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$CD34^+$ circulating cells display signs of immune activation in patients with acute coronary syndrome

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Abstract

Background

Bone marrow derived endothelial progenitor cells (EPC) are released into the peripheral blood in situations of vascular repair/angiogenesis. Regulation of vascular repair and angiogenesis by EPC depends not only on the number of circulating EPC but also on their functionality. As endothelial cells can act as antigen-presenting cells in coronary artery disease (CAD) we postulated that EPC can be immune-activated here as well.

Methods

$CD34^+$-EPC were isolated from peripheral blood of patients with ST-elevation myocardial infarction (STEMI, n=12), non-STEMI/unstable angina (UA, n=15), and stable CAD (SA, n=18). Expression of HLA-DR, adhesion and costimulatory molecules by isolated $CD34^+$-EPC were compared with levels in healthy controls (n=18).

Results

There were no significant differences in VCAM-1 and CD80 expression by peripheral circulating $CD34^+$-EPC between the four groups, yet expression of CD86 was highest in UA ($p<0.05$). ICAM-1 expression was lowest in SA ($p<0.01$). $CD34^+$-EPC constitutively expressed HLA-DR across all groups. Of note, patients pretreated with HMG-CoA reductase inhibitors exhibited lower expression of VCAM-1 by $CD34^+$-EPC throughout all patient groups; furthermore, statins significantly limited ex-vivo induced upregulation of ICAM-1 by TNF-alpha.

Conclusions

To the best of our knowledge this is the first study to examine expression of immune markers in peripheral circulating $CD34^+$-EPC ex vivo. We demonstrate that $CD34^+$-

EPC display different patterns of adhesion and costimulatory molecules in various states of CAD. Expression levels were affected by pretreatment with statins. Hence, immune activity of peripheral circulating $CD34^+$-cells might play a pathophysiologic role in evolution of CAD.

Key words

progenitor cells – atherosclerosis – immunogenicity - cytokines

Introduction

Endothelial cells (ECs) produce regulatory and counter-regulatory factors that control blood vessel flow, tone and the local thrombotic and immune state [1-8]. In the usual quiescent state EC turnover in months to years, but are prone to damage by immunologic, oxidative, and mechanical stressors [9, 10]. The disrupted or diseased artery no longer maintains flow and luminal patency. Endothelial denudation removes biochemical regulation of many vascular events, and signals the initiation and propagation of atherosclerosis [11].

Noteworthy, the endothelium has the ability to repair itself [12]. When a small area of the intima is removed experimentally, ECs at the edges of the lesion proliferate and migrate toward the center. If the endothelium is young and healthy, the local repair process is complete and the intimal layer is reconstituted. If the endothelium is older or receives the assaults of one or more risk factors, such as cholesterol, hypertension, or hyperglycemia, local repair is defective and an atherosclerotic plaque may develop [13]. It has become apparent that endothelial repair is driven not only by local cells but also by circulating endothelial progenitor cells (EPC) [14]. EPC derive from the bone marrow and can be mobilized to the peripheral circulation upon a variety of stimuli including tissue ischemia through release of growth factors [15]. Based on clinical observations a debate exists as to whether circulating EPC levels correlate positively or inversely with disease [16]. Higher levels of $CD34^+KDR^+$ EPC have been associated with decreased cardiovascular related risk of death [17]. Additionally, the percentage of apoptotic $CD34^+$ EPC is significantly increased in patients with acute coronary syndrome (ACS) as compared to healthy subjects and is associated with the extent of coronary stenosis by angiography [18].

Yet, to the best of our knowledge the current understanding of the functional characteristics of circulating EPC is limited, especially in patients affected by

cardiovascular diseases: risk prediction of EPC in ACS has thus far been associated with the number of EPC found at baseline of the index event or during the course of atherosclerotic disease.

Over the course of atherosclerotic lesion development ECs become injured and immune activated [19]. Immunogenicity of ECs is intertwined with quality of basement membrane contact [19-22], as liberation of vasculature-derived ECs from the extracellular matrix and changes in composition and spatial formation have been linked with expression of adhesion and costimulatory molecules, HLA-DR expression as well as thrombogenicity. These findings have been extended to epithelial cells and bone marrow residing ECs [23, 24]. We now aimed to characterize if mobilized EPC would display ‚classical' markers of endothelial immune activation at different clinical stages of atherosclerosis. Central to our work is the idea that EPC substratum interactions might well follow EC-substratum interactions and could explain the variable reports regarding EPC biology.

