The endothelium in sepsis
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER 5

INCREASED CIRCULATING ENDOTHELIAL PROGENITOR CELLS IN SEPTIC PATIENTS: CORRELATION WITH SURVIVAL

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Critical Care Medicine 2007;35:1677–1684
ABSTRACT

Objective: Endothelial damage and detachment of endothelial cells are known to occur in septic patients. Thus, recruitment of circulating endothelial progenitor cells (cEPCs) to these lesions might have a beneficial effect on the clinical course in septic patients. Therefore, we were interested in whether EPCs, detected by flow cytometry, are increasingly mobilized during sepsis and if this mobilization is associated with clinical outcome.

Design: Prospective, nonrandomized study.

Setting: Intensive care unit of a university hospital.

Patients: Patients with (n=32) and without (n=15) sepsis and healthy volunteers (n=15).

Interventions: Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation, and cEPCs were characterized by three-color fluorescence flow cytometry using antibodies against CD133, CD34, and vascular endothelial growth factor receptor-2. Serum concentrations of vascular endothelial growth factor, granulocyte macrophage-colony stimulating factor, and erythropoietin were determined by enzyme-linked immunosorbent assay. Severity of sepsis was assessed according to Acute Physiology and Chronic Health Evaluation II scoring.

Measurements and Main Results: In septic patients, the number of cEPCs was significantly higher than in nonseptic intensive care unit patients (p<0.05) and healthy controls (p<0.02). Nonsurvivors (n=8), defined as death within 28 days after onset of sepsis, had significantly lower numbers of cEPCs than survivors (n=24) (p<0.0001). The number of cEPCs was correlated with survival in septic patients. Serum vascular endothelial growth factor concentrations were significantly higher in septic patients compared with nonseptic intensive care unit patients and healthy controls (p<0.01) and correlated with the cEPC numbers (p<0.0001). Similar findings were observed for granulocyte macrophage-colony stimulating factor and erythropoietin.

Conclusions: Our data suggest that cEPC enumeration in peripheral blood of septic patients might be a valuable marker to assess the clinical outcome in these patients.
INTRODUCTION

Bacterial toxins and pro-inflammatory cytokines initiate a series of immunologic events that alter endothelial function in the macro- and microcirculation. Vasodilation, capillary leakage, endothelial swelling, leukocyte sequestration, thrombosis, and organ dysfunction are well documented in septic patients. Although initially these changes seem not to differ between survivors and nonsurvivors, capillary perfusion only increases in survivors over time, suggesting that altered endothelial function plays an important role in the development of multiple organ failure in sepsis.

Under normal conditions, endothelial cells (ECs) are firmly attached to the extracellular matrix via so-called focal adhesion contacts. In addition, adjacent ECs interact with each other via specialized structures (i.e., adherence and tight junctions), thereby forming a tight barrier to prevent vascular leakage and passive leukocyte migration into the interstitial tissue. However, under pathologic conditions, ECs can be detached from the vasculature and thus appear in the circulation. Inadequate formation of focal adhesion contacts, proteolysis of the endothelial basal membrane, apoptosis of ECs, and the production of anti-angiogenic factors are among other causes for the release of ECs into the circulation.

Simultaneous with these pathologic processes, reconstitution of the endothelial layer is initiated. This can obviously occur via migration and proliferation of surrounding mature ECs. However, terminally differentiated ECs have a low proliferative potential; hence, their capacity to substitute damaged endothelium is limited. Therefore, adequate vascular repair requires additional support.

Many studies have convincingly demonstrated that vascular maintenance, repair, angiogenesis, and neovascularization are partly mediated by recruitment of endothelial progenitor cells (EPCs). These cells are bone marrow-derived and have the propensity to differentiate into mature ECs. Their phenotype is characterized by the expression of the specific hematopoietic marker CD34, the stem cell marker CD133, and the endothelial marker vascular endothelial growth factor receptor-2 (VEGFR-2). However, the identification of EPCs from mature ECs is complicated by the presence of CD34 on both cell types. Therefore, discrimination between circulating mature ECs and circulating EPCs (cEPCs) can be further made by the expression of CD146 and CD105 on mature cells.

