets from healthy controls or patients with CVRF. After 24 h, migrated cells were counted.  $n = 4$ . **c–e** The correlations of the HbA1c of platelet donors with EPC number, colony formation and migration are shown



**Table 1** Dernbach et al. Demographic and baseline characteristics of platelet donors

	Patients	Healthy controls	<i>P</i>
<i>N</i>	14	15	
Age	68 ± 14	42 ± 18	<0.05
Gender (m/f)	9/5	4/11	<0.05
Smoking	7	5	
Hypertension	13	1	<0.05
Hyperlipidemia	9	1	<0.05
CHD	11	0	<0.05
Diabetes mellitus	10	0	<0.05
HbA1c	7.2 ± 1.4	5.3 ± 0.4	<0.05
ASA	9	0	<0.05
Phenprocoumon	7	0	<0.05
Heparin	10	0	<0.05
Clopidogrel	5	0	<0.05
ACE-inhibitor	13	0	<0.05
β-blocker	13	0	<0.05
Statins	11	1	<0.05

Values are ±SD

CHD coronary heart disease, ASA acetyl salicylic acid, ACE angiotensin-converting enzyme

## Discussion

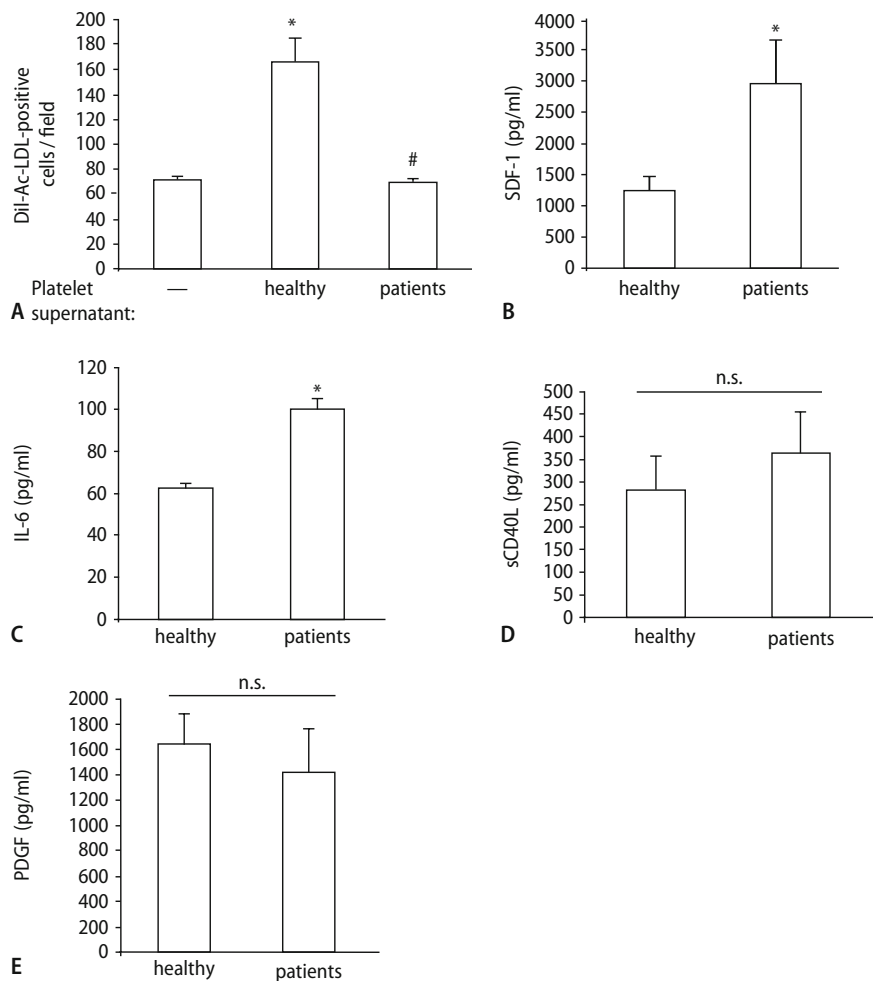
A number of recent studies have demonstrated an *in vitro* and *in vivo* interaction of EPC with platelets, leading to EPC homing and differentiation [4, 21, 24]. The present study indicates that platelet-induced EPC differentiation as well as EPC function is disturbed in patients with cardiovascular risk factors, in particular in patients with diabetes mellitus. Thus, it seems that the documented impairment of the functional activity of EPC in improving neovascularization in patients with coronary artery disease or diabetes [31, 37] also extends to a defective EPC-platelet interaction. Whereas platelets derived from healthy volunteers were able to rescue the reduction in the number and function of patient-derived EPC, platelets derived from CVRF patients actually further impaired EPC numbers and function, again highlighting the importance of platelets in regulating progenitor cell function.