Material and Methods

Study patients

This investigation was performed with approval of the ethics committee of the Ludwig-Maximilians-University, Munich, Germany on research on humans. Written informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

45 patients admitted to our hospital with a diagnosis of coronary artery disease (CAD) and 18 healthy volunteers were included into the study. Healthy volunteers displayed no clinical signs of CAD and were without coronary risk factors. All of these control patients had normal electrocardiography and echocardiograms and no evidence of atherosclerosis by carotid artery sonography. Written informed consent was obtained from each patient and volunteer included in the study. Patients were grouped by presentation (Table 1).

A total of 18 patients had clinical evidence of Canadian Cardiovascular Society class II and III stable angina (SA) and at least one coronary artery stenosis detected at angiography (>50% diameter stenosis). Among these 18 patients 15 received HMG-CoA reductase inhibitors; two were on simvastatin 20 mg once daily (OD), six on simvastatin 40 mg OD, one on simvastatin 60 mg OD, three on atorvastatin 20 mg OD, one on atorvastatin 80 mg OD, and two on rosuvastatin 10 mg OD.

A total of 27 patients were admitted to the coronary care unit of the University Hospital Grosshadern, Munich, Germany, with a diagnosis of ACS and onset of ischemic chest pain at rest within the preceding 6 h. Fifteen of these patients fell into Braunwald class II or III, that is, exhibiting transient ST-segment depression and/or T-wave inversion but no evidence of myocardial infarction by ST-segment elevation or rise in CK or CK-myocardial band (unstable CAD). Among these 15 patients seven

received HMG-CoA reductase inhibitors; three were on simvastatin 20 mg OD, one each on simvastatin 40 mg OD, 60 mg OD, 80 mg OD, or on atorvastatin 20 mg OD. The remaining 12 patients with ACS had STEMI. Identification of STEMI and UH followed the consensus document of the American Heart Association and the American College of Cardiology [25]. Among these twelve patients seven received HMG-CoA reductase inhibitors; two were on simvastatin 20 mg OD, one on simvastatin 40 mg OD, three on atorvastatin 20 mg OD, and one on pravastatin 40 mg OD.

All patients underwent coronary angiography during hospitalization.

Exclusion criteria for this study included previous myocardial infarction within six months, admission more than 6 h from onset of symptoms, inflammatory conditions likely to be associated with an acute-phase response, autoimmune disease, and neoplastic disease. None of the included patients had advanced liver disease, renal failure, or valvular heart disease.

Blood samples (100 ml whole blood) were obtained from all patients with CAD directly after arterial puncture at the beginning of the catheter procedure and strictly before administration of drugs, contrast material, or performance of any percutaneous coronary intervention. Samples were mixed with 4µl sodium heparin and transferred into two 50ml Falcon tubes. Blood samples from healthy subjects were taken via antecubital venous puncture.

Isolation of $CD34^+$-cells

Isolation of mononuclear cells from heparinized blood was performed by Ficoll-Seperating Solution (Biochrom). After isolation, mononuclear cells were washed twice in phosphate-buffered saline (PBS) and re-suspended in PBS. $CD34^+$ ECs were isolated using Flow-Comp-Flexi Dynabeads (Invitrogen) after incubating with

anti-CD34-Mouse-Antibody (Invitrogen). Purity of CD34 isolation reached 97%±2,5% as demonstrated by immunofluorescence staining (FITC-labeled anti-CD34, negative control FITC Mouse IgG_1, κ Isotype Control, both BD Biosciences), of these 90% displayed expression of CD31 (PE-labeled anti-CD31, negative control PE Mouse IgG_1, κ Isotype Control, both BD Biosciences; data not shown).

Isolated $CD34^+$ cells were either left untreated or incubated with 10ng/ml tumor necrosis factor (TNF)-α or interferon (IFN)-γ in EC growth medium (PromoCell) at 37°C for four hours. Both cytokines have been demonstrated to induce expression of costimulatory and adhesion molecules by $CD34^+$-cells [26-29]. Cell viability in cytokine treated cells was analyzed using trypan blue (Thermo Scientific, Pierce Biotechnology, Illinois, USA) following the manufacturer's directions. Viability was >95% in all conditions studied (data not shown).

mRNA isolation, and semiquantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted CD34 cells using Trizol methods (Invitrogen Life Technologies, Carlsbad, California, USA) as described by the manufacturer. RNA concentration and purity were determined spectrophotometrically by measuring fluorescence at 260 nm and 280 nm. RNA (1 µg) was reverse-transcribed to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA), also according to the manufacturer's instructions. The synthesized cDNA as per the manufacturer's instructions was amplified using mRNA primers as shown in table 2. The relative quantitative method was used to detect expression of CD80, CD86, intercellular cell adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, human leukocyte antigen (HLA)-DR, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using SYBR green in an

iCycler IQ multicolor real-time detection system (Bio-Rad, Hercules, California, USA). Amplifications and the thermal-cycle parameters followed the manufacturer's directions. The delta-delta method (i.e., comparative ct [Dct] method) for quantitative analysis of gene expression was used [30]. The results were normalized to those for GAPDH in the same sample and were expressed as fold increase. Were assessed gene expressions were determined based on results of stimulated cells as a fold change compared with basal levels in non-cytokine treated cells.