In healthy individuals, only a small number of cEPCs (~0.002% of total peripheral blood mononuclear cells [PBMCs]) can be found in peripheral blood, whereas this is substantially increased on physical stress and under pathophysiologic conditions.

Mobilization of cEPCs from the bone marrow critically depends on the activation of metalloproteinases and up-regulation of adhesion molecules. This is most likely mediated by soluble factors such as vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), and erythropoietin (EPO).
INCREASED CIRCULATING EPC IN SEPTIC PATIENTS

Serum concentrations of these factors dramatically increase under pathologic conditions, concomitantly with an increase in the number of cEPCs. While metallo-proteinases act to release cEPCs from the bone marrow stroma, adhesion molecules facilitate trafficking of these cells into the circulation. Recently, first evidence was provided that cEPCs play a pivotal role in reendothelialization not only after vascular damage but also after severe inflammation. However, most of the investigators used the colony-forming assay for cEPC enumeration. Although this method could be used to identify and quantify cEPCs, it is time-consuming and likely insensitive for general application in a clinical setting. In the present study we performed a practicable multicolor FACS analysis to detect cEPCs in peripheral blood. Using this method we tested the hypothesis that clinical outcome in septic patients is largely dependent on the ability to reconstitute damaged endothelium. Serum concentrations of VEGF, GM-CSF, and EPO were determined in parallel to the number of cEPCs found in circulating blood and were correlated with Acute Physiology and Chronic Health Evaluation (APACHE) II scoring and mortality.

MATERIALS AND METHODS

Subjects
Patients with sepsis (n=32) were selected from the intensive care unit (ICU) of the University Hospital Mannheim within 48 hrs after sepsis onset or at admission to the ICU. Patients included were enrolled consecutively over a 2-yr period (2004–2005) and met the diagnostic criteria for sepsis of the American College of Chest Physicians/Society of Critical Care Medicine. The rate of bacterial evidence was 70%. Sepsis severity was assessed by the APACHE II score, and mortality was defined as death occurring within 28 days after diagnosis. Exclusion criteria were cardiogenic or hemorrhagic shock, chronic obstructive pulmonary disease, isolated acute respiratory distress syndrome, absence of mechanical ventilation, and use of statins, angiotensin-converting enzyme inhibitors, activated protein C, and hydrocortisone. Clinical data of each patient were recorded.
For controls we recruited 15 patients from the ICU who did require mechanical ventilation, hereafter referred to as ICU controls, and 15 healthy volunteers from our laboratory staff, hereafter referred to as healthy controls. ICU controls, mainly neurosurgical patients, did not meet the criteria for sepsis, septic shock, or systemic inflammatory response syndrome and were not treated with statins or angiotensin-converting enzyme inhibitors. This study was approved by the Ethics Committee of the University of Heidelberg.
Blood Sampling
Blood (25 mL) was obtained from the central venous catheter of septic patients within 48 hrs after sepsis onset or in the case of ICU controls within 24 hrs after admission to the ICU. A second blood sample was obtained from septic patients 5 days after the first. In healthy controls, 20 mL of blood was collected in tubes containing sodium citrate (0.105 M) as anticoagulant by insertion of a 20-gauge cannula intravenously. The initial 5 mL of blood was discarded to minimize EC contamination from the puncture wound of the vascular wall.