To elucidate the potential underlying mechanism, we assessed the effects of supernatants from activated platelets. Indeed, the platelet-induced enhancement of EPC number and function (migration and colony forming ability) was mediated by factors released into the platelet supernatant. The molecular identity of the factor(s) that affected EPC number and function could not be identified. One of the most promising candidates; platelet-derived SDF-1 which is considered to be a critical regulator of progenitor cell homing to vascular injury areas [24], could be ruled out as the concentration of SDF-1 was actually higher in the supernatant of platelets derived from patients with CVRF than from the healthy individuals. The

latter finding of the increased expression of SDF-1 in platelets from patients with CVRF is consistent with a recent report assessing SDF-1 levels in peripheral blood and hearts of patients with manifest cardiovascular disease [32]. However, it is unclear why the increased SDF-1 level detected in the supernatant of patient-derived platelets was not able to induce EPC migration. It is tempting to speculate that the cleavage of SDF-1 by proteases such as matrix metalloproteinases or dipeptidyl peptidase IV, which is reported to result in the generation of an inactive or even toxic molecule may contribute to the present findings [8, 20, 25]. Indeed, MMP-2-dependent cleavage of SDF-1 yielded a neurotoxic remnant lacking part of the biological activities [41].

Likewise, levels of the pro-angiogenic cytokine IL-6 were significantly increased in the supernatants prepared from patient-derived platelets. IL-6 stimulated the endothelial commitment of stem cells and increased eNOS expression and EPC numbers (authors unpublished observation). However, the increased concentration of IL-6 in platelet supernatants did not promote EPC differentiation. In contrast, PDGF-β and CD40L concentrations were not significantly different in healthy volunteer- and patient-derived platelet supernatants. However, sCD40L levels were slightly increased in patient-derived platelets and thus this chemokine might indeed be a potential mediator of disturbed EPC-platelet interaction in patients. Plasma levels of soluble CD40L predict cardiovascular events in patients with acute coronary syndromes and in apparently healthy women [15, 29] and are elevated in diabetic patients [23, 35]. Conversely, reduced EPC numbers are associated with an increased risk of cardiovascular events [28], suggesting that enhanced levels of platelet-derived CD40L in patients with CVRF interfere with EPC number and function. Interestingly, platelets from diabetic patients exhibited higher intracellular CD40L than controls and higher thrombin-induced release of sCD40L, whereas constitutive and inducible surface expression of CD40L on platelets did not differ between diabetic patients and controls [34]. Thus, the modest increase of sCD40L in the supernatant of patient-derived platelets reported in our study may be related to differences in the measurement of released CD40L compared to intracellular CD40L. Moreover, the thrombin-induced release of sCD40L might be helpful for the analysis of patient-derived platelets. It would be also interesting to measure membrane-bound CD40L, which may be released in a microparticle-bound form upon platelet activation in healthy volunteers and patient-derived platelets. Indeed, platelet membrane CD40L is enhanced in patients with chronic heart failure [30] and in patients with unstable angina and myocardial infarction [11]. Thus,

**Fig. 4** Effect of platelet supernatant on EPC number. **a** Platelet supernatant was isolated as described and co-incubated (500  $\mu$ l/1 ml medium) with MNC for 3 days. Adherent EPC were stained with Dil-Ac-LDL and were counted (cells/field),  $n \geq 9$ ,  $*P < 0.05$  Vs. control,  $^{\#}P < 0.05$  Vs. healthy. **b–e** Levels of SDF-1, IL-6, sCD40L, and PDGF- $\beta$  were measured by enzyme-linked-assay in freshly isolated platelet-supernatants of healthy- or patient-derived platelets,  $n = 9$ ,  $*P < 0.05$



one could imagine a dysregulation of platelet membrane CD40L in the pathogenesis of diabetes. Finally, one may think about a potential influence of an platelet inhibitory medication on EPC number or function or platelet activation in patients with CVRF compared to healthy controls. However, aspirin or clopidogrel therapy did not correlate with EPC number in a sub-analysis (authors unpublished observations), thus excluding an impact of an “anti-platelet” therapy on EPC number and function. In addition, although the basal activation status of the patient-derived platelets studied was significantly lower than that of the platelets isolated from healthy donors, the relative increase of CD62P surface expression after thrombin-induced activation was higher in patient-derived platelets (data not shown). A recent study further implies that intake of aspirin did not significantly impair platelet function [6]. Thus, it seems unlikely, that a defective activation of patient-derived platelets mediates the impaired

interaction of platelets with EPC in patients with cardiovascular risk factors.

Taken together, the data obtained in the present study show that platelets derived from healthy volunteers are a source of soluble components that increase the number and improve the function of ex vivo cultivated EPC derived from patients with CVRF. The identification of the distinct factors released by platelets that mediate the effects observed may help to develop novel therapeutic options to improve the severe impairment of EPC function in patients with CVRF, in particular with diabetes mellitus.

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