ELISA (enzyme linked immunosorbent assay)

To evaluate the concentration of TNF-α (in pg/ml) in the serum of control and patient groups an ELISA (R&D Systems) following the manufacturer's instructions.

Statistical analysis

Kolmogorov-Smirnov-test was used to determine whether values followed a normal distribution. As the values for CD86 (SA), ICAM-1 (UA, stimulated), ICAM (SA, increase) and CD80 (UA) did not follow a normal distribution, comparisons between those groups were conducted using the Mann-Whitney-U-Test for unconnected and the Wilcoxon-Test for connected values. For the normal distributed values the t-test was used. If more than two groups had to be compared, the ANOVA-Test was used for normal distributed values and the Median-Test for not normal distributed values. Results are given as arithmetic mean with standard deviation (SD). The program SPSS was used for analysis and all calculations. P-values < 0.05 were considered statistically significant.

Results

Characteristics of Patients

Demographic and clinical data for patients in the 3 groups and healthy controls are summarized in Table 1. There were no significant differences in white blood cell count, C-reactive protein, use of ACE inhibitors/sartans and of acetylsalicylic acid. Use of oral anti diabetics was similar in SA and STEMI patients and significantly higher than in UA patients. Frequencies of patients on 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and ß-blockers were significantly higher in SA patients than in the other two patient groups. All of the UA and STEMI patients were treated with intravenous heparin, acetylsalicylic acid, and morphine.

Assessment of adhesion molecule mRNA expression by $CD34^+$ cells

mRNA Expression of ICAM-1 in peripheral circulating $CD34^+$ cells was significantly lower in SA patients than in controls (SA: 4.35±0.42 vs. CO: 5.29±0.53, $p<0.05$) whereas there were no significant differences when compared to the other groups (UA: 4.75±0.87, STEMI: 5.51±1.21; Fig. 1).

Upon ex-vivo TNF-α stimulation mRNA ICAM-1 expression significantly increased in controls (2.8-fold; $p<0.05$), SA (5.1-fold; $p<0.01$) and UA patients (2.6-fold; $p<0.01$) whereas TNF-α treatment did not result in a statistical significant enhancement of mRNA ICAM-1 expression in STEMI patients (2-fold; $p=0.51$). Overall, extent of increase in expression did not differ between the four groups ($p=0.35$) and resulting mRNA ICAM-1 expression in peripheral circulating $CD34^+$ cells upon cytokine stimulation was similar between the four groups (CO: 6.06±0.64, SA 6.07±0.75, UA 6.04±0.71, STEMI 5.98±0.23; $p=0.45$; data not shown).

There was no significant difference between the three patient groups and controls when VCAM-1 mRNA expression was analyzed in $CD34^+$ cells isolated from

circulating blood (CO: 2.38±1.19; SA 1.78±1.18; UA 2.23±1.53; STEMI 2.12±1.43; p=0,63; data not shown).

TNF-α stimulation of isolated $CD34^+$ cells significantly increased VCAM mRNA expression by $CD34^+$ cells in all groups ($p<0.01$ in all groups). Yet, cytokine-induced levels of VCAM-1 mRNA expression did not differ between the four groups (p=0,926). IFN-γ had no effect on mRNA expression of both adhesion molecules by $CD34^+$ cells ex vivo.

Assessment of costimulatory molecule mRNA expression by $CD34^+$ cells

Transcript levels for CD86 were significantly higher in UA (4.82±0.42; $p<0,05$) than in the other three groups (control 4.14±0.27; SA 4.14±0.62; STEMI 4.03±0.48; Fig. 2). Yet, there was no significant difference between the three patient groups and controls when CD80 mRNA expression was analyzed in $CD34^+$ cells isolated from circulating blood (CO: 1.43±0.65; SA 1.45±0.90; UA 1.22±0.90; STEMI 1.29±0.82; p=0,926; data not shown).

Incubation with cytokines (TNF-α or IFN-γ) failed to induce mRNA expression of CD80 and CD86 in $CD34^+$ cells in each group studied.