Flow Cytometry
All blood samples were processed within 1 hr after collection. PBMCs were prepared by gradient centrifugation using Ficoll-Hypaque (Amersham Biosciences, Freiburg, Germany). The expression of cell-surface antigens was determined by three-color immunofluorescence staining as described previously 31. One hundred microliters of PBMC (containing 1 x 10⁶ cells) was incubated with 10 μL of FcR-blocking reagent (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 10 mins to inhibit nonspecific bindings. Thereafter, the cells were incubated at 4°C for 30 mins with 10 μL of PE-conjugated anti-human CD133 monoclonal antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany), 10 μL of PerCP-conjugated anti-human CD34 monoclonal antibodies (BD Biosciences, Heidelberg, Germany), and 10 μL of APC-conjugated VEGFR-2 monoclonal antibodies (R&D Systems, Wiesbaden-Nordenstadt, Germany). Isotype-matched immunoglobulin G1 and immunoglobulin G2a antibodies (DakoCytomation, Hamburg, Germany) were used for each patient and measurement as negative controls. The cells were washed three times to remove unbound antibodies and finally resuspended in 400 μL of FACS solution (BD Biosciences, Heidelberg, Germany). FACS analysis was performed on a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using WinMDI 2.8 software (Scripps Research Institute, La Jolla, CA). A minimum of 500,000 events were collected. FACS analysis of each probe was performed in triplicate. The frequency of cEPCs in peripheral blood was determined by a twodimensional side-scatter/fluorescence dot-plot analysis of the samples after appropriate gating. EPC counts are expressed as percentage of total PBMC in each patient or control.

Enzyme-Linked Immunosorbent Assay
The serum concentrations of VEGF, GM-CSF, and EPO were assessed using a highly sensitive enzyme-linked immunosorbent assay kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) in triplicate samples obtained from 5 mL of serum. Enzyme-linked immunosorbent assay was performed according to the manufacturer’s instructions.
**Statistical Methods**

All data are presented as mean ± SEM. Both parametric and nonparametric methods were used. Analysis of variance and Kruskal-Wallis test were performed when comparing the three groups. Nonparametric statistical analysis (Kruskal-Wallis) was especially used to compare the three groups regarding GM-CSF, since the concentration in healthy controls was below the detection limit. Student’s t-test and U test were used to compare survival in the septic patient group. Receiver operating characteristic analysis was performed to predict survival probability from cEPC numbers. Correlation analyses (Pearson/Spearman) were considered for all target variables. We considered p<0.05 to be statistically significant. All analyses were performed using the SAS system (version 8.2, SAS Institute, Cary, NC).

**RESULTS**

There was no statistical difference in mean age between the groups: in the sepsis group 55.3 ± 13 yrs, (survivors 53 ± 12 and nonsurvivors 62 ± 16 yrs), in the ICU controls 57 ± 16 yrs, and in the healthy controls 58 ± 15 yrs. Of the 32 patients (18 male and 14 female) fulfilling the sepsis inclusion criteria, eight died within 28 days after diagnosis (i.e., 25% mortality rate). In the survivor group, three septic patients died after 28 days. No differences in leukocytes or C-reactive protein (CRP) were found between survivors and nonsurvivors (survivors, white blood cell count 16.1 ± 8.5 white blood cells/µL, CRP 178 ± 84 mg/L mg/L; nonsurvivors, white blood cell count 20.1 ± 7.2 10E9/L, CRP 166 ± 54 mg/L). Relevant characteristics of all septic patients included in this study are summarized in Table 1.
Table 1. Data of patients with sepsis, including gender, age, mortality, Acute Physiology and Chronic Health Evaluation (APACHE) II score, type of infection, white blood cell (WBC) counts and C-reactive protein (CRP) level at time of first blood sampling

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<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>Days</th>
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<th>Type of infection</th>
<th>WBC, $10^9$/mm$^3$</th>
<th>CRP</th>
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The percentage of hematopoietic stem cells, defined as positive staining for CD34 and CD133, was low in healthy controls (0.15% ± 0.66%). While it was already significantly increased in ICU controls (0.24% ± 0.11%), there was still a significant increase of CD34+/CD133+ cells in septic patients—0.52% ± 0.4% (Fig. 1). Within the septic patients, survivors did not differ from nonsurvivors in this respect.

**Figure 1. Percentage of circulating hematopoietic progenitor cells.** FACS analysis from CD34/CD133-positive cells in the peripheral blood mononuclear cell (PBMC) fraction of healthy volunteers (n=15), nonseptic intensive care unit (ICU) patients (n=15), and septic patients (n=32). Significant differences were found between all three groups. The results are expressed as mean simple linear regression ± SEM; p<0.05 was considered to be statistically significant.

In addition, the percentage of VEGFR-2+ cells within the population of CD34+/CD133+ cells was measured. In healthy controls, only 25% ± 15% of CD34+/CD133+ cells stained positive for VEGFR-2. Both in ICU controls (35.5% ± 22%) and in septic patients (45.5% ± 18%), this was significantly higher (Fig. 2). This corresponds to a percentage of cEPCs (CD34+/CD133+/VEGFR-2+ cells) in the total PBMC fraction: 0.039% ± 0.002% in healthy controls, 0.087% ± 0.04% in ICU controls, and 0.162% ± 0.1% in septic patients (Fig. 3). During the course of sepsis, the percentage of cEPCs remained high; that is, 5 days after the first sampling, cEPC counts were not significantly altered in the survivor group (data not shown). In the nonsurvivor group, only two patients were still alive after 5 days.
Figure 2. Circulating endothelial progenitor cell quantification in peripheral blood mononuclear cell (PBMC) fraction. FACS analysis data representative for each investigated group: healthy volunteers, intensive care unit (ICU) controls, septic patients. A–C, dot plots show negative control; D–F, dot plots show PBMCs stained with anti-CD34 and anti-CD133 monoclonal antibodies; G–I, histograms show the percentage of vascular endothelial growth factor receptor-2 (FLK)-positive cells in the population of CD34/CD133-positive cells. APC, allophycocyanin.
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Figure 3. Up-regulation of circulating endothelial progenitor cells (cEPC). FACS analysis from CD34/CD133/vascular endothelial growth factor receptor-2-positive cells in the peripheral blood mononuclear cell (PBMC) fraction of healthy volunteers (n=15), nonseptic intensive care unit (ICU) patients (n=15), and septic patients (n=32). Significant differences were found between all three groups. The results are expressed as mean simple linear regression ± SEM; p< 0.05 was considered to be statistically significant.

We next investigated if changes in the number of cEPCs were associated with sepsis severity and survival. As shown in Figure 4, sepsis survivors had significantly higher numbers of cEPCs (0.170 ± 0.06) than nonsurvivors (0.06 ± 0.028). However, nonsurvivors had still significantly more cEPCs compared with healthy controls. The patients who died in the survivor group had a lower number of cEPCs than the remaining patients in this group but still a higher number (0.13 ± 0.03) than the nonsurvivor group. The logistic regression revealed a strong association between cEPC numbers and survival in the sepsis group. Septic patients with high cEPC numbers had a higher probability to survive than patients with low cEPC numbers (p<0.036; estimate = 92.03; area under curve = 0.97).

Severity of sepsis was assessed by APACHE II scoring. The mean APACHE II score was significantly higher in the nonsurvivor than in the survivor group (19.6 ± 5 vs. 13.08 ± 4, p<0.01). However, only in the survivor group could a significant correlation between APACHE II score and cEPC numbers (r = 0.44; p<0.03) be detected.
Figure 4. Up-regulation of circulating endothelial progenitor cells (cEPC) in sepsis survivors and nonsurvivors. FACS analysis from cEPCs in the peripheral blood mononuclear cell (PBMC) fraction of healthy volunteers (n=15) and the survivor (n=24) and nonsurvivor groups (n=8) in septic patients. Significant differences were found between all three groups. The results are expressed as mean simple linear regression ± SEM; *p*<0.05 was considered to be statistically significant.