Assessment of HLA-DR mRNA expression by $CD34^+$ cells

Expression of HLA-DR by $CD34^+$ cells was similar between the four groups. Interestingly mRNA HLA-DR expression was on a high level and not different from that of our chosen housekeeping gene GAPDH. Thus, ex vivo HLA-DR could not be induced by TNF-α or IFN-γ in all groups studied.

Circulating serum TNFα levels

Circulating serum TNF-α levels were significantly lower in controls than in patients with SA (8.56±0.92 vs. 10.66±1.95 pg/ml; $p<0.05$). Patients with UA exhibited significantly higher levels of circulating TNF-α than controls and the SA group (17.66±2.54 pg/ml; $p<0.01$), whereas patients with STEMI displayed non-significant increased serum TNF-α levels when compared to serum levels in controls or SA patients (12.81±2.25 pg/ml; $p<0.05$ vs. UA patients; Fig. 3).

There was no significant correlation between TNF-α serum levels and extent of ex vivo cytokine-mediated changes in expression of ICAM-1 ($p=0,45$) or VCAM-1 ($p=0,11$) mRNA by $CD34^+$ cells (data not shown). Inter-assay coefficient of variability was 5,2%, intra-assay coefficient of variability was 2,4%.

Effect of HMG-CoA reductase inhibitor intake on mRNA expression in $CD34^+$ cells

64% of the patients included in this analysis were treated with a HMG-CoA reductase inhibitor – the majority with simvastatin (59%), followed by atorvastatin (28%), rosuvastatin (7%) and one patient treated with pravastatin. There was no significant difference in ICAM-1 mRNA expression by $CD34^+$ cells between patients throughout all groups treated with or without HMG-CoA reductase inhibitors (4.27±0.44 with vs. 4.62±0.36 without statin; $p=0,34$; data not shown).

In contrast, statin intake reduced the ex vivo effect of TNF-α to upregulate ICAM-1 mRNA expression by $CD34^+$ cells ($p<0,05$ vs. TNF-α-induced ICAM-1-expression in patients without statins). VCAM-1 expression by isolated $CD34^+$ cells was significantly lower in patients with UA when treated with HMG-CoA reductase inhibitors (2.04±0.98 without vs. 1.34±0.61 with statins; $p<0,05$). This effect was also apparent ex vivo when $CD34^+$ cells were stimulated with TNF-α. There was no

significant difference in patients with SA or STEMI in VCAM-1 expression by naïve $CD34^+$ cells or cytokine-induced $CD34^+$ cells with respect to statin intake (Fig. 4). With respect to the different dosage of HMG-CoA reductase inhibitors and therefore the small number in each treatment group we only observed tendencies towards greater efficacy of atorvastatin and rosuvastatin when compared to simvastatin or pravastatin influencing expression levels of VCAM-1 and TNF-α induced upregulation of ICAM-1 by $CD34^+$ cells.

Treatment with HMG-CoA reductase inhibitors had no influence on level of CD80 or CD86 mRNA expression by $CD34^+$ cells in neither patient group.

Effect of oral diabetic medications on mRNA expression in $CD34^+$ cells

About fifty percent of SA and STEMI patients and twenty percent with UA were on oral diabetic medications (metformin n=16, sitagliptin n=7, glimepiride n=2, acarbose n=1; Table 1). No significant differences in expression of costimulatory and adhesion molecules by naïve $CD34^+$ cells could be observed between patients with and without diabetic medications throughout the three patient groups. However, treatment with metformin resulted in a non-significant tendency towards reduced levels of induction of ICAM-1 (p=0,09) and VCAM-1 expression (p=0.08) by TNF-α treated $CD34^+$ cells in STEMI but not in SA patients.

Discussion

CD34 is widely regarded as a marker of vascular EPC [31, 32]. These bone marrow-derived cells are found circulating in peripheral blood [33] and have been shown to be important for vessel repair after injury or in atherosclerosis. Cardiovascular risk factors are significantly associated with reduced numbers and impaired functionality of $CD34^+$-EPC [34-37]. An early decline of EPC levels or failure to mobilize EPC from the bone marrow have been described in ACS patients, which in turn is associated with increased mortality [38-40]. Mourino-Alvarez et al. demonstrated differences in proteins implicating coagulation and blood clotting, e.g. expression of coagulation factor XIII A1, fibrinogens, plasminogen or thrombospondin 1 between circulating ECs and EPC in healthy controls and patients with ACS using a large proteomic characterization [41]. Some of these proteins and pathways have been previously identified in plasma from patients with atherosclerosis, aortic stenosis and ACS [42, 43].