The measurement of the circulating growth factors showed the following findings. In healthy controls, the mean serum VEGF concentration was low (56.1 ± 29.8 pg/mL). In both patient groups—that is, nonseptic ICU patients (477 ± 275 pg/mL, *p*<0.01) and septic patients (1351 ± 733 pg/mL, *p*<0.01)—there was a significant increase in serum VEGF concentrations compared with healthy controls. Serum VEGF concentrations were also significantly higher in septic patients compared with the ICU controls (*p*<0.01). Similar to VEGF concentrations, also GM-CSF and EPO concentrations were significantly increased in septic patients compared with ICU and healthy controls (GM-CSF, 3.3 ± 2.2 pg/mL vs. 0.9 ± 0.7 pg/mL vs. 0.049 ± 0.3 pg/mL; EPO, 100.6 ± 75 mUI/mL vs. 19.9 ± 12 mUI/mL vs. 5.7 ± 3 mUI/mL) (Fig. 5). Within the group of septic patients, mediator production of survivors and nonsurvivors was not significantly different (VEGF *p*<0.48, GM-CSF *p*<0.50, EPO *p*<0.91).

A significant correlation between cEPC numbers and serum levels of VEGF (*r* = 0.65; *p*<0.0001), GM-CSF (*r* = 0.58; *p*<0.0001), and EPO (*r* = 0.57; *p*<0.0001) was detected for all the groups. Consequently, high numbers of cEPCs were correlated to high levels of VEGF, GM-CSF, and EPO. Comparing the association between cEPC concentration and
mediator production in septic patients, a significant correlation was only observed for GM-CSF in the nonsurvivor group ($r = 0.73, p<0.04$).

Figure 5. Up-regulation of growth factors in serum. (A) Erythropoietin (EPO), (B) granulocyte macrophage-colony stimulating factor (GM-CSF), and (C) vascular endothelial growth factor (VEGF) concentrations in serum of healthy volunteers, nonseptic intensive care unit (ICU) patients, and septic patients. The results are expressed as mean simple linear regression ± SEM.
and were significantly different between all investigation groups; \( p<0.05 \) was considered to be statistically significant.

DISCUSSION

The present study demonstrated an increased mobilization of cEPCs in septic patients compared with nonseptic ICU patients and healthy individuals. Concomitantly with this, serum concentrations of VEGF, GM-CSF, and EPO were significantly increased. The percentage of cEPCs was correlated with sepsis survival; that is, septic patients with a high percentage of cEPCs had a higher probability of survival.

To our knowledge, this is one of the first studies using standard flow cytometry as a practicable method to detect cEPCs in peripheral blood. Different approaches have been used to quantify EPCs in a variety of patients and controls \(^{11, 19, 22, 32}\). Most of these assays, however, rely on cell separation and subsequent cell culturing, making it difficult to implement these assays in routine diagnostics. In the present study we have used standard FACS analysis to quantify EPC numbers in PBMCs \(^{33}\). Since a FACS apparatus is present in almost every routine lab, and monoclonal antibodies against EPC markers are commercially available, this method has the potential to be more widely used in the near future and find its place in routine diagnostics.

Although the exact phenotype of cEPCs is still controversially discussed, the presence of CD34, CD133, and VEGFR-2 seems to be proven \(^{19, 34}\) and therefore used in this study. Other potential endothelial markers (e.g., CD45, CD14, or VEGFR-1) are likely not specific for EPCs.

Detachment of ECs has been reported under various pathologic conditions that are associated with vascular injury \(^4, 22\). Also, microvascular alterations occur during sepsis, ultimately leading to breakdown of the endothelial barrier function \(^35-38\). Mutunga et al. \(^4\) observed an increase of circulating ECs during sepsis and concluded that endothelial damage occurs. The increased number of cEPCs found in this study might therefore be a consequence of the body’s attempt to limit vascular damage by inducing endogenous endothelial repair mechanisms.

EPCs also seem to play a role under inflammatory conditions, as was suggested in the study by Yamada et al. \(^39\). Those authors observed that inflammatory stimuli induced a rapid release of EPCs into the circulation in humans and concluded that a sufficient number of EPCs is required for proper repair of lung tissue following bacterial pneumonia. Moreover, an association between cEPC numbers and CRP concentrations in human serum \(^21\) also suggests a link between inflammation and EPC mobilization.