As we have seen altered immunogenicity of ECs when deprived from their physiologic cell matrix interaction [21, 22, 44-46] we now aimed to explore the immune phenotype of peripheral circulating $CD34^+$ cells in CAD.

To the best of our knowledge studies on EPC have been conducted on ex-vivo cultivated EPC or EPC mobilized from bone marrow by exogenous cytokine treatment. Thus, these cells might not represent in vivo circulating EPC. Our study is novel as we analyzed freshly isolated circulating $CD34^+$ cells without further ex vivo cultivation. Based on the low resulting count of isolated cells we refrained from further subdividing in different CD34 cellular subsets, however staining revealed that approximately 90% of our isolated cells were $CD34^+/CD31^+$.

Since statins enhance the number and repair function of circulating EPC [47, 48] we

also aimed to characterize if existing statin therapy would influence mRNA expression by EPC in different patient subsets. We furthermore analyzed the ex vivo effect of proinflammatory cytokines (TNF-α, IFN-ɣ) on mRNA expression of HLA-DR, CD80, CD86, ICAM-1 und VCAM-1 by EPC since these cytokines have been demonstrated to modulate markers of endothelial immunogenicity in vivo and ex vivo [49].

We now demonstrate that circulating $CD34^+$ cells indeed express mRNA for CD80 and CD86. The CD28/CD80/CD86-signaling pathway is a pivotal costimulatory pathway for T cell activation by antigen presenting cells [50-52]. Interestingly we found CD86 to be significantly stronger expressed by $CD34^+$ cells in patients with UA than in controls and patients presenting with SA or STEMI. Yet, we could not find a significant difference in CD80 mRNA-expression between the four groups studied. Expression of CD86 by $CD34^+$ cells exceeded that of CD80 in each of the groups studied. Levels of expression of both costimulatory molecules could not be enhanced by stimulation with proinflammatory cytokines (TNF-α or IFN-ɣ). This finding is in marked contrast to mature $CD31^+$ ECs in which expression of costimulatory molecules is enhanced by cytokines [51, 52].

Furthermore, $CD34^+$ cells express high levels of HLA-DR mRNA without significant differences between the four groups studied. HLA-DR is a MHC class II cell surface receptor presenting antigens to effector immune cells; in concert with expression of costimulatory molecules this triggers initiation of an effector immune response. It has been demonstrated that mature $CD31^+$ ECs express HLA-DR depending on the vessel size diameter [53]. Ex vivo cultivated ECs lose their ability to express HLA-DR but can be induced to express HLA-DR by IFN-ɣ [53]. We failed to further induce expression by ex vivo stimulation with IFN-ɣ.

We next explored expression of adhesion molecules by $CD34^+$ cells. ICAM-1 plays an essential role in mediating recruitment of EPC to ischemic tissue [54]. ICAM-1 is expressed by activated ECs thereby mainly recruiting leukocytes into the vessel wall; furthermore, circulating EPC are also attracted to ECs via binding of endothelial ICAM-1 to CD18 expressed by EPC [55, 56].

We now demonstrate that EPC also express ICAM-1 on mRNA level. Our data might further indicate that ICAM-1 expression on circulating $CD34^+$ cells can be induced by TNF-α. Enhanced expression levels might associate with increased surface protein expression and it is to speculate that activated and incorporated EPC within the vessel wall might contribute to the adherence and diapedesis of circulating immune cells [57], thereby further supporting the immune process within the diseased vessel wall. Lowest expression levels of ICAM-1 mRNA could be observed in patients with SA, highest expression level in patients with STEMI with only significant differences between control group and patients with SA. TNF-α stimulation resulted in significantly increased expression of ICAM-1 mRNA in all groups but STEMI patients. Interestingly, upon stimulation with TNF-α no differences in mRNA ICAM-1 expression between the four groups could be detected and no correlation could be observed between extent of mRNA ICAM-1 upregulation and TNF-α serum concentrations within the four groups studied. Based on these results one might extract that the highest TNF-α serum concentration found in UA patients does result in the smallest ex-vivo cytokine-induced upregulation of ICAM-1-expression. It therefore seems conceivable that activation of EPC in UA patients with respect to the markers studied herein might already have reached a peak level, potentially due to the increased concentration of circulating proinflammatory cytokines in states of advanced coronary artery disease.

We further explored VCAM-1 mRNA expression by circulating $CD34^+$ cells. Results

from animal models and human specimens indicated that ECs within atherosclerotic lesions express VCAM-1 [58, 59] resulting in enhanced migration and adherence of T lymphocytes and macrophages [59]. We are now able to demonstrate that circulating $CD34^+$ cells also express VCAM-1 mRNA. Yet, no significant difference in VCAM-1 mRNA expression could be detected between the four groups. Ex-vivo stimulation of $CD34^+$ cells with TNF-α resulted in a significant increase of VCAM-1 mRNA expression in all groups. As with ICAM-1 no correlation could be observed between extent of mRNA VCAM-1 upregulation and TNF-α serum concentrations throughout the groups but there was a tendency towards higher upregulation upon increased serum TNF-α concentration, indicating a certain degree of EPC activation in this cytokine milieu. Overall mRNA expression levels of ICAM-1 exceeded mRNA expression levels of VCAM-1 in all groups.

Earlier studies have demonstrated that HMG-CoA reductase inhibitors augment liberation $CD34^+$-cells from the bone marrow in the peripheral circulation [48, 60]. We are now able to demonstrate that a preexisting therapy with HMG-CoA reductase inhibitors results in lower gene expression of ICAM-1 and VCAM-1 by $CD34^+$-cells. This effect might contribute to the pleiotropic effects of HMG-CoA reductase inhibitors [61] since less expression of adhesion molecules might associate with reduced diapedesis of circulating immune cells from the blood stream into the vessel wall. As one possible mechanistic link, HMG-CoA reductase inhibitor intake has been demonstrated to result in reduced TNF-α serum concentration [61-63]. This effect might translate in less cytokine-induced expression of adhesion molecule expression. This might also explain why we observed a tendency towards increased level of upregulation in mRNA expression of adhesion molecules when $CD34^+$-cells were treated with TNF-α ex vivo in patients pretreated with HMG-CoA reductase inhibitors. Further analysis is warranted to explore if different HMG-CoA reductase inhibitors

differ in their efficacy to influence the pro-inflammatory phenotype of circulating $CD34^+$-cells as we observed tendencies towards more efficacy for ator- and rosuvastatin.

Results presented here within indicate that peripheral circulating $CD34^+$-cells express markers of immune activation on mRNA level in a proinflammatory milieu such as CAD. Expression of adhesion molecules could be further induced by TNF-α. Satoh described an increase in oxidative damage in EPC obtained from patients with CAD when compared to EPC isolated from healthy controls. This was even higher in patients suffering from acute myocardial infarction and under influence of a metabolic syndrome – also indicating an effect of environmental stress on EPC as well as differences within the heterogeneous group of patients affected by CAD [64].
Schwartzenberg et al. described an increased percentage of apoptotic $CD34^+$-cells in patients with ACS as compared to healthy subjects [18]. This associated with the extent of coronary stenosis by angiography. They concluded that functional impairment of progenitor cells through enhanced apoptosis may underlie atherogenesis and cardiovascular events, while improving survival seems to be vital for neovascularization and arterial injury.

Limitations of our study are the overall small sample size. Furthermore, there is still a great controversy on exact definition of EPC and the heterogeneous techniques employed to isolate them (cell cultures, magnetic immunobeads, flow cytometry, etc.) [65]. Such heterogeneity in methods and definition has contributed to obtaining contradictory results between laboratories, increasing the controversy [65]. In fact, some studies have shown that decreasing numbers of EPC are associated to

cardiovascular risk factors and poor outcomes [35, 66, 67], whereas other studies observe increased numbers of EPC in patients with CAD [68].

We aimed to analyze cells ex vivo without ex vivo culturing. We therefore used CD34 as lone isolation marker being aware that the surface marker profile of progenitor cells changes during the process of mobilization and maturation with $CD34^+$-cells to form a more generic population of 'early' progenitor cells [69, 70]. The small number of isolated cells did not allow for co-culture or functional assays to further support our notion of immune activity of the isolated cells.

Another limitation of our study is the age difference between control subjects and patients. Since atherosclerotic lesion development evolves with age we chose a young group of individuals without clinical, functional and morphologic signs of atherosclerosis. On the contrary age seems to have a decremental effect on functionality of $CD34^+$-cells [71, 72]; yet we still observed higher expression levels of immune molecules in our patient groups when compared to the younger control group further underlining the importance of our findings.

To the best of our knowledge this is the first study to examine expression of immune markers in peripheral circulating $CD34^+$-EPC ex vivo. We demonstrate that $CD34^+$-EPC display different patterns of adhesion and costimulatory molecules in various states of CAD. Expression levels were affected by pretreatment with statins. Although the role of EPC in vessel repair in disease is not totally understood our data might adjoin other findings that the activation state of these cells is critical for the vessel repair process.