The importance of vascular repair under inflammatory conditions (e.g., sepsis) has thus far not been studied. Burnham et al. \(^22\) found for the first time an increased number of cEPCs in patients with acute lung injury. They used the colony-forming assay to determine EPC and observed an association between EPC concentration and survival.
INCREASED CIRCULATING EPC IN SEPTIC PATIENTS

Because in some of these patients acute lung injury-associated septic shock was present, we found herein a first confirmation of our results. We observed an increase in cEPC numbers in all investigated septic patients and a correlation between cEPC concentration and survival. The number of cEPCs, however, was still increased in nonsurvivors compared with nonseptic patients, which is in agreement with the observations of Sakr et al. 2. In that study, it was shown that capillary perfusion in septic patients is increased over time only in survivors, but not in nonsurvivors, and might be due to the recruitment of EPCs and subsequent repair of vascular damage. The number of cEPCs was still increased during severe sepsis, even when clinical symptoms of sepsis had been normalized. In our study, the increase of cEPC concentration was found prolonged over an investigation period of 5 days. This suggests that the endothelial alteration and repair are still ongoing and effective in improving microvascular organ perfusion, particularly in sepsis survivors. Any dysfunction in EPC release by bone marrow exhaustion or failure could be excluded, since white blood cell counts were found normal or enhanced, but not suppressed. Apart from the correlation between cEPC concentration and survival in general, we also found in the survivor group that there was a correlation between APACHE II scores and cEPC at time of admission. This score was chosen because it has become one of the most accepted among the general ICU severity of illness scoring systems. To risk-adjust patients with longer and very severe illnesses, several other models of organ dysfunction have become available, including the Sequential Organ Failure Assessment. Because on admission all patients with severe sepsis or septic shock were given an appropriate therapy and hence fulfilled the exclusion criteria, we did not use the Sequential Organ Failure Assessment. The recruitment of cEPCs during inflammation is most likely mediated by cytokines 40. Van der Flier et al. 41 reported a correlation between increased plasma VEGF levels and severity of multiple organ dysfunction during the course of sepsis. They found significantly higher VEGF levels in nonsurvivors than in survivors and concluded a potential role of this mediator in the development of sepsis-associated capillary leakage. We confirmed the increase in VEGF production in septic patients. However, we did not observe significant differences in VEGF levels between survivors and nonsurvivors. The increase in EPC numbers after VEGF administration or endogenous VEGF up-regulation under septic conditions underlines the importance of this mediator in mobilization of EPCs. 13 In addition to VEGF, EPO and GM-CSF are also able to mobilize EPCs with a similar potency 42-44. In line with the observations of Heeschen et al. 25, our data show a correlation between the number of cEPCs and serum VEGF and EPO levels, but not with CRP concentrations. Our data also confirm the previous observation by Torre et al. 45 that GM-CSF serum concentrations are increased in septic patients and patients with noninfectious systemic inflammatory response syndrome.
The clinical relevance of cEPCs in a variety of vascular disorders has already been studied. Hill et al.\textsuperscript{11} reported that levels of cEPCs may be a surrogate biological marker for vascular function and cumulative cardiovascular risk. Werner et al.\textsuperscript{32} found that increased levels of cEPCs were associated with a reduced risk of future cardiovascular events among patients with coronary artery disease. In our study, we observed a correlation between cEPC numbers and survival in septic patients. The level of cEPCs in patients with vascular disorders might have the potential to predict clinical outcome and may help to identify patients at increased risk.

CONCLUSION

EPCs may exert an important function as an endogenous repair mechanism to maintain the integrity of the endothelial layer by replacing denuded parts of the microcirculation. Our study suggests that an increased number of cEPCs during sepsis may predict survival in these patients. Future studies are required to examine the potential of cEPC enumeration in routine diagnostics.
REFERENCES