Since they were discovered, EPC have been considered a promising therapeutic tool to recover ischemic tissue in regenerative medicine. However, it will be necessary to extend our understanding on EPC biology to reconcile findings and to develop new strategies for forthcoming therapies. It will also be of interest to explore if

immunogenicity of peripheral circulating $CD34^{+}$-EPC is being influenced by drug eluting stents.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Figures

Figure 1. ICAM-1 mRNA expression by peripheral circulating CD34^{+}-cells in patients with different stages of coronary artery disease compared to healthy controls

CO: healthy controls, SA: stable coronary artery disease, UA: non-ST-elevation myocardial infarction, STEMI: ST-elevation myocardial infarction
*p<0,05 SA vs. CO

Figure 2. CD86 mRNA expression by peripheral circulating CD34^{+}-cells in patients with different stages of coronary artery disease compared to healthy controls

CO: healthy controls, SA: stable coronary artery disease, UA: non-ST-elevation myocardial infarction, STEMI: ST-elevation myocardial infarction
*p<0,05 UA vs. CO, SA and STEMI

Figure 3. Circulating serum TNFα levels in patients with different stages of coronary artery disease compared to healthy controls

CO: healthy controls, SA: stable coronary artery disease, UA: non-ST-elevation myocardial infarction, STEMI: ST-elevation myocardial infarction
*p<0,05 CO vs. SA
$^{\#}$p<0,01 UA vs. Co and SA
§p<0,05 STEMI vs. UA

Figure 4. HMG-CoA reductase inhibitors modulate VCAM-1 mRNA expression by peripheral circulating CD34^{+}-cells in patients presenting with UA

SA: stable coronary artery disease, UA: non-ST-elevation myocardial infarction, STEMI: ST-elevation myocardial infarction
*p<0,05 vs. UA without HMG-CoA reductase inhibitor

Tables

Table 1. Demographic characteristics and biological parameters of patients and control subjects

	control n=18	SA n=18	UA n=15	STEMI n=12
Age, yrs	36±20	75±6	72±9	66±19
Male (%)	9 (50)	15 (83)	12 (80)	9 (75)
Risk factors				
Hypertension (%)	-	18 (100)	12 (80)	9 (75)
Smoking (%)	-	4 (22)	5 (33)	6 (50)
Cholesterol (mg/dl)	173±17	189±21	189±17	213±38
LDL-chol. (mg/dl)	122±28	137±39	131±19	131±27
HDL-chol. (mg/dl)	45±3	41±7	39±6	39±13
Glucose (mg/dl)	97±11	118±6	124±23	138±28
Laboratory parameters on admission				
CRP (mg/dl)	0.1 (0-0.3)	0.2 (0-0.5)	1.1 (0.3-3.9)	0.8 (0.2-5.1)
White blood count (U/l)	6.8±0.4	7.4±0.6	7.1±1.0	7.4±1.9
Lymphocytes (%)	31±4.3	39±3.7	35±4.3	38±2.8
CK (U/l)	32 (17-54)	36 (18-63)	39 (12-68)	947 (297-1524)*
Troponin I ng/ml	n.d.	n.d.	6.9 (0.9-10.5)	102 (43-178)†
Extension of coronary artery disease				
no. of diseased vessels	-	1.4±0.3	1.3±0.4	1.8±0.6
% diameter stenosis	-	69±18	68±21	89±19
Medication on admission				
ß-Blockers	-	14 (78)†	6 (40)	5 (42)
Aspirin	-	15 (83)	10 (67)	7 (58)
ACE inhibitors/sartans	-	12 (67)	9 (60)	9 (75)
Lipid-lowering drugs	-	15 (83)†	7 (47)	7 (58)
Insulin	-	1 (6)	1 (7)	1 (8)
Oral antidiabetics	-	9 (50)§	3 (20)&	7 (58)#

Values are expressed as mean±SD, median (range), or number (percentage)
LDL-chol. low-density lipoprotein cholesterol; HDL-chol. high-density lipoprotein cholesterol
n.d. not detectable; *p<0.01; †p<0.05 UA vs. STEMI
§metformin (n=4), sitagliptin and metformin (n=3), glimepiride (n=2); &sitagliptin and metformin (n=2), metformin (n=1); #metformin (n=4), sitagliptin and metformin (n=2), acarbose (n=1)

Table 2. DNA-sequences und specific annealing-temperatures of each primer

GAPDH
60°C
forward: 5‘-CGC TGA GTA CGT CGT GGA GTC-3‘
reverse: 5‘-GCA GGA GGC ATT GCT GAT GA-3‘

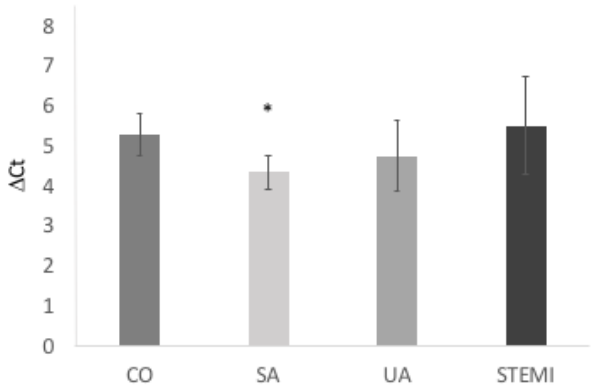
CD80
60°C
forward: 5‘-TAA GGT AAT GGC ACC ACA GCT TC-3‘
reverse: 5‘-TTG TGA TAT GCT GCC TGA CC-3‘

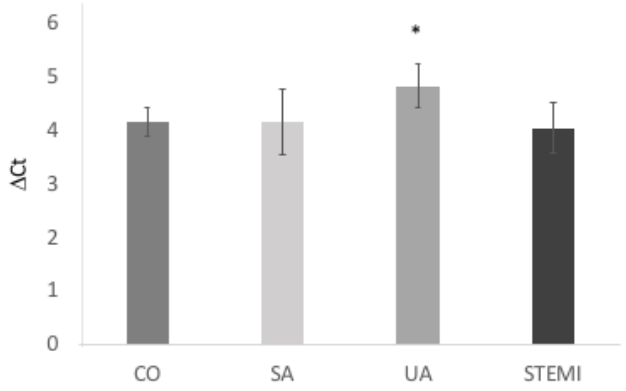
CD86
60°C
forward: 5‘-GCT GCA ACG GAA TTA GGA AG-3‘
reverse: 5‘-TTT CCT CTG GTT GCC TTG AG-3‘

ICAM-1
60°C
forward: 5‘-GGC TGG AGC TGT TTG AGA AC-3‘
reverse: 5‘-TCA CAC TGA CTG AGG CCT TG-3‘

VCAM-1
60°C
forward: 5‘-TAA ATT GCC TGG GAA GAT GG-3‘
reverse: 5‘- GGT GCT GCA AGT CAA TGA GA-3’

HLA-DR
60°C
forward: 5’-AGA CAA GTT CAC CCC ACC AG-3’
reverse: 5‘-AGC ATC AAA CTC CCA GTG CT-3‘

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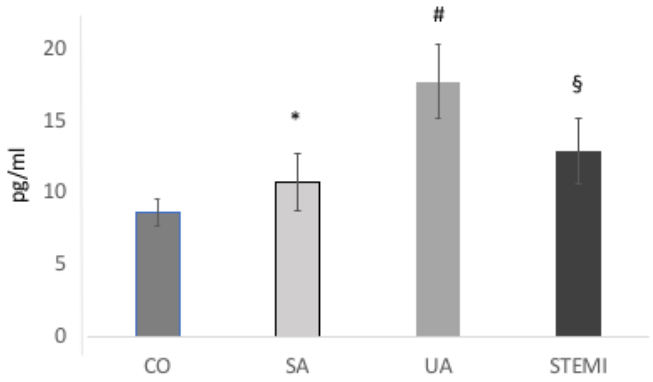
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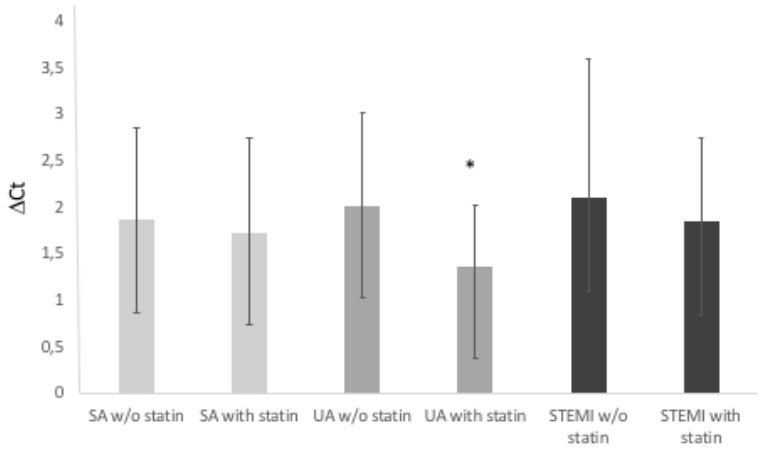
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SA w/o statin
SA with statin
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STEMI with statin